Documentation and molecular analysis of African medicinal plants and naturally-produced chemical compounds that modulate latent HIV-1 infection

by

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Abstract

HIV cure continues to be elusive due to cellular reservoirs that persist despite the highly potent combined antiretroviral therapy (cART) which supresses HIV plasma viremia to undetectable levels. One of the cure strategies currently being pursued is “Shock-and-Kill”, which uses latency reversing agents (LRAs) to activate provirus expression in the presence of cART to avoid reservoir reseeding, while exposing the virus-expressing cells to immune responses for eradication. To date, there is no licensed LRA due to their limited clinical success, i.e., their failure to consistently and significantly reduce the viral reservoir in human clinical studies. New LRAs and LRA combinations are therefore likely needed for the shock-and-kill approach to succeed.

Natural products are a rich repository for novel antivirals and latency modulating agents. My thesis documents and provides molecular analysis of African medicinal plants and naturally-produced chemical compounds that modulate latent HIV-1 infection. First, to highlight the therapeutic potential of naturally-produced chemical compounds, 19 African medicinal plants, whose anti-HIV or latency modulating properties have been documented and are supported by detailed laboratory data in the literature, are discussed in a systematic review (Chapter 2). Furthermore, this thesis, which is interdisciplinary in character, features an exploratory community-based project, involving 13 Traditional Health Practitioners (THPs). This ethnopharmacological study documented 83 medicinal plants used for HIV/AIDS and related diseases management by the BaKalanga Peoples of the Tutume-sub district in Central Botswana. Thirty-eight (45.8%) of the identified medicinal plants have not been previously investigated for HIV-1 specific bioactivities linked to their ethnomedicine use reported in our survey and therefore should be prioritized for bioassay-based studies in the future, in the search for more novel antivirals and LRAs. Finally, this thesis identifies and characterize five (5) novel latency modulating compounds derived from natural sources namely: psammaplin A; aplysiatoxin; debromoaplysiatoxin; knipholone anthrone (KA) and its analogue anthralin (dithranol).

Taken together, my thesis supports the search for novel HIV-1 latency modulating agents and indeed antivirals from natural products to improve “shock-and-kill”-based therapies and eventually finding a cure for HIV.

Keywords: HIV; shock-and-Kill; latency reversing agents; naturally-produced compounds; Knipholone anthrone; psammaplin A

Dedication

This thesis is dedicated to my mother, Thembi Richard, who has always been the backbone of my support system and above all, a trusted and loyal friend.

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List of Acronyms

AIDS	Acquired Immune Deficiency Syndrome
AMPK	AMP Kinase
APC	Antigen-presentation cells
ART	Antiretroviral Therapy
Aza-CdR	5-aza-29-deoxycytidine
AZT	Azidothymidine
BETi	Bromodomain and extra-terminal bromodomain inhibitor
BLT	Bone Marrow-Liver-Thymus
BRD4	Bromodomain-containing protein 4
CA	Capsid
CAR	Chimeric antigen receptor
cART	combination Antiretroviral Therapy
CDC	Centre for Disease Control
cDNA	complimentary Deoxyribonucleic Acid
CITE-seq	Cellular Indexing of Transcriptomes and Epitopes by Sequencing
CPE	Cytopathic effect
CRF	Circulating Recombinant Forms
CTD	Carboxy Terminal Domain
dCA	didehydro-cortistatin A
DIS	Dimer Initiation Site
DMSO	Dimethyl sulfoxide
DMTi	DNA methyltransferase inhibitor

DNA	Deoxyribonucleic Acid
dNTP	deoxynucleotide triphosphate
dQVOA	Differentiation Quantitative Viral Outgrowth Assay
DRM	Drug Resistance Mutation
DSIF	DRB-sensitivity inducing factors
EC	Elite controller
EC_{50}	50% effective concentrations
ELISA	Enzyme-linked immunosorbent assay
Env	Envelope
FBS	Fetal Bovine Serum
FDR	False Discovery Rate
GFP	Green Fluorescent Protein
gp120	Glycoproteins 120
gp41	Glycoproteins 41
HDAC	Histone Deacetylases
HIV	Human Immunodeficiency Virus
HMTi	Histone methyltransferase inhibitor
HPLC	High-performance liquid chromatography
HSCT	Haematopoietic Stem Cell Transplantation
IBA	Ibalizumab
IC_{50}	50% Inhibitory Concentration
IL-2	Interleukin-2
IBD	Integrase-binding domain
IkB	Inhibitory kappa-B

IN	Integrase
IPDA	Intact Proviral DNA assay
IPT	Isoniazid Preventive Therapy
KA	Knipholone Anthrone
LA-ARTs	Long-Acting Antiretroviral Therapies
LMICs	Low- and middle-income countries
LRA	Latency Reversing Agent
LTR	Long Terminal Repeat
MA	Matrix
MBD2	Methyl-CpG Binding Domain Protein 2
MHC I	Major Histocompatibility Complex class I
mRNA	Messenger Ribonucleic Acid
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay
NA	Nucleocapsid
NLS	Nuclear Localization Signal
NF-κB	Nuclear Factor kappa B
NELF	Negative Elongation Factor
NMR	Nuclear magnetic resonance
NNRTI	Non-nucleoside/nucleotide reverse transcriptase inhibitor
NRTI	Nucleoside/nucleotide Reverse Transcriptase Inhibitor
P-TEFb	Positive transcriptional elongation factor b
pANAPL	pan-African Natural Products Library
PBMC	Peripheral blood mononuclear cell
PBS	Primer Binding Site

PCR	Polymerase Chain reaction
PCT	Post-Treatment Controller
PD-1	programmed death-1
PEP	Post-Exposure Prophylaxis
PEPFAR	President’s Emergency Plan for AIDS Relief
PI	Protease Inhibitor
PKC	Protein kinase C
PLWHA	People Living with HIV & AIDS
PMA	Phorbol 12-myristate 13-acetate
PMTCT	Prevention of mother-to-child transmission
Pol	Polymerase
PR	Protease
PrEP	Pre-Exposure Prophylaxis
qPCR	Quantitative Polymerase Chain reaction
QVOA	Quantitative Viral Outgrowth Assay
RDDP	RNA-dependent-DNA Polymerization
RNA	Ribonucleic Acid
RNAP II	RNA polymerase II
RPMI 1640	Roswell Park Memorial Institute 1640
rPRA	Rapid Participatory Rural Appraisal
RRE	REV Response Element
RT	Reverse Transcriptase
SAHA	Suberanilohydroxamic acid
scATAC-seq	assay for transposase-accessible chromatin using sequencing

scRNA-seq	single-cell RNA sequencing
SEB	Staphylococcus enterotoxin B
Smac	Second mitochondria-derived activator of caspases
SIV	Simian Immunodeficiency Viruses
TAR	Trans-activation response
TasP	Treatment as Prevention
Tat	Transactivator protein
TCMs	Traditional and Complementary Medicines
TDR	Transmitted Drug Resistance
TFV	emtricitabine/tenofovir
THP	Traditional Health Practitioner
TILDA	tat/rev-induced limiting dilution assay
TLC	Thin Layer Chromatography
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TOA	Time of Addition assay
TRUCKs	T cells redirected for universal cytokine-mediated killing
TUs	Transcription Units
U=U	Undetectable = Untransmittable
UNAIDS	United Nations AIDS
US FDA	United States of America Food and Drug Administration
VDC	Village Development Committee
vpr	Viral protein r
WHO	World Health Organization

ZFN	Zinc Finger Nuclease
ZFP	Zinc Finger Protein

Chapter 1.
Introduction

1.1. Abstract

In this introductory chapter, background information as it pertains to the Human Immunodeficiency Virus (HIV) and Acquired Immune Deficiency Syndrome (AIDS) will be reviewed. The chapter will also characterize HIV, discussing its taxonomical classification, genetic make-up, life-cycle and pathogenesis. The chapter will extensively discuss HIV therapy and its limitations due to virus evolution; this will include the classes of established anti-retroviral drugs and their mechanisms, newly developed Long-Acting Antiretroviral Therapies (LA-ARTs), Pre-Exposure Prophylaxis (PrEP), Post-Exposure Prophylaxis (PEP), treatment as prevention (TasP) uses of ART and the significance of undetectable = untransmittable (U=U). The chapter will also reflect on the impact of ART in improving management of HIV across the world, and in sub-Saharan Africa in particular, and additionally the progress of HIV 90-90-90 and 95-95-95 strategies. HIV latency and its molecular mechanisms, current cure strategies and their challenges will also be reviewed here. The chapter will end by evaluating the 'shock-and-kill' cure strategy, with more emphasis on the progress made and challenges confronting this latency reversal approach.

Key words: HIV; HIV therapy; HIV latency; molecular mechanisms; cure strategies; shock-and-kill

1.2. HIV & AIDS epidemiology

The Human Immunodeficiency Virus (HIV) is the etiologic agent of Acquired Immune Deficiency Syndrome (AIDS). Since its outbreak, more than 79.3 million people are estimated to have been infected with HIV and approximately 36.3 million people have died of AIDS-related illnesses as of 2020 (1). Globally, it is estimated that 37.7 million people were living with HIV in 2020 while about 1.5 million individuals worldwide became newly infected (1). The burden of HIV & AIDS epidemic is heavily skewed towards Africa, especially Eastern and Southern Africa which account for 44.7% of the global total of new HIV infections. HIV is still most prevalent in Africa, where nearly 1 in every 25 adults (3.6%) are living with HIV, making up 67% of people living with HIV (PLWHA) worldwide (2). However, there have been some advances made in treating and managing the HIV and AIDS scourge. In particular, combination antiretroviral therapy (cART) has been a significant breakthrough that has reduced life-threatening HIV/AIDS to a clinically manageable chronic disease. Globally, 73% of people living with HIV (PLWHA) were accessing antiretroviral therapy (ART) worldwide in 2020, whereas ART-access in Eastern and Southern Africa was reported to be 77% in 2020 (1-2). However, despite these recent advances in cART development and accessibility, HIV continues to remain a global health threat. For example, just in 2020 alone, 680,000 people died of AIDS-associated illnesses worldwide (1-2).

1.3. Human Immune Virus (HIV): The Pathogen

1.3.1. Taxonomical classification

Taxonomically, HIV belongs to the family and subfamily of Retroviridae and Orthoretrovirinae, respectively. The Human Immunodeficiency Virus is classified as a Lentivirus, which is its genus, and additionally comprises Simian Immunodeficiency Viruses (SIV) which infects non-human primates (3-4). HIV exists as two species being HIV-1 and HIV-2 whose genetic variance in nucleotide sequence is 50%, and with HIV-1 being the most pathogenic and infectious strain (4-6). HIV-2, the less-virulent type, is thought to have originated from sooty mangabey, and resembles SIVsmm infections prevalent in West-African sooty mangabey species, though these are non-pathogenic in

its natural host (3). HIV-2 is further divided into 9 groups recognizably different lineages: A-H, and only two of these groups (A & B) seems to infect human beings (3,4) (**Fig. 1.1**).

On the other hand, as shown in **Fig. 1.1,** HIV-1 has four groups: M (Main), N (New), O (Outlier) & the recently discovered P (3, 7-9). Phylogenetic data show that all the four groups of HIV-1 cluster with Simian Immunodeficiency Viruses, and share approximately 70% genetic sequence similarity (3-4,7-9). Whereas Group M and N clusters with SIVcpz strains isolated from chimpanzees in Cameroon, group O and P are genetically close to the gorilla-linked SIVgor viral strains. However, in as much as these HIV-1 groups are phylogenetically related, they are genetically distinct from each other. For example, the similarity of envelope and pol sequences between HIV-1 group O and M strains are as low as 50% and 30% respectively (9). Group M, is the deadliest group of the HIV-1 species and the main contributor to the global HIV/AIDS pandemic. Group M is further divided into ten subtypes (A–D, F–H, J, K-L) which are slightly genetically different from each other. However, HIV-1 subtypes are characterized by 70-90% shared genetic sequence identity (4). These subtypes are spread in different parts of the world in varying proportions. For example, subtype C is predominant in Southern-Africa and India; subtypes A and D are prevalent in eastern and central Africa, and subtype B strains are mostly in Europe and North America (3). Furthermore, viral mutations of several HIV-1 subtypes have resulted in the emergence of multiple distinct circulating recombinant forms (CRFs) (3-4, 10-11).

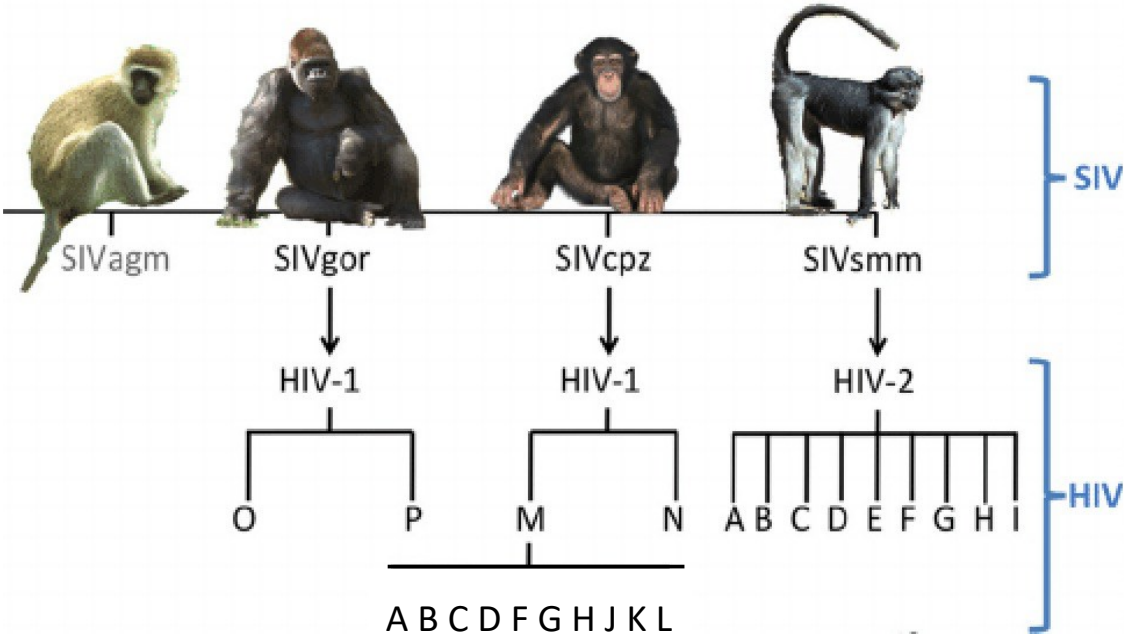

Figure 1.1: Diagram showing the phylogeny-related HIV and SIV lentiviruses;
(adapted from Letko, *et al*., 2013 (8))

1.3.2. HIV Structure and function

HIV measures approximately 100nm in diameter and comprises a lipid envelope along with Matrix, Capsid, and nucleocapsid proteins (**Fig. 1.2**). The nucleocapsid houses two copies of HIV's single-stranded, positive-sense RNA genome, whose size is approximately 10 kilobases (kb) (12-13). The viral genome is flanked by Long Terminal Repeat (LTR) sequences at both ends, whose interaction with various host factors drives viral gene transcription (**Fig. 1.2**). Briefly, LTRs, one of the most conserved regions of the viral genome, are a pair of identical viral DNA sequence measuring about 634 bp, segmented into U3, R and U5 regions — that contain several binding motifs for an array of transcription factors (14). In particular, the LTR situated in the 5' end acts as the promoter region for HIV-1 transcription, whereas the 3' end is responsible for RNA polyadenylation. Importantly, the HIV LTR consists of almost all regions necessary for viral gene expression including the stem-loop forming TAR (Trans-activation response) element, which binds to the viral Transactivator protein (Tat) to activate full-scale viral transcription (15-16). Also present in LTR is the 18 nucleotides primer binding site (PBS) within which lies the $tRNA^{Lys}$ primer specific binding site near the 5' end of the HIV-1 genome, which is needed in the initiation of the HIV-1 reverse transcription process

(17,18). The LTR also contains the Poly A region which facilitates genome packing and dimerization. Other regions contained in the LTR are Psi packing element and DIS (Dimer Initiation Site) required for HIV genome packaging and RNA encapisdation (15, 19).

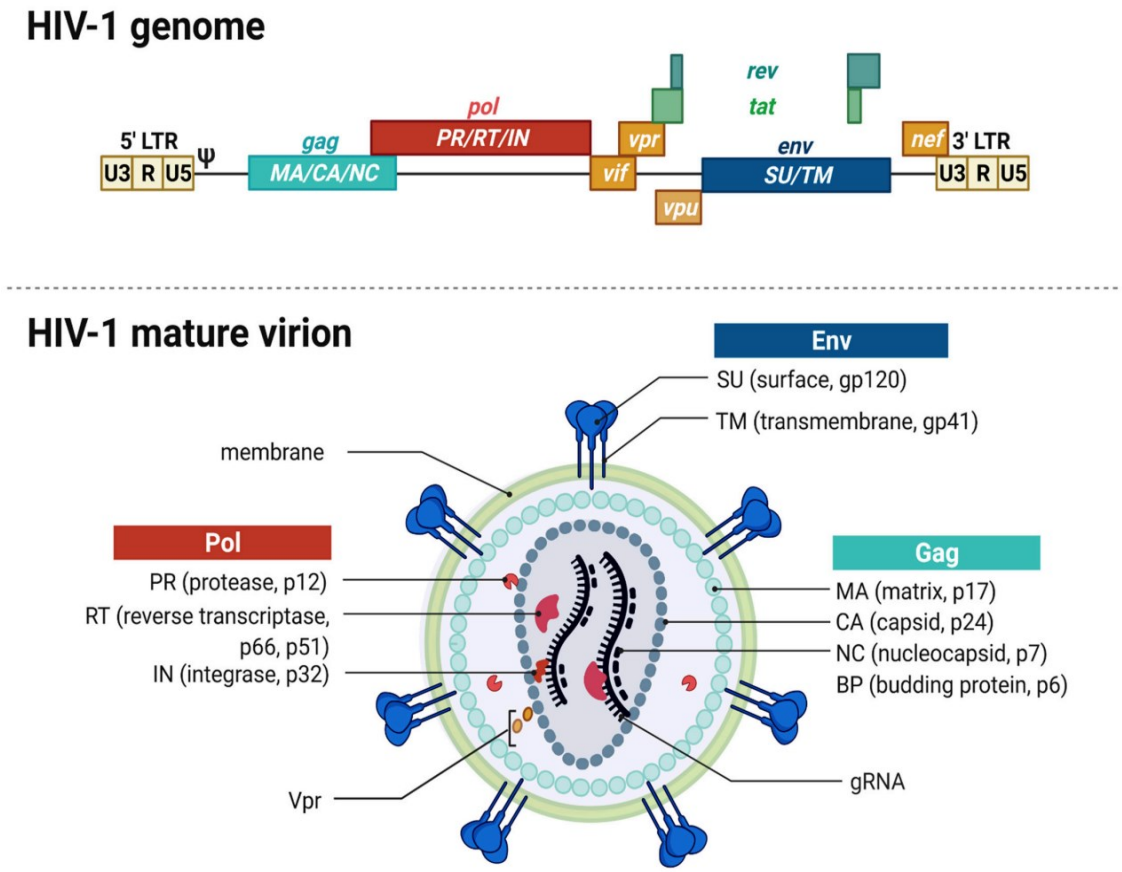

Figure 1.2: Genomic organization of HIV and the structure of mature virion;
(adapted from Heuvel, *et al*., (2022) (21))

The HIV genome codes for 9 open reading frames: Env, Pol, Gag, Vpu, Nef, Tat, Vif, Rev and Vpr whose transcription makes up various proteins used in the production of a new infectious virus (12-13, 20) (**Fig. 1.2**). The three structural genes of the HIV genome (env, pol, gag) encode for the structural proteins. Specifically, the Env gene encodes viral envelope glycoproteins: the gp120 (glycosylated cap) and gp41, which is a knob-shaped hydrophobic component. The gp120 has high affinity for the host CD4 receptor and chemokine co-receptors CCR5 and CXCR4 (22-24). gp41 is a transmembrane protein that anchors gp120 and mediates the fusion of the viral membrane to the host's target cell membrane. The transcription and translation of the Gag gene results in 4 distinct structural

proteins including Matrix (MA, p17) — a myristilated polypeptide that constitutes the HIV's inner membrane layer and holds the viral core in place; capsid (CA, p24) — responsible for the formation of conically-shaped viral capsid, nucleocapsid (NA, p7) whose primary role is the formation of the RNA complex; and p6, a 52-amino acids protein involved in the viral particle release and egression (12-13, 20).

The Pol gene gives rise to enzymatic proteins including reverse-transcriptase (RT, p51), integrase (IN, p32) and protease (PR, P10) (25). RT catalyzes the biochemical process of converting the single-stranded RNA viral genome to the double-strand complimentary DNA (cDNA) following viral entry. IN performs an enzymatic function in the incorporation of the viral cDNA into the genome of the host, while PR facilitates the proteolytic cleaving of Gag-Pol and Gag polyproteins, ensuring that the virion particle matures into an infectious virus (13,20, 25). Tat and Rev reading frames encode for two regulatory proteins including transactivator protein (p14) and RNA splicing regulator (p19), respectively. Tat promotes and amplifies viral replication by way of stimulating viral genes transcription (13, 26). Rev, a viral component that shuttles between the cytoplasm and the nucleus, is involved in the exportation of the unspliced HIV mRNA out of the host's nucleus (27).

Four genes, nef, vpu, vpr and vif, are responsible for producing accessory proteins: negative regulating factor (p27), viral protein unique (p16), virus protein r (p15) and viral infectivity protein (p23) respectively. Accessory proteins generally help in promoting and enhancing viral infectivity, viral replication and viral budding (13, 28-31). Specifically, Nef increases HIV-1 pathogenicity by way of downregulating CD4 expression on the target cells, promoting swift endocytosis. In addition, it reduces Major Histocompatibility Complex class I (MHC I) on the HIV-infected cells, thus shielding the virus-infected cells from the killer-immune responses by CD8+ T cells (30-33).

Vpu is a 16-kilodalton protein that enhances the release of the virus particles from HIV-infected cells. It also modulates the degradation of the newly synthesized CD4 through its cytoplasmic domain, in addition to downregulating existing CD4 on the cell surface (28-29, 34-35). Vif measures 23 kD and helps in viral replication by targeting and neutralizing the antiviral activity of the human enzyme, APOBEC-3G, by ubiquitinating it to mark it for degradation (31, 36). Viral protein r (vpr) is a 15 kD HIV regulatory protein that enhances viral replication by way of transactivating the HIV-LTR as it interacts with the trans-

activator protein (Tat) and the cyclin T1/Cyclin Dependent Kinase 9 complex, SP1 and p53, in addition to activating other cellular transcription factors such as NF-κB (31, 37, 39). Vpr also plays a role in the conduction of the HIV-1 pre-integration complex to the host nucleus. Vpr, through its C-terminal domain, also stimulates a prolonged pro-apoptotic G2 cell cycle arrest, a phenomenon that inhibits proliferation in HIV-1 infected cells (31, 40).

The transcription of the viral genome yields full-length HIV-1 RNA transcripts. Typical of retroviruses, the splicing of the full-length HIV-1 RNA transcript occurs by way of alternative RNA Splicing, a process that results in over 40 fully spliced and unspliced mRNA species (28). Some of the unspliced full-length viral genome transcripts evade splicing due to rev and are utilized as mRNAs for the Gag-Pol proteins and as RNA genome for the new viruses being produced (41-43) (**Fig. 1.3**). However, all other viral proteins are products of RNA splicing. The full-length genomic transcript undergoes complete splicing to produce mRNA transcripts of early regulatory proteins Tat and Rev, whereas mRNA transcripts that translate into proteins such as Vif, Vpr and Env are the results of incomplete splicing which is enabled by the shuttling of these transcripts from the nucleus by the early protein Rev (41-42).

Viral splicing, which is a highly organized and sequential process, takes place from 5' splice sites (5'ss) and 3' splice sites (3'ss), about four and five sites respectively. Using the splice donor D1 found in the 5' UTR, the transcripts splice in the direction of A1 to A5 downstream acceptors. As mentioned, viral RNA splicing is a highly orderly process; for example, the first step of splicing, to the A1 downstream acceptor gives out the vif transcript, A2 makes a vpr transcript and the process continues in the same way yielding various HIV-1 transcripts that would be translated to different viral proteins (41-42). The viral proteins from different mRNAs are determined by the selection of the alternative 3'ss in the primary HIV genomic transcript.

The vpu, env and REV Response Element (RRE) viral mRNAs results from the splicing of D4 to A7, which can succeed the D1 splicing. Nef coding transcript is generated from the splicing activity at A5, coupled with the splicing at D4 to A7. Tat viral transcripts are produced from the splicing activity that occurs at A3 together with splicing of D4 to A7; whereas Rev mRNA, measuring approximately 1.8kb, is made from splicing that takes place at A4a, A4b or A4c alongside D4 to A7 (41-43). Rev, which basically shuttles between the cytoplasm and nucleus, attaches cooperatively to RRE-containing RNA

transcripts in the env gene and conduct the partially spliced and the intron-containing (unspliced) transcripts out of the nucleus, to be translated for proteins that are packaged into new virus particles (44-45).

Basically, Vif, Vpr, Vpu, and Env precursor protein represent the HIV-1 proteins that are generated from partially spliced mRNA transcripts, whereas Tat, Rev and Nef are made from fully spliced transcripts. However, Genomic RNA, Gag and Gag/Pol precursor proteins are generated from unspliced full-length transcript as shown in **Fig. 1.3** below.

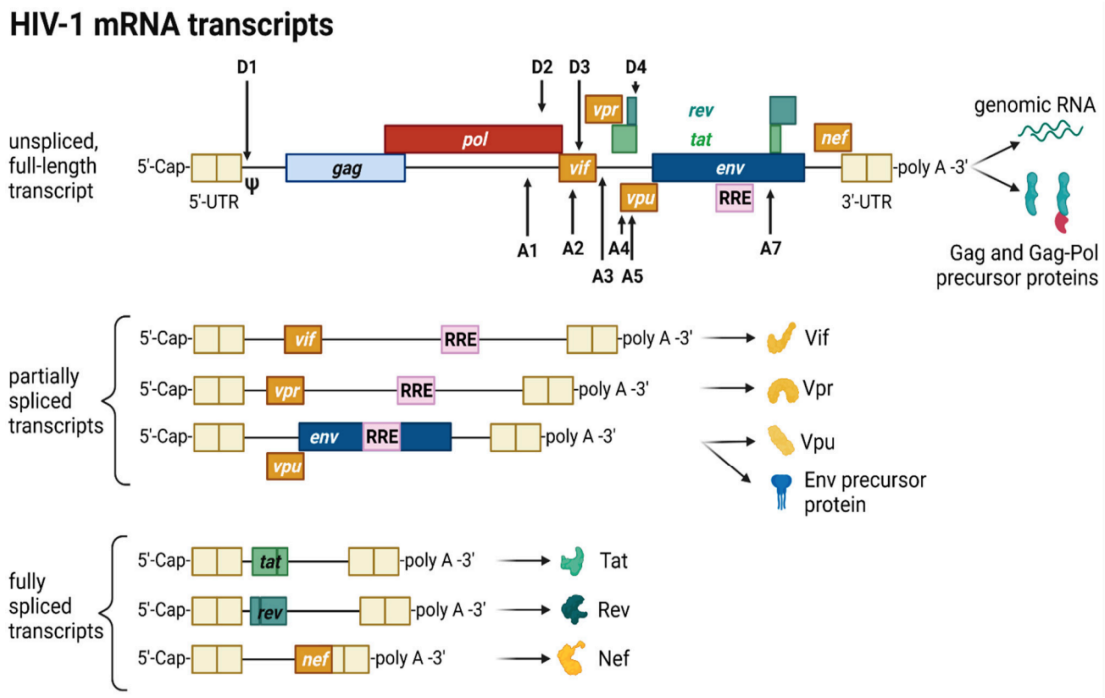

Figure 1.3: HIV-1 mRNA transcripts and splice sites;
(adapted from Heuvel, et al., (2022) (21))

1.3.3. Life cycle, replication stages and pathogenesis of HIV

As a lentivirus, HIV cannot replicate on its own but requires 'hijacking' of a host cell's transcription and translation machineries to produce copies of its own. The life-cycle of HIV is generally characterized by several steps which include fusion, entry, reverse transcription, integration, transcription, translation, assembly, budding & maturation (**Fig. 1.4**.).

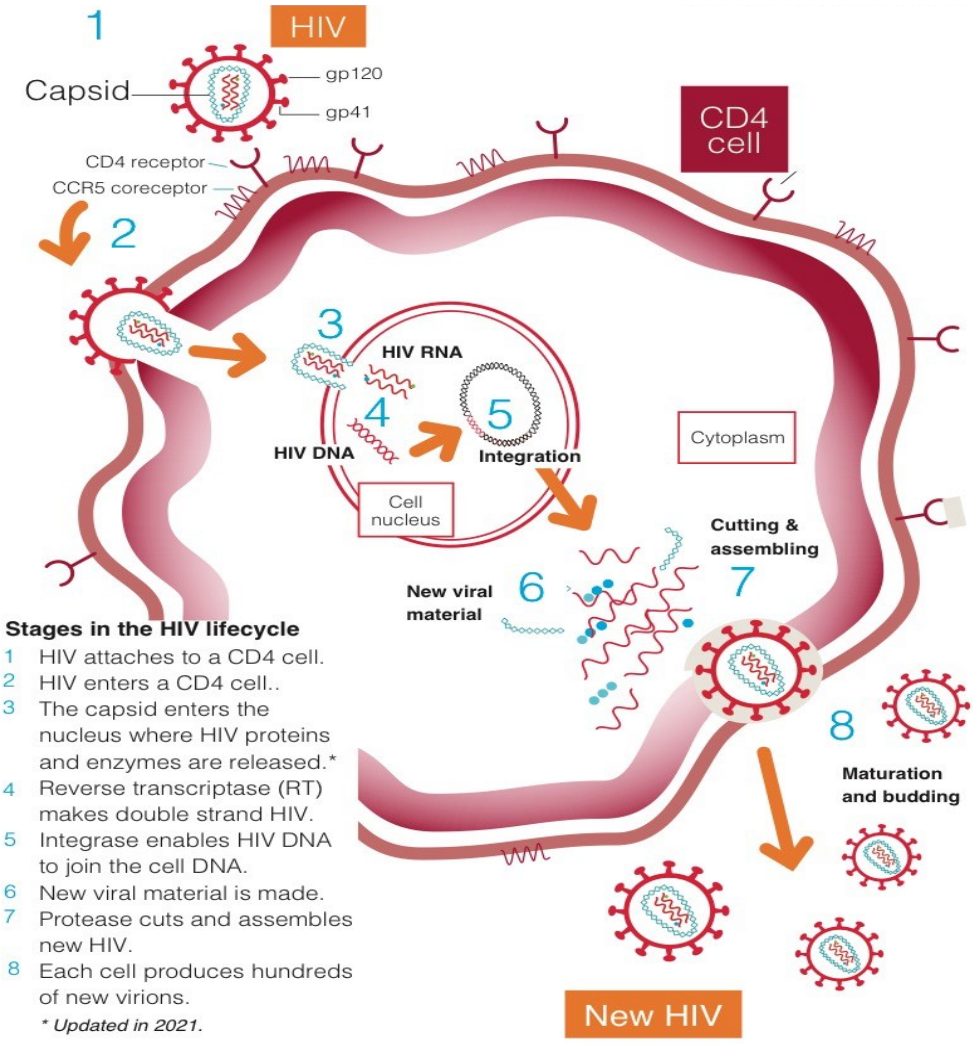

Figure 1.4: HIV replication cycle;
(adapted from https://i-base.info/guides/art-in-pictures/the-hiv-lifecycle)

HIV specifically infects T-helper cells (CD4+ T cells), macrophages and dendritic cells (13, 20). First the virus, using gp120, attaches to and binds with the host CD4 protein, which acts as the primary host receptor (22-24, 47). CD4 protein is abundantly expressed by T-helper cells, even though it is expressed — in lesser quantities — by other immune cells such as macrophages and dendritic cells which also makes them targets for HIV-1 (47-49). HIV additionally requires binding to CXCR4 or CCR5 chemokine co-receptors (50-52).

Thus, the interaction of gp 120 (glycosylated cap protein) with CD4 receptor causes some alteration in the structural conformation of the viral envelope that exposes its binding site to the HIV co-receptors CXCR4 or CCR5 (53). The type of chemokine coreceptor the virus binds to, determine its tropism. Thus, HIV-1 virion particles that interact with CXCR4 are X4 tropic, while those that bind to CCR5 are R5 tropic (54). HIV may also exhibit dual/mixed (D/M) tropism or R5/R4 tropism, when it binds both CXCR4 and CCR5 coreceptors (54-55). The newly transmitted HIV-1 preferentially uses CCR5, but at later stages of infection, about 50% of the virion particles switch to CXCR4, a situation characterized with a sharp decline of CD4+ cell count and rapid disease progression in HIV-1 infected individuals (55-58).

Following binding to host receptors, the hydrophobic component of the virus surface glycoprotein (gp41) penetrates the host plasma membrane, and the virus injects the pre-integration complex into host cell cytoplasm (59-60). The pre-integration complex contains the HIV genome (HIV RNA) and enzymatic proteins IN, RT, and PR (13, 20, 58, 61).

Upon entry, RT converts the viral RNA into a double-stranded complimentary DNA (cDNA): a process known as reverse transcription (59-60). RT protein facilitates this process through RNA-dependent-DNA Polymerization (RDDP) reactions and through its RNAse H-specific motif, which enables the Ribonuclease H (RNase H) to cleave and degrade the HIV-1 RNA strands in the resulting RNA/DNA complex (62-64).

Following reverse transcription, the cDNA is then transported to the host nucleus where IN mediates integration which results in the cDNA being incorporated into the host genome (64-65). The IN initiates this highly coordinated integration process by cleaving nucleotides of each cDNA strand at the 3' end, a reaction that exposes the 3'-OH group. Next, IN facilitates its DNA strand transfer activity, cutting the host genomic material and joining it to the cDNA, in a biochemical reaction known as a transesterification (66-67). Thereafter, host DNA enzymes including the ligase, are recruited to repair DNA of host and fill in the gaps (66,68).

Notably, HIV preferentially integrates in actively transcribing genes, presumably to promote productive viral gene expression (69). Thus, the nuclear architecture, as it pertains to the accessibility of an 'open chromatin', influences the sites of integration. It has been shown that HIV associates around the nucleus (at the nuclear pore), an area

containing active cellular genes (70). Cellular tethering proteins also plays a role in dictating and directing the HIV-1 integration sites. For example, the lens epithelium-derived growth factor, or transcriptional co-activator p75 (LEDGF/p75), is a tethering protein implicated in the HIV-1 integration process (66-67, 70). LEDGF/p75, a transcription coactivator harbouring a ProTrp-Trp-Pro domain used in protein-to-protein interactions, and an AT-hook motif and the nuclear localization signal (NLS) bind the IN at the integrase-binding domain (IBD) to direct the IN to the host DNA of actively transcribing genes and around transcription units (TUs) and in AT-rich genomic hotspots (66-67). Cell cycle-directed DNA replication is also thought to dictate the site of integration at the time of HIV-1 infection, although this may not be essential as HIV-1 can infect both dividing and non-dividing cells (66-67, 70).

After the proviral DNA has been integrated into the host cell DNA, the virion 'hijacks' the host transcription and translation machineries replicate and produce new viral RNA and proteins, using the cDNA as a template (13, 20). Important to note, viral transcription can either happen immediately after integration or could occur later — in latently infected cells habouring an incorporated proviral genome.

For example, following HIV-1 integration, activated CD4+ T-cells may revert back to their resting physiological state. The ability of the CD4+ T-cells to switch between active transcription state and latency, coupled with its distinctive properties to undertake effector-to-memory transition, fosters the establishment of a viral reservoir (71). The highly proliferative naive CD4+ T cells, after being exposed to the viral antigen, differentiate into various subsets of memory T cells, including a population of Central Memory T cells, transitional memory T cells, effector memory T cells and terminally differentiated T cells, in addition to functional subsets such as follicular helper T cells and regulatory T cells, all which have been reported to contain an integrated HIV-1 provirus (72,73). The Central Memory T cells are largely the ones that account for the latent reservoir, which can bounce back to produce new infectious virion particles upon reactivation. The viral reservoir is highly stable, with a half-life of approximately 44 months; and may persist for an estimated period as long as 60 to over 73 years in the presence of cART that suppresses viral load to undetectable levels (74-77). While the majority of infected cells productively transcribe virus following successful incorporation of the viral DNA, the small fraction of infected cells that enter latency after viral integration are sufficient to generate this lifelong latent HIV reservoir.

In the instance where transcription occurs immediately, which is largely the most common occurrence, the viral 5' LTR acts as the binding site for transcription factors that initiate transcription (14-16). For example, transcription factors present in the host cells such as nuclear factor kappa B (NF-κB) attach to the HIV-1 LTR (78-79). NF-κB is made up of five units that binds to the promoter regions of different genes as dimers, the p50/p65 being the most abundant (78-79). NF-κB is found in the cytoplasm and typically exists in an inactive state bound to IκB (80). Upon stimulation, the IκB is phosphorylated, ubiquitinated and degraded by the proteasome, in the process freeing NF-κB and allowing it to translocate into the nucleus to bind the promoter region of target genes (81) and, in the case of HIV-1, the 5' LTR to help induce the complex viral transcription process (79).

This initiated viral transcription is however stalled by several factors such as NELF (negative elongation factor) and DRB-sensitivity inducing factors (DSIF) (82-83). The virion circumvents this transcription termination using viral Tat protein, which firmly binds to the nascent viral Trans-activation Responsive (TAR) element (13, 26, 84). As mentioned earlier on, TAR, the stem-loop RNA structure, is found in the R region of 5' LTR of all HIV mRNAs and mediates effective Tat-driven HIV-1 transcription (14-16, 84). Tat recruits and interacts with P-TEFb (Positive transcriptional elongation factor b), a complex made up of host Cyclin T1 (CycT1) and the cyclin-dependent kinase 9 (CDK9), to activate and elongate viral transcription by way of phosphorylating the carboxy terminal domain (CTD) of stalled host RNA polymerase and productive viral transcription.

Following transcription, virion proteins assemble into immature virus particles that then egress out of the host cell membrane. The viral replication cycle is completed by way of proteolytic cleaving of Gag-Pol and Gag polyproteins which is mediated by HIV-1 PR enzyme contained within the viron particle, a process that delivers a mature infectious virus (13).

As mentioned above, viral egress results in the eventual loss of CD4+ T cells, which are important components of the human immunological defense system. As CD4 is also expressed by macrophages and dendritic cells, all of these cells can be lost following HIV infection (90-93). Dendritic cells are important members of the innate immune system, which serve as antigen-presentation cells (APC), in addition to secreting cytokines and activating T cells (91-92). Macrophages, also an innate immune member, are recruited to infection sites to phagocytose and eliminate the pathogen and cell-derived debris; they

also exhibit both pro- and anti-inflammatory immune responses. Macrophages are also antigen-presentation cells (APC) that avail antigen peptides to the CD4+ T cells through the MHC-II, to elicit antibody responses (93). T helper cells, on the other hand are key members of the adaptive immune system. T helper cells have several functions including activating cytotoxic T cells (CD8+ T cells), B-lymphocytes as well as innate immune cells (90).

Macrophages, Dendritic cells and CD4+ T cells are all critical in HIV infection, pathogenesis and disease progression because they express CD4, the HIV-1 primary receptor and HIV-1 co-receptors (CCR5 and CXCR4), all required for HIV-1 attachment, fusion and entry in the host cells through their interaction with HIV-1 Env 120 subunit. Their ability to induce cytotoxic T cells and their involvement in viral antigen presentation also makes them obvious targets by the activated apoptosis-inducing CD8+ T cells (90-93). Eventually, CD4+ T cell count of the host drops drastically – a hallmark of an HIV infection. The progressive, inexorable depletion of CD4+ T helper cells population, in the absence of treatment that durably suppresses viremia, inevitably collapses the host immune-defense system, with the HIV-infected person advancing and succumbing to Acquired Immune Deficiency Syndrome (AIDS) (94-95).

1.4. HIV Therapy

1.4.1. Historical perspective

Although HIV/AIDS was recognized in the 1980s, to date there is still no cure. However, several forms of antiretroviral therapy have become available to suppress viremia and convert AIDS to a chronic, manageable condition in much of the world. The first ART was discovered and introduced in the late 1980's, with azidothymidine (AZT), a Nucleoside/nucleotide Reverse Transcriptase Inhibitor (NRTI), being the first clinically approved HIV medication in 1987 (96-98). AZT monotherapy was reported to cause adverse clinical side effects including macrocytosis, bone marrow suppression, myalgia and anaemia. For example, a double-blind, placebo-controlled study, found out that 24% of the study participants who received AZT developed anaemia that was characterized by disturbingly low amounts of haemoglobin, that dropped below 7.5g per deciliter (99). The AZT monotherapy was associated with moderate efficacy and heamatologic toxicity (99-

101). HIV-infected people also had to take AZT pills multiple times a day, so there was need to develop more effective anti-HIV medication that was associated with reduced pill-burden. Another major issue with AZT monotherapy, pertained to the problematic HIV-1 drug resistance due to mutations on multiple amino acids including codon 67, 70, 215 and 219 that were reported less than 2 years after the approval of the drug (102). Approximately 50% of AZT-taking patients were reported to rapidly develop resistance immediately after 6 months post-ART initiation (103). This AZT-associated drug resistance underscored the urgent need for more therapies that were less toxic and more effective. Thus, Didanosine and Zalcitabine monotherapies, both NRTIs, would quickly be added to the HIV-1 armamentarium in 1991 and 1992 respectively (104-105), to circumvent some of these shortfalls of AZT, while widening the options of ART medication.

As the search for better HIV medication continued, Protease Inhibitors (PIs) and Non-nucleoside/nucleotide reverse transcriptase inhibitors (NNRTIs) were discovered, with Saquinavir (1995) and Nevirapine (1996) being the first PI and NNRTI respectively, to be approved for clinical use (106-107). It was until recently, post the year 2000, that another class of HIV drugs was approved: Integrase strand transfer inhibitors (INSTIs). Raltegravir became the pioneer INSTIs to be clinically approved as ART in 2007 (108). All these drugs are discussed in detail hereinunder, in the next sub-topic: classes of antiretroviral drugs and their mechanisms. The fact that these drugs targeted different stages of HIV-life cycle was a great advancement that definitely improved the success rate of ART in purging HIV/AIDS, but the efforts were still insufficient due to HIV-drug resistance. For example, Saquinavir monotherapy was associated with mutations at positions 48 and 90, with an incidence of about 45% in 12 months (109). More anti-HIV medication with improved efficacies and low pill burden were still needed. The discovery of these drugs from different functional classes paved way for combination HIV therapy.

To this end, HIV-1 medication has since evolved and developed drastically. The evolution of ART has since seen the frequent administration of monotherapies being replaced with combined therapies that allow the patient to take the medication on a reduced frequency and indeed, reduced pill-burden. Part of the evolution and revolution of HIV therapy have seen the development of more tolerable cART, one which is associated with higher efficacies and a significantly improved quality of life for HIV patients. Furthermore, combined therapy (cART) replaced the single-drug administration as evidence showed that combined therapy resulted in higher efficacies and lowered progression to AIDS in

HIV-infected individuals (104-105). Thus, zidovudine and lamivudine became the first approved combined HIV-1 regimen, following the completion of a double-blind randomized clinical trial in which a 2-drug combined therapy (zidovudine-lamivudine) achieved superior clinical outcomes when juxtaposed with zidovudine, as measured by HIV-1 viral load, p24 antigenemia and CD4 cell counts (110).

However, it was until mid and late1990's, that researchers focused in triple drug ART regimen, which would later become a monumental breakthrough achieved with regard to combination therapy. For example, Montaner *et al.* (1998) conducted a double-blind, controlled, randomized clinical study to assess and compare the antiviral efficacies of various combinations of nevirapine, didanosine, and zidovudine in 153 HIV-1 patients (111). The 3-drug regimen of nevirapine, didanosine, and zidovudine notably reduced plasma HIV-1 RNA by log 2.18-fold following drug initiation, which was far higher than double therapies of zidovudine-didanosine and zidovudine-nevirapine, which attained log 1.55, and 0.90-fold reductions in HIV-1 RNA, respectively. By the end of 52 weeks administration, 51% of the patients treated with triple drug therapy had plasma viral RNA levels below 20 copies per milliliter; while combined therapies of zidovudine-didanosine (12%) and zidovudine-nevirapine (0%) showed low virologic effects as indicated. Montaner *et al.* (1998) reported that 79% of the patients who took the 3-drug regimen had an undetectable viremia; no virus could be isolated and amplified in those patients. The results far outstripped patients enrolled in dual therapies of zidovudine-didanosine, and zidovudine-nevirapine in which only 53% and 31% patients had no virus to isolate in their plasma, respectively. Overall, the triple-drug therapy treatment was evidently far superior than double-drug therapies (111).

The triple drug therapy would later become a major development and milestone towards transforming the deadly HIV/AIDS into a manageable chronic health condition. The co-administration of multiple HIV drugs, of various mechanisms targeting different HIV life-cycle made its way into the guidelines of HIV-1 medication. It became what was called Highly active antiretroviral therapy (HAART) and now combination antiretroviral therapy (cART) (112-113). HAART was introduced for the following purposes: to reduce HIV and AIDS related death rates and morbidities; lower plasma viral load and improve immune system function of people living with HIV; prevent transmission of HIV between individuals and obviate the issue of drug resistance — all with an overall aim to improve the quality of life in PLWHA (111-114). Nevirapine (NNRTI) and Rotanavir and Indinavir (both PIs)

were the first cocktail of HIV-1 antiretroviral medication to be approved by US Food and Drug Administration (FDA) in 1996 (114). In this way, HIV was eventually reduced to a chronic disease, thanks to the triple drug therapy. HIV-1 drug classes and their mechanisms are discussed in detail below.

1.4.2. Classes of antiretroviral drugs and their mechanisms

There presently exists 5 broad classes of HIV antiretroviral drugs which have been approved by US Food and Drug Administration (FDA) for clinical use. The FDA-approved classes are: Reverse Transcriptase Inhibitors which exists as Nucleoside/nucleotide Reverse Transcriptase Inhibitors (NRTIs) and Non-nucleoside/nucleotide reverse transcriptase inhibitors (NNRTIs); Entry Inhibitors comprising of Post-Attachment Inhibitors, CCR5 antagonists, Fusion inhibitors; Integrase strand transfer inhibitors (INSTIs), and Protease Inhibitors (PIs) **(Table 1.1).**

Table 1.1: FDA-approved HIV-1 antivirals and their classes

(adapted from 20, 115-117)

Non-Nucleoside/nucleotide Reverse Transcriptase Inhibitors (NNRTIs)	Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs)	Integrase strand transfer inhibitors (INSTIs)	Protease Inhibitors (PIs).	Entry Inhibitors
Azidothymidine – Zidovudine (1987)	Nevirapine (1996)	Raltegravir (2007)	Saquinavir (1995)	Enfuvirtide (T20) (2003)
Didanosine (1991)	Efavirenz (1998)	Elvitegravir (2013)	Indinavir (1996)	Maraviroc (2007)
Zalcitabine (1992)	Delavirdine (2007)	Dolutegravir (2013)	Ritonavir (1996)	
Stavudine (1994)	Etravirine (2008)	Cabotegravir (2021)	Nelfinavir (1997)	
Lamivudine (1995)	Rilpivirine (2011)		Lopinavir + Ritonavir (2000)	
Abacavir (1998)	Doravirine (2018)		Atazanavir (2003)	
Tenofovir (2001)			Fosamprenavir (2003)	
Emtricitabine (2003)			Tripanavir (2005)	
			Darunavir (2006)	

The first generation of HIV drugs to be approved by FDA were NRTIs — these include azidothymidine, Didanosine and Zalcitabine which were introduced in 1987, 1991 and 1992 respectively (96, 104-105). The latest NRTIs to be approved were Tenofovir (2001) and Emtricitabine (2003) (118-119). NRTIs competitively bind to the active site of the HIV RT enzyme. Because NRTIs typically lack the 3' hydroxyl group, their binding to the incipient viral DNA molecule prevents the incorporation of the next DNA nucleotides to continue reverse transcription (96, 118-119).

However there have been concerns relating to the emergence of HIV-1 resistance to this class of NRTI-based medication (102-103, 120-123). Generally, there are two reported biochemical mechanisms associated with viral resistance of NRTIs monotherapies. One of the mechanisms is selection for genetic mutations that allow RT to avoid binding the NRTIs agent without affecting intrinsic RT enzymatic activity (124-125). RT discrimination against the chain terminating NRTIs means the enzyme continues its role of reverse transcription and cDNA elongation uninhibited, even in the presence of the antiviral (124-125). Another mechanism of NRTI-based HIV-1 drug resistance is through mutations that mediate the increased hydrolysis-based expulsion of the NRTI agent from the RT active site (pyrophosphorolysis), thus reducing the competitive binding of NRTIs and promoting cDNA synthesis (124-125).

HIV-1 isolates from individuals taking AZT features several mutations that confer resistance to this antiviral. To this end, a multiplicity of genetic alterations at codons 41, 67, 70, 210, 215 and 219 acting both individually and collectively, have been associated with AZT resistance (120-123). Mechanistically, this zidovudine-linked resistances have been shown to occur by way of AZT mono-phosphate pyrophosphorolysis from a terminated viral DNA, thus enhancing RT catalytic performance and efficiency. The alteration in the above codons, by extension, drive resistance not only to AZT medication, but other NRTI-based single-drug therapies such as Didanosine, abacavir, and stavudine (102-103, 120-123). Some of the reported mutations in this class of antivirals drugs include T215Y/F, T69D and M184V (126-128). T69D, a mutation at position 69 of the RT including one or two amino acid insertions is also a notable genetic alteration that confers HIV-1 resistance to all NRTIs (123). Position 69 is part of the RT's finger region (position 64-72) that interact with the incoming deoxynucleotide triphosphate (dNTP) during DNA synthesis (126). T215Y/F, emanating from 2-base pair alterations, represents another known mutation driving drug resistance in patients who receive dual NRTIs antiviral

regimen or zidovudine monotherapy. The standard phenotypic drug susceptibility assay showed that T215Y/F cause approximately 20-fold AZT resistance (127-129). HIV drug resistance testing is conducted using phenotypic drug susceptibility assay and genotypic-susceptibility assay — that identifies known drug resistance-associated mutations. Using standardized recombinant virus assay, the *in vitro* drug inhibition of HIV-1 can be quantified and data is reported as fold-change in drug susceptibility calculated through this formular: (IC_{50} for sample virus/IC_{50} for reference virus) (123, 130-131). M184V, a mutation at the conserved position 184 near the catalytic site of RT has been profiled as a cause for HIV-1 drug resistance in patients taking lamivudine therapy and indeed other NRTIs. Previous studies found out that M184V induces over 100-fold viral resistance in patients using lamivudine single-drug therapy (123, 132-133).

Non-Nucleoside/nucleotide Reverse Transcriptase Inhibitors (NNRTIs) are another form of HIV-1 drugs targeting the RT enzymatic function. Efavirenz and Rilpirine (approved in 1998 and 2011 respectively) are some of the examples of the clinically-approved Non-Nuclear Reverse Transcriptase Inhibitors (NNRTIs). The mode of action for NNRTIs is such that they non-competitively attach to the non-catalytic site of the viral RT situated near the active site – this interaction alters the structural shape of the RT substrate-binding site to diminish its enzymatic activity (134-136). NNRTI resistance has also been investigated and documented. Among the NNRTI-associated mutations occurring between codons 98-108, K103N is of high clinically significance primarily because it induces 20-50-fold resistance across all currently available NNRTI HIV-1 medication, including nevirapine, efavirenz and delavirdine (123, 131), as measured by phenotypic drug susceptibility assay and genotypic- susceptibility assays. NNRTI resistance is also caused by the V106A mutation, particularly in nevirapine, where it has been shown to confer approximately 30-fold resistance (123, 131, 137). Other mutations causing NNRTI resistance occur between codons 179-190 and include Y181C/I (inducing 30-fold increase to delavirdine and nevirapine therapies), Y188C/L/H and V179D at codons 181, 188, and 179 respectively (123, 136, 138). Other mutations occur between codons 225-236, with the rare M230L mutation for example conferring high-level resistance to several NNRTIs — 60-fold, 40-fold and 20-fold resistance to delavirdine, nevirapine and efavirenz, respectively (123, 139).

Another class of HIV antiretroviral drugs targets the integration stage: Integrase strand transfer inhibitors (INSTIs). Four licensed drugs are members of this class — Raltegravir,

Elvitegravir, Dolutegravir and Cabotegravir. These drugs interfere with the integrase enzymatic activity by competing for its catalytic site, thus inhibiting the process of viral cDNA incorporation into the host genome (140-146). HIV-1 drug resistance against Integrase strand transfer inhibitors (INSTIs) has been observed and reported in both first generation (raltegravir and elvitegravir) and second generation (dolutegravir, bictegravir, and cabotegravir) of INSTIs, though second generations of INSTIs generally show lower resistance susceptibility. For example, Oliveira et al, (2018) found out that T66I/A, R263K and E92G/V/Q mutations were associated with viral resistance to elvitegravir all causing approximately 100-fold resistance (147). Patient-derived HIV-1 isolates treated with cabotegravir were also shown to feature Q148R/K mutations, capable of inducing significant cross-resistance in other types of INSTIs (147). Previously, Q148R/K mutations were shown to cause raltegravir resistance, alongside N155H (147-149). Y143R/C mutations also conferred high-level resistance to raltegravir drug (148-149).

Some of the HIV-1 antiviral drugs that are classified as Protease Inhibitors (PIs) include Ritonavir (1996); with Tripanavir and Darunavir being the latest to be approved in 2005 and 2006 respectively (150-154). PIs are both peptide-derived and non-peptide molecules that biologically mimic the viral protease substrate, and thus bind to the catalytic site of the Protease. This prevents the HIV protease enzyme from cleaving the Gag-Pol and Gag polyproteins; as a result, the maturation of the virion particle into an infectious virus is blocked (150-154).

Like other classes of antivirals, clinically significant PI-linked resistance has been reported, as a result of mutations in several positions of the protease enzyme. Viral variants that are known to exhibit high drug resistance against several PIs including nelfinavir, indinavir and ritonavir, feature mutations at codons 48, 54 and 82, within the protease's substrate-binding cleft (154-157). HIV-1 samples isolated from indinavir and ritonavir-taking patients have been found to feature a protease substrate cleft mutation at codon 82 known as V82A/T/F/S, a mutation that is also present in individuals exposed to sustained saquinavir therapy (123, 155). G48V, represents another PI-associated mutation on in HIV-1 isolates from patients treated with saquinavir conferring about 10-fold resistance (158). Other mutations are found within conserved residues of PR enzyme at codon 73 e.g., G73S associated with resistance of indinavir and saquinavir monotherapies (159). The PR flap mutations, situated within positions 46-56, particularly I54V, I54T/L/M and F53L, represents another type of mutations also linked to HIV-1 PR resistance to several PI

inhibitors, including ritonavir, amprenavir and indinavir (123, 157, 160). F53L is mostly found in patients, at a rate of more than 10%, using many PR inhibitors than those using single-drug therapy (123, 161).

Entry inhibitors, another group of HIV-1 medication, is the broad and less common class of ARVs that target fusion events between HIV-1 and the target cells. Post-Attachment Inhibitors, CCR5 antagonists and Fusion inhibitors (FIs) belong to this class of HIV-1 medications. Fusion inhibitors represent function extracellularly to block the interaction of the incoming infectious virus with the receptor cell (CD4+ T cell). The peptide-based Enfuvirtide (T20) (2003) is an example of an FDA-approved fusion inhibitor (162-163). The mechanisms of fusion/entry inhibitors may vary and are drug-specific. For example, Enfuvirtide (T20) acts by way of binding to the viral gp41, in the process, impeding its fusogenic function (162-163). Another entry inhibitor which has been clinically approved is Maraviroc (2007), an antiretroviral drug that targets CCR5-tropic virion particles, hence it is classified as a CCR5 antagonist (164). Post-Attachment Inhibitors attache to the CD4 receptor in CD4+ T cell, where the interaction prevents HIV-1 from binding with the required chemokine co-receptors. Examples of FDA-approved HIV-1 Post-Attachment Inhibitors include Rukobia (Fostemsavir) (165-167) and Ibalizumab (IBA) (168-169) — the humanized immunoglobin (Ig) G4 monoclonal antibody. Specifically, IBA binds to the CD4 receptor and prevent the conformational changes in gp120 which are necessary for the viral-chemokine coreceptor interaction following the exposure of gp41(168-173). There has been reports of clinical resistance to HIV-1 entry inhibitors, particularly, Enfuvirtide (T20) monotherapy (174). V38A mutations within the HR1 domain in HIV-1 isolates of Enfuvirtide-taking patients, conferred 45-fold resistance, while a dual mutation at position 36 (G36D) induced 9.1-fold resistance to T20 monotherapy (174).

1.4.3. Long-Acting Antiretroviral Therapies (LA-ARTs)

FDA and Health Canada recently approved landmark LA-ARTs whose drug delivery method is novel in as far as HIV treatment is concerned: intramuscular, subcutaneous injection. The approved LA-ARTs, which will be used in people who are virologically suppressed, are a combinatorial regimen of rilpivirine (a potent NNRTI) and cabotegravir (a potent INSTI) which are currently the most investigated and best characterized long-acting ARVs both for HIV treatment and prevention (175-176). Cabotegravir, a structural

analogue of Dolutegravir, is readily absorbed in the body and is estimated to have an elimination half-life of about 40 days, a pharmacological property that makes it a suitable long-acting anti-HIV injectable (177-178). Rilpivirine, whose effective daily dose as anti-HIV pill is 25 mg, has a similarly long half-life *in vivo* (in the range of 13–28 weeks) (177-180).

Human clinical trials conducted to date, including the ATLAS-2M, FLAIR, and LATTE-2 studies, suggest and support use of combined HIV therapy of cabotegravir and rilpivirine as LA-ARTs (175, 181-182) For example, a randomized, multicentre phase 3b study (ATLAS-2M) conducted in 13 different countries including Canada, South Korea, USA and South Africa, revealed that the combination of cabotegravir plus rilpivirine long-acting therapy at an interval of 2 months was adequate to maintain viral suppression (HIV-1 RNA ≥50 copies per mL) (175). Adverse events recorded in these clinical trials included pain at the injection site for at most 7 days, and this was reported by 88% of study participants in FLAIR (175). In LATTE-2 trial 84% study participants had mild injection site reactions, whereas 15% experienced moderate injection site reactions (182). Additionally, there are several other candidates of LA-ARTs which are still in different stages of clinical trials including islatravir (a first-in-class nucleoside reverse transcriptase translocation inhibitor) (178, 183), and lenacapavir (GS-6207), a capsid inhibitor (178). The method of delivery for islatravir, will be by way of a year-long implant in the body of the HIV-infected individual (178).

Indeed, the newly-approved long-acting ARTs, are an attractive HIV-1 treatment option and a promising development which has the potential to change the outlook HIV treatment and management by improving HIV-1 medication adherence and reduce the pill burden which patients had to endure daily and, in many cases, amid social stigmatization. LA-ARTs are also a significant step towards achieving HIV-1 remission, a state where HIV-infected can control viremia below undetectable levels even in the absence of ART (184).

1.4.4. Use of ARTs for Pre-Exposure Prophylaxis (PrEP), Post-Exposure Prophylaxis (PEP), and TasP, and the significance of U=U.

Pre-Exposure Prophylaxis (PrEP), which is the use of antiretroviral drugs by individuals at high risk of contracting HIV-1, specifically prevent HIV acquisition and transmission. PrEP

was first approved by FDA in 2012 as Truvada, an oral combinatorial single-pill of emtricitabine (200 mg)/tenofovir disoproxil fumarate (300 mg) taken on a daily basis (185-186). Truvada use is supported by several randomized placebo-controlled human clinical trials. For example, the iPrEx Study involving 2,499 participants from several countries including Brazil, Ecuador, Peru, South Africa, Thailand and USA, reported for the first time that PrEP reduced HIV-1 acquisition by 44% relative to placebo over a period of ~2.8 years (187). A follow-up study showed that PrEP significantly decreased HIV-1 incidence by up to 99% (188). The difference in efficacy of Truvada-based PrEP in these two studies was attributed to the drug concentrations and adherence levels. In the iPrEx, drug concentration was found to be low, specifically in cryopreserved PBMCs and blood plasma. The efficacy of PrEP was high in study participants who had better adherence to the medication compared to those with lower adherence (188).

Subsequent studies would further support prophylactic efficacy and safety of PrEP, in particular, emtricitabine/tenofovir (TFV) disoproxil fumarate (189-190). For example, the TDF2 study conducted in Botswana, involving 1,200 heterosexual men and women who took oral Truvada once per day resulted, in 62% PrEP efficacy (189). Emtricitabine (F)/tenofovir alafenamide is now also prescribed after been shown in a clinical study to confer 90% HIV-1 incidence reduction in study participants, and most importantly, in juxtaposition with emtricitabine/tenofovir (TFV) disoproxil fumarate, its efficacy was achieved with low bone and renal risks (191-192).

There is also emerging evidence that HIV drugs can be used as injectable PrEP. For example, a recent clinical study (HPTN 076), indicate that as a long-acting therapy, rilpivirine can effectively achieve suppression of viremia in 8-week dosing intervals at concentration of 1200 mg (193-194). In 2021, the US FDA approved a long-acting injectable PrEP medication called Apretude, which is an extended-release formulation of a potent integrase inhibitor, cabotegravir (600 mg) to be administered every 2 months, into gluteal muscle, for individuals weighing > 35kg (195). This approval followed a randomized clinical trial that compared the efficacy of long-acting injectable cabotegravir every 2 months with a daily oral pill of tenofovir disoproxil fumarate–emtricitabine (TDF–FTC) as PrEP medications, with former displaying superior prophylactic antiviral activity (196). The ability of PrEP to protect against HIV-1 infection is directly associated with adherence to medication prescriptions (197).

On the other hand, Post-Exposure Prophylaxis (PEP), whose efficacy has been confirmed in several studies (198-202), refers to the use of ARVs by persons who have been exposed to HIV infection risk. PEP is an essential part of the HIV-1 prevention strategy and has been shown to reduce HIV acquisition by 81% in a case-control study where participants (health workers) were taking zidovudine as a post-exposure medication following percutaneous exposure with HIV-positive blood (200). However, to achieve efficacy, PEP must be taken early, i.e., immediately after HIV risk exposure — ideally within 2 hours (the earliest) and no later than 72 hours over a course of 4 weeks (200, 201). PEP is given to adults as combined, fixed-dose 3-drug pill of tenofovir (300 mg), and emtricitabine (200 mg) with dolutegravir (50mg) or raltegravir (400 mg) (200, 204).

Indeed, the use of anti-retroviral medication continues to be explored under different approaches, all with an intention to prevent, treat and manage HIV and AIDS. One of the approaches is TasP (treatment as prevention). TasP is a biomedical HIV preventative intervention that involves the use of antiretrovirals in HIV-positive individuals irrespective of their CD4+ cell count to diminish chances of HIV transmission (205-206). HIV-1 transmission is associated with high viral load, as such reducing plasma viremia, precisely to undetectable levels, helps to avert transmission of the viral pathogen between persons (207-208). For example, a clinical study (HPTN 052) previously showed that antiretroviral therapy (ART) reduced genetically-associated viral transmission by 96% among HIV-serodiscordant couples (209-210). Prevention of mother-to-child transmission (PMTCT) which target maternal transmission is another example where TasP has been demonstrated by reducing perinatal HIV infections (211-212). For example, a double-blind placebo-controlled study evaluating the efficacy and safety of orally-administered ART (zidovudine: 100 mg, 5 times a day) in preventing perinatal HIV infections demonstrated that AZT lowered vertical transmission by 67.5% (211). Another study, the HIVNET 012 randomized trial showed that nevirapine decreased mother-to-child transmission by approximately 47% when juxtaposed with AZT (212). Taken together, these results demonstrate that the use of HIV ART in TasP can contribute immensely towards the battle of ending HIV/AIDS.

In fact, it is now considered commonplace that a person who consistently takes and adheres to antiretroviral medication should eventually attain a state of viral suppression, defined by Centre for Disease Control (CDC) as an HIV concentration below 200 copies per milliliter in the blood (213). Furthermore, HIV viremia can further be suppressed

beyond detection (≥50 copies per milliliter), which simply means the viral load cannot be detected by currently available viral load standard tests. This is what is commonly referred to as undetectable viral load. There is also overwhelming evidence as demonstrated by several studies such as the HPTN 052 clinical trial, PARTNER, and the Opposites Attract study that an HIV-infected individual with undetectable viral load does not transmit HIV (190, 210, 214) — hence the concept Undetectable = Untransmittable (U=U). Globally, there is a scientific consensus that people enrolled in an effective ART and are durably suppressed will not sexually transmit HIV. This is, indeed one of benefits of TasP in clinical practice, and it is significant in reducing HIV stigmatization. It is clear that TasP may contribute to an HIV-free generation, in addition to reducing AIDS-related morbidity and mortality rates.

1.4.5. HIV 90-90-90 and 95-95-95 strategies

In 2016, in an admittedly ambitious effort to end AIDS epidemic, the United Nations, through its arm UNAIDS, coined and committed countries to what was known as 90–90–90 strategy (215). The strategy sought to ensure that by the year 2020, 90% of all people living with HIV (PLWH) know their status; 90% of people diagnosed with HIV are reliably accessing ART; and 90% of people who are receiving ART are virally suppressed (215). The strategy was moderately successful, because the countries individually and collectively put in tremendous work in the quest to achieve the set targets. Similarly, the HIV cascade, 95–95–95 strategy, aims to ensure that 95% of individuals living with HIV know their HIV status; 95% of people tested positive for HIV infection receive sustainable ART and 95% of those enrolled in ART have suppressed viremia by the year 2030 (216).

The 90–90–90 strategy progressed well but the world did not meet the 2020 targets. At the end of 2019, UNAIDS statistics for HIV cascade of care and treatment indicated that globally, 81% of people living with HIV knew their status while 67% of individuals diagnosed with HIV infection enrolled in ART. Data collected further showed that the global efforts would not meet the 90% target in terms of viral suppression attainment by people receiving ART, as only 59% of people who were enrolled in ART were virally suppressed, though this was reflective of a 44% gain in terms of percentages of virally-suppressed people between 2015 and 2019 (217). Global UNAIDS update 2021 indicates that there

has been a slight improvement in the 90–90–90 strategy since the 2019, with the latest HIV testing and treatment cascade data showing that 84% PLHWH know their status, 73% PLHWH have access to ART while 66% are virally suppressed (218). These results indicate that, while the 90-90-90 targets were not achieved, there was however no obvious loss in progress since 2020 due to the COVID-19 pandemic.

To this end and to hit the 95–95–95 strategy target, UNAIDS, through the Fast Track approach, aims to accelerate action towards ending HIV by 2030 by evoking a paradigm shift that will see the world adopt more effective management approaches. For example, in order to fast track efforts on HIV treatment delivery, the world will reduce over-reliance on medically trained staff and facility-based treatment and adopt a more flexible treatment delivery approach such as community-based and rights-based treatment delivery approach including offering treatment immediately after a positive test (216). UNAIDS will aim to reassess current approaches of testing options with a deliberate intention to widen their scope by including community-based testing, self-and home-based testing, and event- and location-based testing. In this Fast track approach, countries will also intensify combination prevention through initiatives such as high coverage of key populations and pre-exposure prophylaxis for specific populations (216).

1.4.6. The impact of ART in improving management of HIV across the world, and in sub-Saharan Africa in particular

Antiretroviral therapy (ART) has significantly improved HIV/AIDS management across the world, particularly in Sub-Saharan Africa, a region that is disproportionately affected by the epidemic. ART roll-out which started slowly was scaled up and its accessibility exponentially increased. For instance, the number of HIV-infected people accessing reliable ART shot-up to 28.2 million as of June 2021, rising from 7.8 million in 2010 (1). The scaling up of ART has yielded enormous gains, which are reflected by the number of lives saved from AIDS-related deaths. Globally, since 2010, HIV ART is roughly calculated to have prevented approximately 16.6 million AIDS-associated deaths, while reducing AIDS-linked mortality rates by 47% (218).

Such gains have been made possible by collective efforts and support from various stakeholders including President’s Emergency Plan for AIDS Relief (PEPFAR) which is

reported to have assisted over 20 countries to attain the control of HIV/AIDS global epidemic (219-220). For example, through PEPFAR aid, many countries have reached the 90–90–90 HIV cascade of care targets. By the end of September 2021, 18.9 million people were benefitting from PEPFAR-aided ART (220). Additionally, PEPFAR, through its financial aid, has helped prevent vertical transmission, ensuring that around 2.8 million children born to HIV-infected mothers are born HIV free (as of September 2021) (220). Since its creation in 2003, PEPFAR is reported to have contributed over $100 billion to fight HIV/AIDS, with the United States of America's (USA) government being the largest contributor (218). For example, USA's contribution to the Global Fund to Fight AIDS was $10.8 billion in the fiscal year 2021, rising from $2.2 billion in the fiscal year 2004 — funding that supports several life-saving HIV treatment and prevention strategies (219-220).

Thus, using a combination HIV prevention approaches such TasP strategy including PMTCT, PreP, PEP, and coupled with early ART, the world and Sub-Saharan Africa in particular, have not only incredibly averted a catastrophic incidence of AIDS-related deaths but also significantly reduced global HIV transmission. Latest UNAIDS data suggests that globally HIV incidences have reduced by 31% since 2010, while East and Southern Africa recorded a 28% decline in HIV new infections in the last two decades. AIDS-related mortality rate also fell by 44% in East and Southern Africa between 2010 and 2020 (1, 213). Some African countries such as Botswana, Namibia and Eswatini have even exceeded the 2020 UNAIDS 90-90-90 targets. Botswana for example made remarkable progress in achieving UNAIDS 90-90-90 targets — with the latest data indicating that 91% of PLHWH in the country know their HIV status, 95% of them are on ART and 98% of people on treatment in the Botswana have a suppressed viral load (218). Even though there has been great progress in the region, there are considerable variations between countries, with others such as Madagascar and Mauritius still lagging behind, with approximately a score card of 25% in PLHWH who know their HIV status — evidence that gaps and challenges still exist (218).

1.4.7. Limitations of antiretroviral therapy (ART)

The introduction of cART as the clinically-accepted and certified medication for HIV & AIDS is conspicuously a great success. While cART has advanced to a point that it can

suppress HIV viremia to undetectable levels and expand life by decades, an HIV cure is hampered by the lifelong presence in HIV reservoir-containing cells which include T-helper cells (CD4+ T cells) but also macrophages, dendritic cells, and others (74-76, 221-222). These reservoir cells bear an integrated viral DNA that can reactivate at any time to produce new virus and infect new host cells and are not targeted by existing cART (74, 215-216). The half-life of this viral reservoir during sustained cART is approximately 44 months, which would require 60-70 or more years to achieve full elimination. As a result, people living with HIV (PLWH) must maintain a lifelong cART treatment regimen (74, 223-225).

The life-long treatment also has its own implications and adversities including the potential of long-term drug-induced toxicities, increased risk of chronic inflammation, and other non-AIDS comorbidities (226-227). For example, long-term use of some drugs like protease inhibitors is associated with metabolic disorders like dyslipidemia (228-229). Additionally, as the population of PLWH ages, comorbidities such as renal disorders, cancers, and cardiovascular disease, among others, are occurring at higher rates than in people aging without HIV (230). This is because although current cART can inhibit viral replication below limits of detection, they do not block low levels of viral protein expression from already infected cells. Moreover, the existence of immune and pharmacologically-privileged sites may allow for persistent, low level viral replication from the reservoir even in patients adhering to cART (231-233).

Viral resistance to ARVs also continues to be a global concern, particularly in areas with restricted access to multiple classes of ARVs (234-235). Generally, what amplifies and exacerbates this HIV-1 drug resistance is the nature of viral gene transcription. HIV-1 Reverse Transcriptase in executing its enzymatic function of facilitating the conversion of the viral RNA to cDNA is prone to errors, largely because it lacks the 3’-5’ exonucleolytic proofreading mechanism. The errors in the reverse transcription process could result in significant mutations that allow the virus to adapt and escape immune-system responses and antiretroviral treatment pressures. As shown and discussed above, viral resistance has been recorded and reported in several classes of HIV antiviral drugs (138-140, 147-148, 154-156, 158, 174). Finally, HIV stigma is also associated with lower treatment adherence, depression, and anxiety in PLWH (236-237).

1.5. HIV latency and Cure strategies

1.5.1. Types of HIV latency

HIV latency and the consequent viral reservoir remain a major obstacle to an HIV cure. Two types of viral latency are known to exist: pre-integration and post-integration latency (238). The pre-integration latency results from an extrachromosomal HIV-1 DNA that comes from the incoming virus in the cellular protoplasm — which over time will deteriorate and decay (239-240). Latency under this circumstance may be established when the infected, quiescent T-cell is restimulated before the pre-integration complex fully decays, thereby leading to its resurgent integration into the host DNA, viral replication and infection of other cells (239-241).

However, the more-characterized form of viral latency is post-integration latency which is primarily responsible for establishment of the latent reservoir and occurs when an activated T cell containing integrated viral DNA enters a quiescent state (74-76,238). In this state, the host's latently infected cells ($CD4^+$ T cells) containing the incorporated replication-competent viral genome is both transcriptionally-inert and immunologically-invisible to cellular immune-response mechanisms for clearance and cannot be targeted by ARVs (74-76). Thus, this phenomenon is one of the major contributors to viral persistence even in the face of the combined retroviral therapy that is able to suppress viraemia to undetectable levels (74-76). As discussed above, activated CD4+ T cells differentiate into various subsets of memory T cells such as Central Memory T cells, transitional memory T cells, effector memory T cells and terminally differentiated T cells, follicular helper T cells and regulatory T cells (72, 73), which carry an incorporated, inducible, replication-competent HIV-DNA, with the Central Memory T cells responsible for the large part of the latent reservoir.

The latent reservoir is made up of intact proviruses and non-intact proviruses/ 'defective' proviruses (242-245) as shown in **Fig 1.5**. More than 90% of the integrated proviruses in the pool of latently infected cells are defective, they feature harmful genetic mutations and deletions and as such do not encode replication-competent, infectious viruses (246-247). Only a subset of HIV-1 proviruses, which are referred to as intact proviruses, can be transcribed, spliced correctly and actually produce replication-competent, infectious

viruses (242-245). Whereas defective HIV proviruses are biologically-relevant in that they can trigger immune responses, inducible intact proviruses are clinically-significant because of their ability to make infectious virion particles that can cause viral rebound upon ART cessation (242-248).

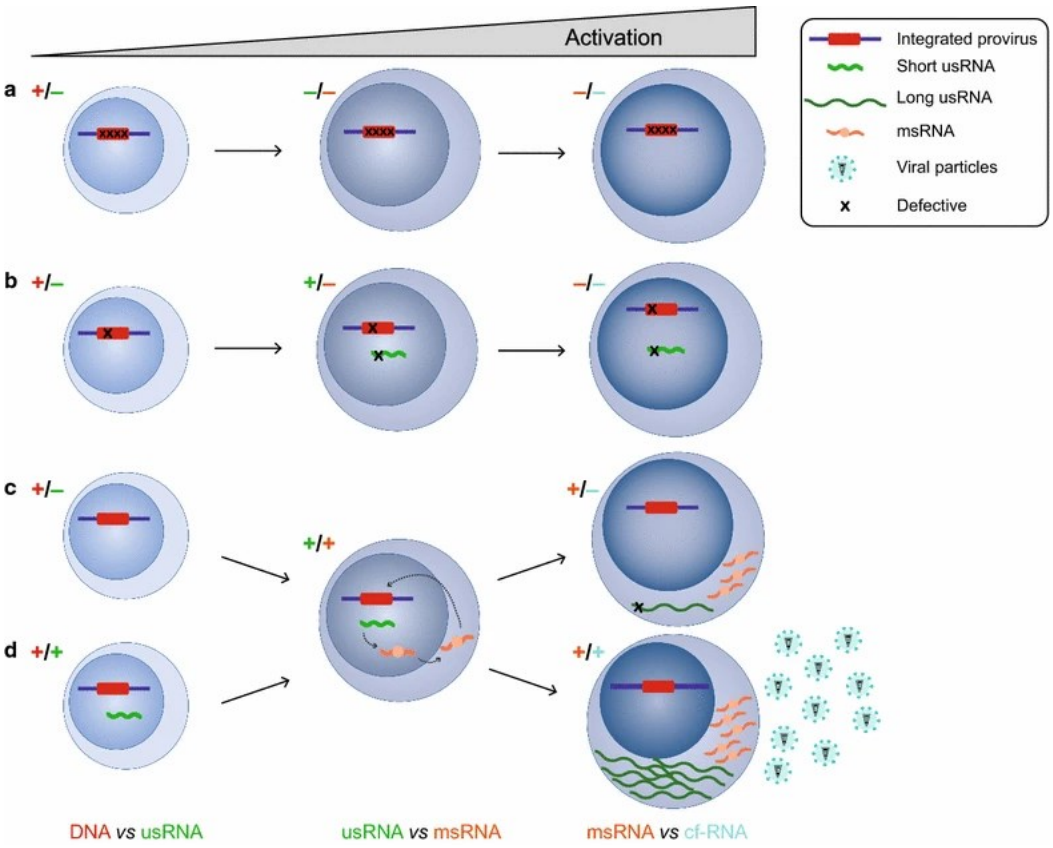

Figure 1.5: Types of HIV proviruses that makes up the reservoir and their transcriptional profiles upon activation;
(adapted from Plantin, et al., (2018) (245))

In addition to comprising intact and defective proviruses, the HIV reservoir is characterized by heterogeneity (249) and varying size between individuals, and it can be found in different sites in the body including the lymph nodes, latently infected, testes, brain, and follicular dendritic cells (250-254).

There are significant challenges in identifying these latently infected cells and/or reservoir. Lack of biomarkers to identify the rare cells that contain the latent HIV-1 reservoir

represents one of the most challenging hindrances to finding accurate ways of eradicating HIV-1 viral persistence. Previously, Descours *et al*., (2017) proposed that CD32a is a biomarker of latent HIV-infected CD4 T-cell which is highly associated with inducible replication-competent proviruses (255). In this study, *in vitro* screening showed that there are a total of 103 differentially upregulated genes, including 16 genes that encode transmembrane proteins in latently infected cells. *FCGR2A* gene, encoding for Fc-gamma receptor FcγR-IIa (CD32a) was the mostly activated and expressed gene (255). Apparently, the researchers showed that a subpopulation of CD4 T cells (0.012%) from ART-enrolled HIV-infected donors with undetectable viremia, preferentially expressed CD32a and enriched HIV DNA, harboring inducible replication-competent proviruses (with a median of 0.56 HIV DNA per cell) (255). However, several studies would later present what apparently became a corrigendum to the finding that CD32a is a biomarker of latent HIV-infected CD4 T-cell (256-260). For example, Badia *et al*., presented evidence that CD32a is not necessarily a marker of the HIV-reservoir, but it is rather associated with T-cell activation (260). They showed that CD4+ T cells treated with several stimulants such as IL-2, IL-7, PHA, activated T cells and elicited expression of CD32a in addition to CD69 and HLA-DR, all which are known markers of T-cell activation. Additionally, CD32a-positive or CD32a-negative CD4+ T cells from HIV-infected donors showed no observable or quantifiable variation in inducible HIV provirus (260). Taken together, evidence suggest that a CD32a is not a signature of HIV-1 reservoir (256-260).

Efforts aimed at identifying possible biomarkers of HIV-1 reservoir continues. Recently, there have been reports that suggest that inducible replication-competent proviral-DNA is preferentially enriched in programmed death-1 (PD-1)-expressing cells (261-263). For instance, Banga *et al*, (2016) found out that lymph nodes (LN) PD-1 (+) cells constituted 46% of the total subset of memory CD4 T cells that harboured an integrated, inducible, replication-competent HIV provirus, and indeed 96% of all the memory CD4 T cells that carried infectious virus in the lymph node (262). Other proposed markers reportedly enriched in the latent HIV-reservoir include chemokine receptors CXCR3 and CCR6 (264-266) and the activation marker HLA-DR (Human Leukocyte Antigen-DR isotype) which the MHC class II cell surface receptor (267). However, none of all these putative markers are specific for latent HIV-1 reservoir.

In a quest to understand and characterize the latent reservoir, several techniques have been developed, investigated and evaluated. For example, the tat/rev-induced limiting

dilution assay (TILDA) measures multiply-spliced HIV-1 mRNA transcripts produced after cellular stimulation (245, 268). The limitation of TILDA is that the measurement of the cell-associated HIV-1 mRNA does not discriminate against or exclude defective proviruses (269).

Quantitative Viral Outgrowth Assay (QVOA), is another technique that has been used to explore and investigate HIV-1 provirus HIV-1(270-271). It is culture-based assay that is able to quantify reservoir size and inducible replication-competent virus, albeit with a disadvantage of requiring a large number of latently-infected cells, (269-272). Hence, the development and introduction of differentiation QVOA (dQVOA), an improved, more accurate version of (QVOA that has the ability to differentiate effector memory CD4+ T cells (273). In the contrary, the Intact Proviral DNA assay (IPDA) represents a more accurate method to measure and characterize HIV-1 viral reservoir, in addition to being highly scalable; it competently distinguishes between defective and intact proviruses (269, 274).

However, all these assays have a fundamental limitation of not being able to pass the test of the ability and capability to analyze HIV-1 latency at a single-cell level and resolution — away from bulky pool of latently infected cells (269). This is against the backdrop of cell-to-cell variations of latently-infected cells upon stimulation, the heterogeneity of the HIV-1 viral reservoir, its paucity and low frequency in patients (269). To this end, methods and techniques, such as high-resolution single-cell RNA sequencing (scRNA-seq), used in tandem with epigenome and proteome-evaluating technologies including Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-seq) and the assay for transposase-accessible chromatin using sequencing (scATAC-seq), have been developed and are being leveraged to characterize HIV-1 latency at a single-cell level, and is already showing cell-specific differences in as far as HIV-1 latency modulation is concerned (269, 275-276). That said, mechanisms underlying HIV-1 latency modulation remains an attractive area for research interest that need to be and is being continually explored.

1.5.2. Molecular mechanisms of HIV latency

HIV-1 latency is maintained and modulated through several complimentary molecular and/or cellular mechanisms that either directly or indirectly affect viral gene expression.

For example, epigenetic regulation, through molecular mechanisms such as DNA methylation, Histone Deacetylation, and Histone methylation has been shown to silence proviral transcription (277-279). These epigenetic pathways play a role in the modification of the chromatin architecture in the HIV-1 promoter region (5' long terminal repeat (LTR)) leading to the suppression of viral gene transcription and thus viral protein expression. Histone acetylation and deacetylation are important for gene regulation as they ensure the 'opening' and 'closing' of the chromatin structure for gene transcription and expression respectively. Proviral Histone Deacetylation occurs when Histone Deacetylases (HDAC1 and HDAC2) are recruited to the HIV-1 LTR promoter in the process promoting the formation of heterochromatin structure which keeps the HIV-1 LTR away from transcription machineries and this promotes long-term viral gene repression. HDACs recruitment and the resulting viral gene silencing is mediated by several transcriptional repressors such as Sp1, COUP-TF, late SV40 factor (LSF) and YY-1 (278-284).

DNA methylation, usually occurring in or around CpG-rich regions, refers to the addition of the methyl group on the cytosine base — a DNA modification that effectively remodel the chromatin structure. Thus, the methylation of the HIV-1 LTR promoter within the host's genomic material of the infected CD4+ T-cells is associated with gene silencing and induction of viral latency (279, 285-286). Histone methylation also modulates gene expression and/or silencing. Depending on the site of methylation, this epigenetic modification can either activate or repress gene transcription. For example, the methylation of H3K4 stimulate gene transcription whereas histone H3 lysine 9 (H3K9) around the HIV promoter region silence viral transcription (287-288).

There are also cellular signalling factors that modulate viral latency, these include the cell size and cell cycle progress in the four stages of the cell cycle, the site of proviral integration and cell activation state (223, 289-291). Furthermore, HIV latency at cellular level may also be perpetuated by the inadequate expression/unavailability and/or weak sequestration of critical host transcription factors such as P-TEFb, NF-κB, and Protein Kinase C, which are important in cell activation and induction of transcription (223, 292-294). Essentially, when an activated provirus-containing cell is unable to have adequate access to these transcription factors, it might revert to its quiescent (resting) state and thus establish and maintain viral latency (292-294).

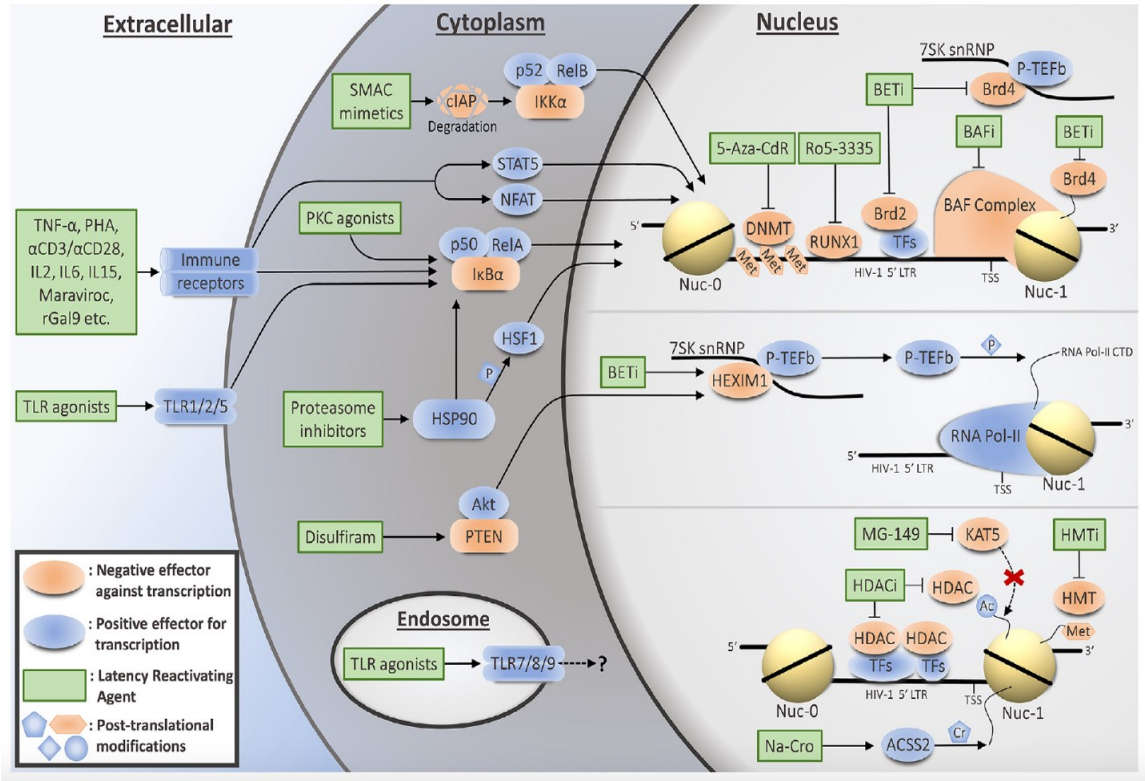

Figure 1.6: Representative signaling pathways that regulate HIV transcription and latency at the LTR;
(adapted from Abner & Jordan., (2019) (295))

HIV gene silencing may also result from viral factors that impact the process of gene transcription. For example, the repression of Tat, the transactivator protein which acts as the viral elongation factor promotes the establishment of HIV latency (26, 296-298). As explained earlier on, upon the termination of transcription, that would have been initiated when NF-κB binds to the HIV-1 LTR, Tat recruits the host factor P-TEFb which then phosphorylates the carboxy-terminal domain (CTD) of the paused RNAP II complex to restarts and elongates viral transcription (26, 296-299). Sometimes, these host transcription factors that interact with Tat (e.g., P-TEFb) are inadequately expressed, a situation that result in poor recruitment of Tat or its suppression, leading to the initiation and maintenance of HIV-1 latency. Also, the stochastic fluctuations in Tat play a role in gene expression and silencing (300-302) (**Fig 1.6**)

1.5.3. Current cure strategies and their challenges

To date, after decades of HIV-directed intensive research there is still no cure for HIV/AIDS. Studies have shown that despite the highly effective antiretroviral therapy, HIV cure remains elusive due to viral persistence in latently infected cells (mainly CD4+ T cells) that contain an integrated viral DNA or provirus that is capable of rebounding upon treatment cessation (74-75, 221-222). It is now well-understood that ART cannot target the HIV proviral-containing resting cells. Several cure strategies are now being pursued toward ending HIV-1 (**Fig.1.7**).

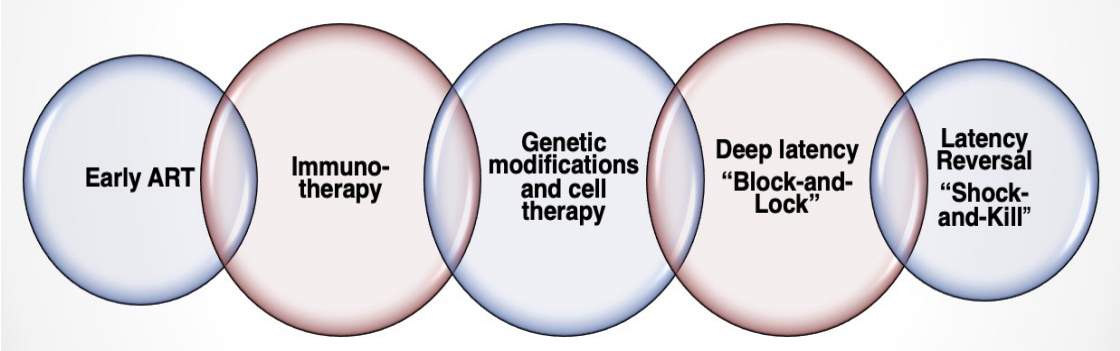

Figure 1.7: Current HIV-1 cure therapies being pursued;
(adapted from Ndung'u, *et al*., (2019) (303))

Broadly, there exist two forms of HIV cure: the functional and the sterilizing cure (304). HIV-1 functional cure refers to a state of long-term remission whereby the viral load would be silenced and reduced to undetectable levels in the absence of an antiviral regimen. On the other hand, sterilizing cure is aimed at absolutely eradicating the replication competent HIV-1 in the human body regardless of the state of the cell — whether activated, dormant and/or reactivated (304).

Presently, few cases of successful sterilizing cure have been reported and include the Berlin Patient (305-306) and the London Patient (307), with potentially others. The sterilizing cure in these cases was achieved using chemotherapy to wipe out the infected immune system of the patients. Mechanistically, this procedure's curative properties hinges on the discarding of the whole HIV-seropositive immune system and then replacing it using allogeneic Haematopoietic Stem Cell Transplantation (HSCT) from the donor whose chemokine co-receptor gene features a notable CCR5Δ32 mutation. Individuals who have received HSCT CCR5Δ32-based therapeutic intervention become almost

impervious to the CCR5-tropic virion particles infection (305-307). Although HSCT represents hope and inspiration towards finding cure, two challenges limit it. Firstly, the procedure is risky; it is associated with high death rates (40-55%) and unsuccessfulness, thus limiting its scalability and applicability to the millions of HIV-1 infected people. Secondly, donors whose chemokine co-receptor genes feature the CCR5Δ32-homozygous deletion are rare, in addition to not being readily available for donation (303, 308-313). However, these individual cases do demonstrate that eradicative cell therapy cure is possible. In some instances, viral rebound might be recorded even after allogeneic haematopoietic stem cell transplantation (314-315).

Most importantly, the success of HSCT CCR5Δ32-based sterilizing cure inspires hope that gene modification could be an attractive curative strategy. A candidate for the gene therapy could be the haematopoietic stem cells. For example, with emerging gene-engineering technologies, the CCR5 gene could be edited to make it unable to function as an HIV coreceptor (316-317). Genetic editing could also target the genomic regions that feature integrated HIV-1 DNA. The techniques for targeted genetic engineering include zinc finger nuclease (ZFN) technology (316), whose therapeutic potential has been tested in CCR5 gene of an HIV-infected T cell, at the C-C motif and shown in several studies to significantly reduce and control HIV-1 viremia in patients, in addition to helping preserve and repopulate the human T cells in the body (305, 319). This ZFN technique uses an artificially-created sequence-specific endonuclease, containing a zinc finger protein (ZFP) blended within the *Fok*I restriction enzyme's cleavage domain, to effectively cut the DNA at the selected targets (318). HIV-associated CRISPR/Cas9 genetic editing, which cleaves the incorporated HIV-1 DNA in target cells has also been investigated and shown to both deactivate the provirus and reduce viral load in the blood and tissues non-human primates (320-321). The question of safe delivery methods of the edited products still remains a major hurdle, and CCR5Δ32 genetic edits may still be vulnerable to the CXCR4-vriron particles. Additionally, there are concerns that gene-editing approaches may suffer yet-to-be known off-target effects, notwithstanding that none of the current gene-editing studies and approaches have prevented viral rebound (303, 314-318, 323).

HIV-seropositive individuals who naturally control the viraemia, otherwise known as 'Elite controllers (ECs)' are hypothetically viewed as potential leads to how functional cure could be achieved or work (324-326). The properties of the viral reservoir in ECs and typical HIV-infected individuals on ART have been studied and continue to be investigated.

Several studies in analyzing the viral reservoir size using IPDA and/or full-length HIV-1 proviral DNA sequencing have revealed that the level or frequency of total and intact HIV-1 DNA in ECs, when juxtaposed with that of ART-enrolled HIV-infected individuals, is approximately 20-fold lower (327-329). ECs viral reservoirs are also characterized by high frequency of clonal expansion as compared to ART-taking individuals (328-330). For example, when analyzing 99 env sequences amplified from CD4+ T cells of a cohort of predominantly spontaneous HIV controllers, Boritz *et al*., (2016) found out that the frequency of clonally-expanded env was within a range of 32.7% — 96.8% (330), consistent with finding Jiang, et al., (2020), who also observed that level of clonal expansion of the replication-competent provirus was higher in ECs than durably-suppressed ART-taking individuals (328). Furthermore, natural viremia controllers have also been shown to contain lower HIV DNA and cell-associated viral RNA than ART-suppressed HIV-1 subjects (330-331). For instance, a study by Hatano *et al.*, (2013) demonstrated that viremic controllers had a median of 496 copies of rectal HIV DNA/10^6 CD4+ T cells and 19 copies of HIV-RNA/10^6 CD4+ T cells, while in the contrary, virally-suppressed ART-enrolled patients were shown to have significantly higher levels on both viral DNA and cell-associated HIV RNA, scoring 6116 copies/10^6 CD4+ T cells and 1625 copies/10^6 CD4+ T cells, respectively (332).

The control of viraemia in HIV-1 ECs has also been attributed to their expression of protective Human Leukocyte Antigen (HLA) alleles that elicit potent HIV-specific cytotoxic T lymphocytes (CD8+ T cell) that kills the intracellular viral pathogen (333-334). Thus, there has been a substantial interest in finding a functional cure that is dependent on or linked to CD8+ T cell immune responses. Attempts to genetically modify stem cells, CD8+ T cells and NK cells to elicit HIV-specific chimeric antigen receptor (CAR) responses have been tried even at clinical stage, (335-342).

Previously, a study representing the first generation of CAR, used chimeric T-cell receptor (CD4ζ)-modified HIV-specific CD4+ and CD8+ T cells connected to CD3 T-cell receptor intracellular signaling chain and reported that they antagonize viral replication and eliminated virus-expressing cells in *vitro* (337). In this study, comprising of 40 HIV-infected people, rectal biopsy HIV DNA analysis indicated a significant reduction of gut-viral DNA in CD4ζ-engineered cohort (–0.50 log; P = .007) (337). Additionally, quantitative HIV coculture analysis also showed that patients who got the CD4ζ-modified HIV-specific CD4+ and CD8+ T cells showed a decrease in cultured virus from baseline (> 0.5 log

decrease) than those who did not receive genetically-engineered CAR-T-cells (337). Both second and third generation CAR T cells, bearing the costimulatory 4-1BB domain and (CD3ζ-CD28-41BB) respectively, have been shown to yield optimized results in viral replication inhibition and killing of HIV-infected cells (338-339). For instance, second generation CAR T cells bearing the costimulatory 4-1BB domain were shown to be 50-fold superior in viral replication inhibition potency *in vitro* than the first generation of CAR T cells (338); comparable potency was also demonstrated *in vivo*.

Engineered CAR T cells known as TRUCKs (T cells redirected for universal cytokine-mediated killing) can directly recognize, target and kill virus-infected cells even without the help of antigen-presenting MHC, thus decreasing chances of viral escape upon activation (340-343). Hence HIV-specific CAR therapeutic strategy is now being tried in combination with latency reversing agents to maximize cell stimulation, viral surveillance and elimination of latently-infected cells — so as to optimize the efforts of reducing or clearing the reservoir, an outcome that is yet to be achieved clinically. CAR-modified T cells are thought to present an enhanced 'kill' element which is lacking so far in the 'shock-and-kill' therapeutic approach described below (341).

Early ART administration is another approach to reduce or eliminate viral replication. This has been tried on non-human primates and found to curtail viral reservoir establishment such that viral rebound is hindered, though this feat is yet to be achieved clinically on human patients (344). Generally, studies show that starting HIV-1 cART on early stages of infection not only reduces latent reservoir size but restricts viral load in the host, consequently preventing immune system deterioration which usually leads to progression of the infection to a deadly clinical stage (AIDS) (345-346). Early ART is also associated with limited viral mutations and diversity resulting in reduced HIV-1 quasispecies and heterogeneity (344). The so-called Post-Treatment Controllers (PTCs), such as 'Visconti Cohort', who have been able to attain virological control even after ART discontinuation have been associated with early ART enrolment (346). Once off therapy, PTCs in this 'Visconti Cohort' study had cell-associated HIV-1 DNA levels (median 1.71 log copies/10^6 PBMC) that was comparable to those of ECs, and significantly lower than the non-controllers who initiated ART during HIV chronic infection stage (346). Another example is the so-called 'Mississippi Baby' who was born to a newly-diagnosed mother with HIV and initiated ART immediately after birth. After being lost to medical follow-up and ART discontinuation, this individual was subsequently relocated and found to have

gone for 27 months without detectable virus after ART discontinuation, before HIV viraemia rebounded (347). Thus, early ART remains an attractive approach towards attaining a state of HIV-1 long-term remission, though its mechanisms are yet to be fully understood.

Vaccine antibody-based interventions are also being actively explored (348-352). However, successful vaccine efforts face major hurdles such as overcoming exceptional HIV-1 diversity and variability resulting from antibody-driven viral mutations. Furthermore, extensively glycosylated Env and the ability of the virus to also escape immune responses represent one of the challenges of vaccine development (353-354). To date, there is no licensed vaccine that successfully controls HIV (353-354). Although the ALVAC-HIV (vCP1521) and AIDSVAX B/E vaccines in RV144 trial showed an efficacy that stood at 31.2% as shown and measured by the avidity of the antibody (IgG) and antigen (Env), but could not reduce HIV viral loads, hence ALVAC-HIV (vCP1521) and AIDSVAX B/E vaccines activity was deemed a modest HIV-1 infection prevention (355-357).

Deep latency, commonly called ‘Block-and-Lock’ has been suggested as a possible HIV-1 functional cure strategy. This relatively nascent strategy is hinged on the supposition that the heterochromatin structure harbouring HIV-1promoter in latently infected cells can be transcriptionally and irreversibly silenced using deep latency agents to achieve HIV-1 remission (358). Given the fact that some integrated replication-competent HIV-1 DNA cannot be pharmacologically-stimulated and/or reversed from their latency state, researchers are looking into finding molecules that could target infected resting cells with an aim to ‘block’ viral transcription and ensure that the cells perpetually remain ‘locked’ into a state of deep latency, in the absence of ART (358-359), without affecting overall T cell function. The ‘Block-and-Lock’ strategies obviously have to target various viral proteins, host factors, repressors and epigenetic modifications that have a role in viral transcription and it’s silencing thereof. These proteins and/or factors include NF-κB, P-TEFb, NF-AT and Tat just to mention but a few (292-294, 296-299, 358-359) Various candidates for the induction of deep latency have been reported and are under investigation. For example, didehydro-cortistatin A (dCA) has been identified as a potent Tat inhibitor in both *in vitro* and *ex vivo* models that blocked Tat-driven HIV-1 transcription and reactivation induced by latency reversing agents (LRAs) as part of “shock-and-kill” described below (360-361). dCA also modestly delayed viral rebound in mice compared to control mice. However, didehydro-cortistatin A is yet to be tested clinically in humans

(361). Another Block-and-Lock' strategy is bromodomain-containing protein 4 (BRD4) modulators. A molecule named ZL0580 has been found to be block HIV transcription, interfering with Tat -P-TEFb interaction, by selectively targeting BD1 domain of BRD4 (362). ZL0580 was found to hinder Tat's known roles of transactivation and viral transcription elongation (362).

There has also been interest in pre-integration inhibitors such as LEDGINs, which could be potential block-and-lock agents. LEDGINs are pre-integration inhibitors that abrogate and impair the interaction of HIV integrase enzyme and LEDGF/p75 cellular transcription cofactor, which is necessary for directing viral integration to the preferred actively transcribing genes (integration sites) (363-366). Thus, LEDGIN inhibitors modify and disrupt the viral integration, forcing it to occur at regions that are not easy to transcribe (363-366). For example, Vranckx *et al*, (2016) in their investigation demonstrated that LEDGF/p75-depletion promotes HIV-1 transcriptional quiescence, while simultaneously decreasing the ability of proviruses to reverse latency. Using an *ex vivo* model of primary CD4+ T cells, the group showed that LEDGINs prevent viral integration, promote the establishment of latent HIV-1 reservoir, and hamper viral reactivation in those HIV proviral-containing cells (363).

A question remains as to whether this 'Block-and-Lock' cure approach would reach and target each and every latently infected cell and/or viral reservoir with the host reservoir sites. Also, it is not clear yet if 'Block-and-Lock' approaches would overcome viral rebound in the HIV-infected individuals. For example, dCA only delays but does not eliminate viral rebound in humanized mice model to date. Furthermore, 'Block-and-Lock' agents are yet to be tested in clinical trials, as such their efficacies and safety remain unknown (358-366).

Finally, Latency Reversal also called 'Shock-and-Kill' strategy, is hypothetically thought to help attack and eradicate latent viral reservoir by exposing the infected resting cells to molecules called latency reversal agents (LRAs) (366-368). LRAs stimulate proviral reactivation in latent reservoirs resulting in the provirus expression for clearance by cellular immunity, in the presence of ART regimen to prevent the inevitable reseeding of the reservoir. This strategy, which is also the basis of this thesis, is discussed in detail below.

1.6. Towards an HIV Cure: The “Shock-and-Kill” approach

HIV-1 persists in cellular reservoirs despite ART regimen which suppresses viremia to undetectable levels (74-76). The latent reservoir, which is capable of reactivating and producing infectious virus upon ART interruption, is a major barrier towards both an HIV-1 functional and sterilizing cure. The ‘Shock-and-Kill’ therapeutic approach entails using latency reversal agents (LRAs) to induce HIV-1 provirus expression (**Fig. 1.8**). HIV reactivation, coupled with immunotherapy support (366), could render infected cells “visible” to the host immune system, whereas co-administration of cART would prevent further seeding of viral reservoirs (367-369).

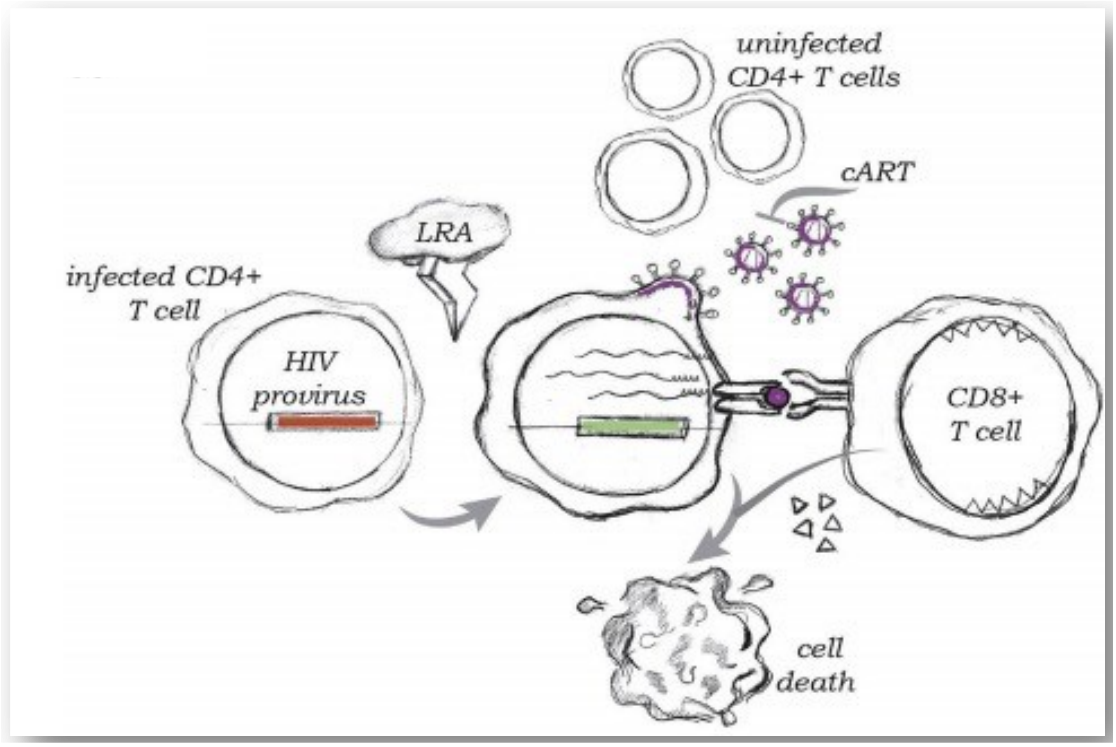

Figure 1.8: HIV latency reversal therapeutic strategy (shock-and-kill);
(adapted from Andersen et al., (2018) (370))

Several ‘Shock-and-Kill’ agents are reported to target different molecular pathways and factors that are associated with HIV-1 latency establishment and modulation with an ultimate aim to reverse and eliminate the latent reservoir. Inevitably, LRAs have to target mechanisms and/or cellular factors involved in both host and viral transcription activation, transcription elongation and epigenetic modulation (discussed above) if they are to

effectively induce provirus transcription (277-300, 371). Numerous first generation LRAs from various functional classes have been investigated and the non-exhaustive list includes Protein kinase C (PKC) activators, DNA methyltransferase inhibitors (DMTis), BET bromodomain Inhibitors, Histone methyltransferase inhibitors (HMTis) and Histone deacetylase inhibitors (HDACis) (372-374).

HDAC inhibitors are LRAs which prevent gene-silencing induced by histone deacetylation but instead trigger histone acetylation at different points of HIV integration (369). Histone deacetylation by histone deacetylases (HDACs) promotes the formation of transcription-repressing heterochromatin – which impedes the transcription machinery from reaching the integrated HIV-1 promoter LTR within the host genomic material. This results in the establishment and maintenance of HIV-1 latency (372-381). Examples of HDAC inhibitors include Panobinostat, which has been reported to target class I, II and IV HDACs and induce latent viral expression (366-367). For instance, Tsai, et al., (2016) investigated the latency reversal activities of Panobinostat both *ex vivo* using $CD4^+$ T cells from aviremic HIV-infected patients taking suppressive ART, and *in vivo* using 13 humanized BLT mice infected with HIV-$1_{JR\text{-}CSF}$. Stimulation of latently-infected $CD4^+$ T cells with 20 nM panobinostat resulted in 6.2-fold increase in levels of cell-associated HIV RNA in one of patients, relative to untreated cells; while the other two recorded 3.6- and 3.7-fold increase (376). However, treatment of the virally-suppressed humanized mice with panobinostat (2 mg/kg) showed no observable statistically significant difference in viral RNA expressions between treated and untreated animals, neither was there reduction of the latent reservoir (376).

Vorinostat, also called Suberanilohydroxamic acid (SAHA), is another intensively-studied HDAC inhibitor which activates the HIV promoter and induces hyperacetylation (378-380). SAHA was subjected to a clinical trial and found to induce HIV-1 Gag RNA expression in some patients. However subsequent studies showed that the induction of this HIV-1 Gag RNA expression was not linked to HIV-1 provirus transcription in latently infected T-cells but a result of host-gene readthrough transcriptional mechanisms. At any rate Vorinostat, only induced a minimal percentage (0.079%) of proviruses in a clinical trial (379-381), further indicating that though pharmacological-based ‘shock-and-kill’ research has been intentionally skewed towards non-T-cells activating LRAs, evidence show that such molecules do not target and induce adequate transcription of proviruses, as such they wouldn’t significantly reduce or clear the viral reservoir.

Romidepsin, a bicyclic peptide produced by *Chromobacterium violaceum* is another potent HDACi that has been shown to induce the reactivation of HIV-1 from latency. Wei, *et al*., (2014), conducted a study in which HIV-1 latency reversal activities of HDACis, vironostat, panobinostat and romidepsin were assessed and compared (382). Romidepsin displayed superior efficacy in reactivating HIV provirus *in vitro* (EC_{50}=4.5 nM), compared to panobinostat (EC_{50}=10 nM) and vironostat (EC_{50}=3,950 nM) (382). The *ex vivo* data generated using CD4 T cells of aviremic ART-enrolled HIV-infected donors corroborated the potency observed *in vitro*, as Romidepsin at 40nM elicited 6-fold increase in cell-associated HIV RNA expression relative to the vehicle-control; whereas vironostat induced at most 3-fold increase in viral RNA levels (382). Romidepsin was further shown to reactivate HIV-1 *in vivo*, in a clinical trial that enrolled 6 HIV-1 infected study participants receiving the suppressive ART and with undetectable viremia. Administration of 5 mg/m^2 of Romidepsin to the patients stimulated as high as 5.0-fold-increase of copies of cell-associated un-spliced HIV-1 RNA relative to the baseline (383). Plasma viral RNA quantification showed a sharp rise from the <20 copies/mL at baseline to higher levels in the range of 46–103 copies/mL in 5 out of 6 aviremic individuals who received Romidepsin (383).

Recently, Gunst, *et al*., (2022) in a randomized multicentre-controlled study that enrolled 60 participants, assessed the impact of coadministration of romidepsin as an LRA and broadly-neutralizing antibody (bNAb) 3BNC117 on establishment of HIV-1 latency in patients initiating first-line INSTI-based ART regimens. Study participants were divided into 4 different cohorts exposed to the following conditions: 1.) ART treatment 2.) ART + 3BNC117 (30 mg/kg), 3.) ART+ romidepsin or 4.) ART+ 3BNC117 + romidepsin. Cell asssocited-HIV-1 mRNA and p24 measurements indicated that the co-administration of ART + 3BNC117 had superior effect than ART treatment alone, in eradicating HIV^{mRNA+} and HIV^{p24+} CD4 T cells, especially central memory CD4 T cells (384). 80% of the patients who were sensitive to bNAb-3BNC117 prior to ART treatment, as indicated by PhenoSense and HIV env sequencing, were able to control viremia better than those who were not sensitive to 3BNC117 (384). Furthermore, romidepsin was shown to significantly reverse HIV latency in the groups exposed to this potent LRA. This recent data, that used romidepsin combined with bNAbs in HIV-infected patients, suggest that scientists may be on a track to reduce viral reservoirs when the right LRA regimen is paired with good

effector kill agent, further justifying the need to continue to search for more novel LRAs, with better potency.

Prostratin, a non-tumor causing phorbol ester, along with Bryostatin, are some examples of protein kinase C (PKC) activators (385-386). PKC activation by PKC agonists leads to IκB phosphorylation and degradation, an event that enables translocation and/or sequestration of NF-κB to the nucleus and binding to the HIV-1 5' long terminal repeat (LTR) promoter region to induce HIV-1 provirus transcription (387). Prostratin-dependent activation of PKC allows RNA polymerase (Pol) II to load on the HIV LTR promoter to transcribe the viral genes allowing for an upregulation of provirus expression (372, 387-388). Bryostatin, a marine natural product isolated from *Bugula neritina*, modulates HIV-1 latency by way of PKC stimulation and AMP Kinase (AMPK) signaling (373, 389-392). Synthetically-produced derivatives of bryostatin 1 have been shown to possess comparable or greater potency than naturally-available bryostatin in reversing HIV-1 latency *in vitro* (J-Lat 10.6 cell line) when compared to activity of prostratin by 1000-fold (392). Subsequently, Marsden, *et al*., (2017), evaluated the latency reversing activities of the bryostatin analogue (bryolog SUW133) *in vivo* using a humanized BLT mice and primary CD4+ T cells isolated from ART-treated aviremic patients and HIV latency U1 cell line model (393). They demonstrated that the bryostatin-1 analogue is more tolerated and exhibited superior efficacy in virus production compared to a several established LRAs such as Panobinostat, JQ1, bryostatin 1 and vorinostat, reaching nearly one-third of HIV-RNA expression levels occasioned by maximal stimulation using anti-CD3+anti-CD28 antibody (393). Following 24 hours stimulation in humanized mice, SUW133's activated higher levels of CD69 expression (above 80%) in murine splenocytes compared to bryostatin 1 which induced 60% CD69 expression — this induction was dose dependent. SUW133-induced proviral expression is also accompanied by or causes apoptosis of the virus-expressing cells (393), evidence that the bryostatin derivatives could help achieve the "kill" component which has been lacking in the "shock-and-kill" approach.

Bromodomain and extra-terminal [BET] bromodomain inhibitors [BETis] are another class of LRAs. BET bromodomain proteins competitively inhibit Tat-SEC attachment, thus blocking Tat transactivation. BET bromodomain inhibitors such as JQ1 displace and expel Brd4 from the HIV-1 promoter to allow Tat-transactivator protein to bind to the promoter and elongate the RNA polymerase II-dependent viral transcription (394-396).

DNA methyltransferase inhibitors (DMTis) represents another class of latency reversal agents that purge HIV-1 latent reservoirs. DNA methylation has been shown to contribute to viral gene silencing *ex vivo* using HIV-positive PBMCs as well as in other cell-line models (279, 285-286). Methylation inhibition therefore alters the chromatin structure to open the genes for transcription. 5-aza-29-deoxycytidine (Aza-CdR) is an example of a DNA methyltransferase inhibitor that blocks the cytosine methylation, but instead promotes histone acetylation induction to allow for gene expression (397). Aza-CdR has been reported to repudiate the recruitment of Methyl-CpG Binding Domain Protein 2 (MBD2) and HDAC2, all which are known to be epigenetic modulators that repress gene transcription (397).

Histone methyltransferase inhibitors (HMTis) have also been reported as putative latency reversal agents targeting the HIV-1 reservoir. Examples of HMTIs that have been studied include Chaetocin and BIX-01294. Chaetocin specifically antagonizes the SUV39H1-regulated methylation of histone H3 lysine 9 (H3K9) around the HIV LTR promoter region to overcome viral gene repression (398-399). Studies show that Chaetocin induced 25-fold of provirus expression in Jurkat T cell lines, and worthy noting is that this induction of HIV-1 latency reversal was bereft of global T-cell activation and significant cytotoxicity (398). Chaetocin was also reported to synergize with other LRAs of different functional classes on $CD4^+$ resting T cells isolated from HIV-1 patients who are on ART (399).

Another Histone methyltransferase inhibitor, a diazepin-quinazolin-amine derivative called BIX-01294 binds to active (catalytic) site of histone lysine methyltransferase (HKMT) G9α and effectively blocks its enzymatic function, and reportedly yielded 80% HIV-1 latency reversal ex *vivo* (399).

So far, first-generation LRAs have been overall unsuccessful in reducing the viral reservoir *in vivo* as indicated above, in addition to other limitations as it would be discussed hereinunder. Toward improving shock-and-kill two approaches are being pursued: one is combination LRAs and the other approach is second-generation LRAs, all which are discussed below.

The continued exploration of the shock-and-kill strategy in HIV-1 cure research has birthed the second generation of LRAs that include Toll-like receptors (TLRs) agonists, IL15 super-agonists, and SMAC mimetics and Programmed cell death protein-1 (PD-1)

inhibitors (400-415) **(Fig 1.6)**. TLR agonists have been shown to reactivate HIV-1 in latently infected cells, in addition to boosting innate immune responses. Toll-like receptors (TLRs) are expressed by various immune cells including macrophages, dendritic cells, epithelial and endothelial cells, natural killer cells, T and B cells, thus the proviral reactivation is specific for and dependent on the TLR-expressing cells (401). For example, Equils, *et al*., (2003) showed that treatment of human THP-1 monocytic cell line with TLR2, TLR4, and TLR9 ligands (STF, LPS, and CpG DNA respectively), result in HIV-LTR *trans*-activation as quantified by luciferase activity (404). Furthermore, in this study, spleen cells isolated from HIV-1 transgenic mice and treated with TLR2, TLR4, and TLR9 agonists elicited and upregulated HIV-1 replication in a dose-dependent pattern as measured by the supernatant p24 Ag production *ex vivo* (404). In a different study, TLR-1/2 agonist Pam3CSK4 was also shown to reactivate latent HIV-1 in central memory $CD4^+$ T cells, without inducing T cell activation and/or proliferation (405).

Whereas *in vitro* and *ex vivo* data demonstrated efficacy of TLR agonists in reversing HIV-1 latency, clinical studies are yet to yield any significant results. For instance, Toll-like receptor-3 agonist, Poly-ICLC which had shown significant potency in activating HIV-1 replication in preclinical studies, though well-tolerated, could not reproduce the same efficacy when evaluated in a randomized, double-blinded human clinical trial of virally suppressed HIV-infected people taking ART (406). No significant difference in total HIV-1 DNA in CD4+ T-cells was observed in placebo-controlled group and the Poly-ICLC-treated group, much as there was no significant enhancement of innate immune responses noticed (397).

IL-15 superagonist N-803 (formerly ‘ALT-803’), another example of second generation of LRAs, has also been reported as a latency modulating agent that reactivates proviral HIV-1 expression in a primary cell model, in addition to priming the viral reservoir for CD8+ T-cell recognition (407). However, subsequent *in vivo* studies revealed that N-803 could not reverse latency in aviremic ART-taking, SHIV-infected macaques; there was minimal activation of latently-infected CD4+ cells; no significant difference in plasma viral load and viral reservoir size between animals treated with N-803 and those that were untreated (408).

Smac mimetics also represent second generation of LRAs that target and induce latent HIV-1, through non-canonical NF-κB pathways. These drugs are derivatives of the highly

conserved binding motif of Smac (second mitochondria-derived activator of caspases), an apoptosis modulating mitochondrial protein (409) **(Fig. 1.6).** Smac mimetic compounds have been shown to activate HIV-1 reservoir in a dose-dependent manner, both *in vitro* (J-Lat 10.6 cells) and *ex vivo* models, using CD4+ T cells isolated from virally suppressed ART-enrolled HIV-infected donors – and their latency reversal activities synergized with HDACis (Panobinostat and Vorinostat) (409-410). Subsequently, a proof-of-concept, preclinical study by Pache, *et al*., (2020) evaluated the latency reversal efficacies of a modified Smac mimetic (Ciapavir) using humanized mouse (411). The researcher demonstrated that bivalent Ciapavir potently reactivate latent HIV-1 *in vivo*, in the liver, bone marrow thymus. The study showed that Ciapavir's latency reversal activities were comparable to phorbol 12-myristate 13-acetate (PMA)/ionomycin positive control, as it matched its efficacy by >65%. The latency reversal activity of Ciapavir occurred with negligible T cell activation and the drug was well tolerated by the animal (411).

Programmed cell death protein-1 (PD-1) inhibitors are another example of latency reversing agents, that target latently infected cells and induce HIV proviral expression and virus production (412-415). HIV-1 reservoir of HIV-infected people on suppressive ART is reported to preferentially express PD-1 (261-263), making this immune checkpoint protein an attractive therapeutic target for purging the latent reservoir. For example, a study by Uldrick, *et al*., (2022) involving 32 ART-enrolled HIV and cancer patients, showed that Pembrolizumab, an antibody that blocks PD-1, reversed HIV latency *in vivo* (412). The intravenous administration of Pembrolizumab to this cohort induced 1.32-fold increase (median) in unspliced HIV RNA and 1.65-fold increase in plasma viral RNA. The researchers also observed an increase in the frequency of $CD4^+$ T cells harbouring inducible HIV proviral DNA relative to the baseline after 6 cycles, as measured by *tat/rev* limiting dilution assay (412). Anti-PD-1 therapy is also reported to yield better results in purging the viral reservoir when combined with other interventions, including T cell activation (414). Another study, that used an *ex vivo* model of PBMCs isolated from aviremic HIV-infected individuals on ART, reported that co-administration of pembrolizumab and bryostatin resulted in 36-fold increase in HIV production relative to the isotype control (415). Taken together, PD-1 blockade activates HIV latency reversal and viral production, especially when combined with other therapeutic interventions.

In summary, while substantial progress has been made towards finding an HIV-1 eradicative cure within the realms of 'shock-and-kill' approaches, substantial challenges

exist. As indicated above, LRAs tested to date in humans have shown limited clinical success and are not effective enough to activate and/or eliminate all cellular reservoirs. Current LRAs are not fully effective due to a variety of reasons that include extensive toxicity, poor efficacy, inconsistent viral reactivation, and/or insufficient engagement of cellular "kill" mechanisms (295, 374, 416). Past *ex vivo* studies and clinical trials have also demonstrated limitations in some of the currently available LRAs. In some cases, there is evidence of viral reactivation but no reductions in viral reservoirs (379, 417-423). For example, Sarabia, *et al*., (2021), using primary T_{CM} Cell Model of Latency, demonstrated that several potent and clinically-applicable LRAs including HDACi (SAHA and MS-275), SMAC mimetic (AZD-5582), PKC agonists (Ingenol 3,20-dibenzoate), though inducing viral expression from different HIV strains, their stimulation is inadequate as it only induces less than 50% of the intact provirus (417). The latent HIV-1 could not be depleted even with maximal stimulation using TCR engagement. The same pattern was shown in another study, where the PKC activator, Bryostatin-1, could not substantially decrease HIV-1 reservoir *ex vivo* using $CD4^+$ T cells isolated from durably-suppressed ART-enrolled patients, even when combined with the potent PMA/ionomycin (418). Non-specific cell activation is also a potential issue for LRAs, as there is need to target only those cells which are latently infected, as opposed to global activation of T-cells (422). Also, some LRAs are less potent after multiple dosing, which is a further concern (422-426).

Thus, new LRAs, or combinations of LRAs, are needed to circumvent these issues. Notably, LRA combinations may have synergistic effects in HIV latency reversal. Synergism, usually calculated through bliss independence model, refers to correlated action of two or more compounds so that the combined action is greater than the sum of each acting separately (426-427). LRAs with similar mechanisms of action tend to exhibit additive effects when applied in combination, while LRAs with different mechanisms of action tend to exhibit synergistic effects (426-427).

Several recent studies showed that multiple drug combinations of LRAs resulted in significantly enhanced latency reversal compared to individual drug treatments — in a way justifying that combinatorial approach might be the next step toward viable shock-and kill therapies (425, 427). For example, Jiang *et al*. described that the PKC activator ingenol-3-angelate (PEP005) and the BET bromodomain inhibitor JQ1, which each alone stimulated an ~25-fold increase in HIV transcription in vitro, could induce a 250-fold

increase when applied in combination (425). Similar results are also reported using primary CD4 T cells from HIV-infected donors (425, 427). These observations suggest that optimized LRA combinations may promote broader latency reversal at lower concentrations, thereby maximizing virus reactivation while limiting drug toxicities and other off-target effects. Indeed, discovery of novel LRAs that can enhance the efficacy of current LRAs is much needed, especially those acting through unique mechanisms.

This thesis is anchored on the 'Shock-and-Kill' pharmacological approach. The search for clinically-relevant molecules that could purge HIV-1 latent reservoir has been extended to natural products — both from marine sources and medicinal plants. As such, this thesis seeks to search, document and mechanistically characterize novel latency modulating agents derived from natural sources including African Traditional Medicine, that could inform combinatorial therapeutic efforts. Hence the next chapter reviews some of the reported anti-HIV compounds and latency modulators of botanical origin in the African continent, which is the hot spot of HIV & AIDS epidemic.

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Chapter 2.

African Traditional Medicines used for HIV-1 Management: A Review

2.1. Abstract

Combination antiretroviral therapy (cART) is the gold standard in the treatment of HIV-1 and it suppresses viremia to undetectable levels. However, the inability of cART to eradicate the quiescent CD4+ T cells containing an HIV-1 proviral genome highlights the need for both inhibitory agents of HIV (i.e., similar to current ARTs) and latency reversing agents. Compounds derived from natural sources such as medicinal plants remain a very promising reservoir for finding more antivirals and HIV-1 latency modulators. This is more relevant in low- and middle-income countries (LMICs) who are still face issues of ART affordability and accessibility. As such in the absence of ART, a productive use of medicinal plants could be helpful as an alternative especially in Africa where Traditional and Complementary Medicines (TCMs) is widely used and relied upon. In this chapter we review the scientifically-documented TCMs of botanical origin used in Africa as adjuvants to ART and/or for HIV/AIDS management and are evidently supported by biomedical data. I discuss nineteen (19) African medicinal plants which have been shown to be HIV-1 antivirals or latency modulators, in a way, corroborating that TCMs within 'reverse pharmacology' model remains a rich avenue for novel HIV antivirals and latency modulators.

Key words: HIV; cART; traditional and complementary medicines (TCMs); reverse-pharmacology; antivirals; latency modulators

2.2. Introduction

Whereas combination antiretroviral therapy (cART) has been a great success, it still has limitations. Some of the limitations of cART, as discussed in chapter 1, include drug resistance, high toxicities and most importantly failure to eradicate the latently infected cells to clear the viral reservoir, hence HIV-1 patients have to remain enrolled in a life-long treatment. In low- and middle-income countries (LMICs), affordability and accessibility of cART continue to remain limited and, as of 2021, 9.5 million of 37.7 million people living with HIV (PLWH) still lack reliable access to cART (1-3). Africa, a continent that accounts for two thirds of PLWH globally, continues to remain the global epicenter of the HIV pandemic, where roughly 1 in every 25 adults (3.6%) are HIV-positive (3). In this region, 5.8 million (22.9%) of the 25.3 million PLWH, lack reliable access to cART (3).

To this end, improvements to cART remain warranted, particularly toward the goals of improving accessibility to PLWH in many LMIC, targeting latent HIV, and reducing long-term viral antigen production. Toward these goals, traditional and complementary medicines (TCM), frequently in the form of medicinal plants and indeed marine products, may serve as an additional source of therapeutic leads, particularly in Sub-Saharan Africa where TCM is widely accessed. When proper methodologies are used, TCMs can be extensively documented for their traditional collection, preparation, administration, and use by PLWH while ensuring that local communities benefit from documentation and disclosure of plant use (4). TCM documentation, when properly performed, can also generate testable hypotheses to be assessed in biomedical laboratory studies. This approach, frequently referred to as “reverse pharmacology,” relies of detailed documentation of *existing and ongoing* TCM preparation and use and humans, which in turn informs bioassay studies to confirm antiviral properties, isolate active chemical constituents, and elucidate molecular, cellular, and antiviral mechanisms of action (5). Here we review **TCMs of Sub-Saharan Africa with documented use for HIV management to supplement cART and are supported by biomedical data.**

2.3. HIV replication cycle and therapeutic targets

For the purpose of emphasis, and to avoid repetition of chapter 1 contents, this section summarizes the HIV-1 replication cycle and highlights the therapeutic targets thereof —

in which TCM products may be used as adjuvants to ART, and where applicable, as novel antiviral armamentarium. HIV-1 genome is a positive-sense, single-stranded RNA that encodes 9 proteins: The structural proteins gag and envelope (Env); the enzymatic proteins protease, reverse transcriptase (RT), and integrase; the regulatory proteins tat and rev; and the accessory proteins vif, vpr, vpu and nef. HIV particles contain two copies of the RNA genome, along with protease, RT, and integrase enzymes, enclosed within a capsid (viral protein p24) that is further surrounded by the viral envelope. This envelope is comprised of a host-derived lipid bilayer studded with the viral glycoprotein Env. Mature Env features two subunit proteins, a surface, heavily glycosylated cap protein (gp120) and a hydrophobic stem subunit (gp41) (6).

Viral entry into target cells requires binding of gp120 to the main entry receptor CD4, expressed at high levels on T-helper cells but also dendritic cells and macrophages (7,8). Initial binding of gp120 to CD4 triggers a conformational change in Env that allows binding to an additional CCR5 or CXCR4 co-receptors. This co-receptor binding stabilizes virion attachment and allows insertion of the hydrophobic gp41 into the host cell membrane, the formation of a fusion pore, and delivery of the viral capsid into the host cell (6). Following entry, RT converts the single-stranded RNA genome into double-stranded cDNA, which is then transported to the nucleus and integrated into the host genome by integrase. Integration preferentially occurs in actively-transcribing genes (9,10) and from here, viral transcription, virion assembly, and progeny release proceeds. However, in rare cases, proviruses enter a largely transcriptionally silent, latent state, resulting in formation of the latent reservoir, although this reservoir can reactivate at any time to produce infectious virus (1, 11). **TCMs have been identified that target one or more of these processes, providing the opportunity to supplement existing cART and/or provide stopgap measures in the absence of or use of established cART.**

2.4. Therapeutic targeting of HIV latency: an avenue to test TCMs within ‘reverse pharmacology’ approach

At a cellular level, many factors control or contribute to HIV latency including cell activation state (12), availability of transcriptional host co-factors such as NF-κB and/or

P-TEFb (13), and histone deacetylase-mediated epigenetic silencing (14). Stimulation of latently-infected cells with cytokines, protein kinase C signaling, or histone deacetylation can reverse latency and stimulate virus production (15-20). Consequently, an experimental therapeutic approach to identify and eliminate these latently-infected cells involve inhibition of the epigenetic factors responsible for transcriptional repression at the LTR and/or stimulation of other proviral cellular pathways such as PKC activation (21-23). For example, HDAC inhibitors are being pursued clinically due to their ability to trigger HIV transcription, potentially leading to identification and elimination of latency infected cells (24).

This approach, as mentioned in Chapter 1 is frequently referred to as “shock-and-kill” or “kick-and-kill”, involves reactivation of latent viruses by latency reversing agents (LRAs) combined with immune-boosting strategies (21-22). In this approach, reactivated viruses would be expected to produce viral proteins, allowing the immune system to recognize and eliminate infected cells. This would be conducted in the presence of cART to prevent further infection of new cells and new reservoir seeding (25). HDAC inhibitors such as vorinostat or Panobinostat relieve repressive heterochromatin at the HIV LTR, increasing NF-kB and P-TEFb activity (26,27). PKC agonists such as bryostatin or prostratin activate the PKC signaling pathway, ultimately leading to increases in NF-kB activity in the nucleus (15-16, 28). Other LRAs act by increasing the amount of free P-TEFb available for viral transcription (29-31) or induce NF-kB signaling through nonclassical, PKC-independent pathways (32) and TLR7 agonists (33). However, despite robust *in vitro* results, this approach has not yet succeeded in clinical trials (34). For example, although HDAC inhibitors have been shown to transiently increase viral transcription in patients, no resulting decrease in the size of the latent reservoir has been observed (35,36). **However, some TCMs have been identified that reverse HIV latency, indicating a reverse pharmacology approach to potentially improve these methods.**

2.5. Traditional and Complementary Medicines as a potential repository for HIV therapies

TCM are well established worldwide to treat a variety of diseases and conditions. For example, individuals throughout the world regularly consult traditional health practitioners (THPs) to manage HIV, tuberculosis (TB), malaria, and other infectious diseases. It is a knowledge system and a health care model that people across the globe frequently access. Statistically, over 80% of people worldwide use traditional medicine (TM) as part of their primary health care (37-39). In Sub-Saharan Africa, this percentage ranges between 70% and 90% (40,41). For example, studies show that Africans who have acquired HIV-1 infection have consulted THPs even after enrolling in cART programme, thereby raising the concept known as medical pluralism (41-45). While medical pluralism contributes to the delay of patients' progression through the HIV cascade-of-care, including delays in accessing care and treatment; mixing of care and treatment options; interruptions of care and treatment (44), the embedded use of TCM in many communities and particularly those in LMIC means that it will continue to be a major source of health care which will not disappear simply by wishing it away. To this end, World Health Organization (WHO) Traditional Medicine Strategy 2014–2023 aims to assist countries to harness the potential contribution of TM to health, wellness and people-centred health care. It also seeks to achieve TM regulation in order to promote its safe and effective use with an overall goal of integrating TM products, practitioners, and practice into health care systems wherever appropriate (46). Countries in Africa share the WHO vision of improving and developing traditional medicine. For example, in 1996 Botswana set a national vision (Vision 2016) which aimed to bring TM within an appropriate regulatory framework and make TM accessible in cooperation with modern medical facilities (47). These goals also enable the exploration, documentation, and validation of indigenous knowledge which can additionally benefit humanity, for example in the form of improved value chains of medicinal plants, validation of local medicinal knowledge, and building of community and ecological resilience.

Here we review medicinal plants found in Africa that are reported to be used traditionally to target HIV, its symptoms, and/or AIDS-related opportunistic diseases. We particularly

focus on those where TCM documentation includes a clear definition of study site, defined type of study, and clear description of research tools and techniques to obtain information including bioassays. We also prioritized studies that meet at least one of the following criteria:

1. Isolation of the active chemical compound (s) guided by a traditional use report, as we want to highlight established links between TM and biomedical research.
2. Elucidation or hints towards a possible molecular mechanism of the plant-derived antivirals
3. Reports of cell-based bioactivity assays and/or *in-vitro* enzyme assays. However, for studies where only enzyme assays were performed, due to the potential for false positives (whose results can vary considerably in a viral, cellular or *in vivo* context), we included only those plants whose activity was similar or within 10-fold of a control antiretroviral therapy drug.
4. *in vivo* data, for example from animals or nonhuman primates
5. Biomedical measures in individuals who use a traditional medicine for HIV/AIDS management
6. Any patient-oriented studies (observational or clinical) regarding the respective anti-HIV plant-derived traditional medicines

2.6. Medicinal plants traditionally used for HIV/AIDS management in Africa

2.6.1. *Azadirachta indica* A. Juss. (Meliaceae)

Azadirachta indica A. Juss. has been shown to possess *in-vitro* immunomodulatory properties against HIV-induced CD4+ T-cell stimulation (48). *A. indica* belongs to the Meliaceae family and it is commonly known as neem. Its ethnopharmacological value include treatment include arthritic health conditions, diabetes, skin diseases, with its aqueous leave extract having been reported to have activity against Dengue virus type-2 (49,50). In Nigeria and across Africa, A. *indica* is used in folk herbal medicine to treat bacterial, fungal and protozoal diseases including malaria. Previously, human clinical trials of A. *indica*'s leave extract, blocked the attack of lymphocytes by HIV-1 and improved the CD4+ cell count of HIV-1 subjects in an *in-vitro* model (51,52). A past study, showed

that *Azadirachta indica* inhibited both HIV-1 fusion into the host cell and the activity of HIV-1 reverse transcriptase *in vitro*, in addition to downregulating activation markers (CD69 and CD38) in an *ex vivo* model using phytohemagglutinin A-induced peripheral blood mononuclear cells (PBMCs) (53).

In this study, Olwenyi *et al.* (2021) obtained the fresh leaves of *A. indica* from the Mabira Central Forest Reserve in Uganda and prepared an ethanol-water plant extract. The researchers recruited both HIV-1 patients (who were enrolled in ART and were durably suppressed) and healthy donor's, of which they obtained Peripheral blood mononuclear cells (PBMCs). As part of the enrollment process, researchers administered a questionnaire that aimed to capture data around the use of plant-based medicine by the recruited donors.

Study patient's PBMCs were subjected to HIV-1 infection screening which was carried out using Determine ™ HIV-1/2 AG/AB COMBO and Chembio HIV 1/2 STAT-PAK® Assay and subsequently, had their HIV-1 viremia quantified using Chembio HIV 1/2 STAT-PAK® Assay). PBMCs) from HIV-1 negative donors were exposed to Staphylococcus enterotoxin B (SEB) and then treated with *A. indica* ethanol-water mixtures (0.5 µg/mL). The positive control for this experiment was 1µg/mL SEB and the negative control was cyclosporine (0.015 µg/mL). In addition, as part of the treatment conditions, PBMCs were exposed to 0.5 µg/mL *A. indica* + 1µg/mL SEB. Cells were stained to assess the expression of cell surface markers (CD38 and CD69); and in addition, Programmed cell death protein 1 (PD-1), Human Leukocyte Antigen, interferon gamma (IFN γ), Tim-3, IL-2 and CD4 + T. These were evaluated using BD FACS Canto II methods, wherein flow cytometry data was generated and analyzed.

A. indica's ethanol-water mixture was also evaluated for its ability to downregulate CD4+ T cell activation & depletion in HIV-1 positive donors exposed to multiple concentrations of test agent extracts ranging from 0.0 to 0.5 µg/mL, in addition to the assessment of its immunomodulatory activities, specifically HIV specific CD4+ T cell, and interferon gamma and IL-2 (48).

Olwenyi *et al.* (2021) found out that *A. indica* downregulated CD4+ T cell activation in SEB-stimulated PBMCs in a concentration-dependent manner, measured within the range of 0.0 to 0.5 µg/mL. Precisely, PBMCs (from HIV-1 negative study participants) treated

with positive controls alone yielded 1.470 (0.400–2.870) % CD4 + CD38 + HLA-DR+ cells and this value decreased to 0.640 (0.079–1.210) % when cells were treated with 0.5 µg/mL *A. indica* + 1µg/mL SEB (p = 0.027).

Also, in a manner that mirrored the results of the positive control (cyclosporine A), 0.5 µg/mL *A. indica* + 1µg/mL SEB downregulated the expression of CD69 (the early activation marker) in HIV negative individuals, dropping the expression value from 11.95% (positive control) to 8.12% (*A. indica* + SEB). This trend was replicated in HIV-1 positive individuals undergoing cART, whereat both the positive control and *A. indica* showed 1.4 folds CD69-expression reduction.

When tested for its ability to inhibit CD4+ T cell exhaustion, *A. indica* extract gave out results that are comparable to the positive control (cyclosporine A) as it reduced Tim-3 and PD-1 expressions by 1.7 – 4.2 folds, in both healthy donors and HIV infected participants. *A. indica* had no effect on gag-specific CD4+ T-cell cytokine responses as measured by IFN-γ and IL-2 expressions.

2.6.2. *Bridelia micrantha* (Hochst) Baill. (Phyllanthaceae)

Bridelia micrantha grows in Limpopo Province of South Africa and it is part of the Venda folk medicine, locally known as Munzere. This herb has some traditional medicinal use such as treatment of abortifacient, stomach aches and diarrhoea (54). Bessong *et al*. (2006) performed a screen to determine the anti-HIV properties of the water, butanol and acetyl acetate isolates of the crude methanol extracts of *B. micrantha* roots on the enzymatic function of HIV-1 Integrase and RNA-dependent-DNA Polymerization (RDDP) activity of HIV-1 Reverse Transcriptase (RT).

The inhibitory effects of this plant's fractions at concentrations of 1, 10 and 100 µg/ml, were assessed by way of quantification of the incorporation of methyl-3H thymidine triphosphate ([3H] TTP) by RT enzyme using polyA-dT as a template-primer. For the *B. micrantha*'s HIV-1 integrase activity inhibition, 0.1, 1, 10 and 100 µg/ml concentrations were used to evaluate the fractions against the bioactivity of integrase.

Bioassay-guided fractionation and phytochemical screening, using thin layer chromatography (TLC) and spectroscopic analysis were employed an attempt to isolate active ingredients and determine possible phytoconstituents present in *B. micrantha*

extract. Bessong *et al*. (2005) report that of all the fractions tested, n-butanol fraction of *B. micrantha* exhibited the highest inhibition against the RNA-dependent-DNA Polymerization (RDDP) activity of HIV-1 Reverse Transcriptase (RT), with an IC_{50} of 7.3 µg/mL. The water-soluble fractions were not inhibitory to the RDDP function of HIV-1 RT. Acetyl acetate fraction and n-butanol fractions of *B. micrantha* showed 91±3.2% and 98.6±2.1 RT activity inhibition respectively. The IC_{50} of Acetyl acetate fraction was 10.4 µg/mL.

Phytosterols friedelin and ß-sistosterol were found in *B. micrantha* as some of the phytoconstituents but had no activity in both the reverse transcriptase and integrase activities when tested against RNA-dependent-DNA Polymerization (RDDP) activity of HIV-1 Reverse Transcriptase (RT) and HIV-1 Integrase activity (54).

The Acetyl acetate fraction isolated the methanolic extract of *B. micrantha* was inhibitory to RT function and recorded an IC_{50} value of 9.6 µg/mL – this fraction however did not inhibit HIV-1 integrase activity. The subsequent phytochemical screening and analysis revealed that *B. micrantha* contained flavonoids and tannins, but had no alkaloids and saponins. Flavonoids are chemicals that has been profiled as anti-HIV RT compounds (55).

2.6.3. *Cassia abbreviata Oliv. Oliv*. (Leguminosae)

Cassia abbreviata Oliv. has been shown to significantly inhibit of HIV-1c (MJ4) replication in a study conducted by Leteane *et al.* (2012). The researchers noted that *Cassia abbreviata* root and bark is commonly used in Botswana to treat various conditions including the treatment of menstrual pains, uterus and abdominal pains. It is also used as a general blood cleanser, it also improves appetite, used in the treatment of syphilis and gonorrhea and lowered HIV levels in its users. It is locally known as Monepenepe (56).

This plant was identified through an ethnomedical survey that involved in-depth interviewing of 11 traditional healers practicing in North-West District of Botswana on the medicinal plants they use to treat/manage HIV/AIDS. Thus, *Cassia abbreviata Oliv. Oliv* was investigated for inhibitory activities on HIV replication. The methodology for this study included preparation of ethanolic extracts, tannin dereplication, detection of HIV-1c p24 antigen using enzyme-linked immunosorbent assay (ELISA), HIV-1c binding and entry

assay and phytochemical screening and thin layer chromatography (TLC) profile of the medicinal plant ethanolic extracts.

PHA (1 µg/ml)/IL-2 (100 U/ml) activated PBMCs were infected for 3 h with 0.01 M.O.I. HIV-1c (MJ4) in the presence or absence of crude or tannin-free plant extracts at various concentrations. *C. abbreviata* displayed inhibitory activity against HIV-1c (MJ4) p24 antigen production by PBMCs which were activated by PHA (1 µg/ml)/IL-2 (100 U/ml) before and after tannin dereplication. Leteane *et al.* (2012) reports that in this study the root extract of *C. abbreviata* showed an inhibition of 55.1% in the presence of tannins and this inhibition dropped to 38.5 ± 2.1% after tannin removal. The active extract of *Cassia abbreviata* recorded an EC_{50} of 102.7 µg/ml, which was considerable higher than the control, AZT, an antiretroviral medication.

The cytopathic effect (CPE) protection assay, 7 days after infection of PBMCs with HIV-1c (MJ4) revealed that *C. abbreviata* ethanolic tannin-free extract displayed a protective effect by way of blocking cell death to 35.8 ± 1.2% — a figure that matched with the initial *C. abbreviata* extract's inhibition of p24 antigen production under the same conditions (56). This demonstrated that its anti-HIV activity isn't due to cytotoxic effects. In fact, the MTT assay showed that *C. abbreviata* extract was not toxic to PBMCs over a concentration range of 0.0001–1000 µg/ml, a signal for a good therapeutic index.

In terms of mode of action tests through HIV-1c binding and entry assay, the researchers found out that *Cassia abbreviata* tannin-free extracts (root and bark) do not block HIV at binding - or entry stage suggesting a different mechanism of inhibition. In accounting for the possible mechanism of action, Leteane *et al.* (2012) noted other groups that found out that *C. abbreviata* had some inhibitory activity on an enzyme (glucosidase) whose inhibition has been linked to a reduced infectivity of HIV virions resulting from the suppression of the functional glycosylation of HIV gp120. *C. abbreviata* could not block HIV-1 at binding and entry stage (56).

The qualitative phytochemical screening of root extracts of *C. abbreviata* indicated the presence of anthocyanins, anthranoids, anthraquinones, polyphenols and tannins. However, the detailed isolation and structure elucidation of these compounds was reported to be under way.

Subsequent studies that followed by several isolated at 28 chemical compounds from *C. abbreviata* and 6 of them were associated with HIV-1 inhibition into target cells (57, 58). The 6 phytoconstituents were: oleanolic acid, palmitic acid, taxifolin, piceatannol, guibourtinidol-(4α→8)-epiafzelechin, and cassiabrevone which was a novel compound. For example, guibourtinidol-(4α→8)-epiafzelechin recorded an IC_{50} of 42.47 μM, while cassiabrevone's inhibitory activity showed an IC_{50} of 30.96 μM, and targeted the HIV-1 gp120 and CD4 interaction at the entry stage and events (57, 58).

2.6.4. *Cassia sieberiana D.C*. (Leguminosae)

Leteane *et al.* (2012) tested medicinal plants from North-West District of Botswana for anti-HIV bioactivities selected from an ethnobotanical study and discovered that *Cassia sieberiana D.C* ethanolic root extract hinders HIV-1c replication in PBMCs *in vitro*. *C. sieberiana* is otherwise locally known as Mororwe in Botswana. It has a long-standing tradition for being used medicinally to manage a variety of health conditions among others, weight loss, skin rashes, sores all over body, loss of appetite and diarrhoea (56). Following in-depth interviews with the local traditional medicine practitioners (THPs), Leteane *et al.* (2012) embarked on exploration of the inhibitory properties of this suggested traditional herb.

Using HIV-1c (MJ4)-infected PBMCs, the researchers showed that the ethanolic extract of C. *sieberiana* blocks HIV replication by quantifying the viral p24 antigen using enzyme-linked immunosorbent assay (ELISA). *C. sieberiana* bark extract inhibited viral p24 production in PBMCs recording 95.1 ± 3.9% ($P < 0.05$) inhibition before, and 80.1 ± 1.0% ($P < 0.05$) after tannin dereplication (56). The root extract also exhibited significant blockage of viral p24 antigen production in supernatants of PBMCs infected with HIV-1c (MJ4) as 98.1 ± 3.1% and 94.5 ± 1.0% inhibition was achieved in tannin-containing extract and tannin-free extract respectively. The EC_{50} for HIV-1 inhibition by *C. sieberiana* root and bark extracts were reported to be 65.1μg/ml and 85.3μg/ml respectively.

The mechanism for *C. sieberiana* investigated using an HIV-1c binding and entry assay, was found to be inhibition of HIV-1 at binding and entry stage. Tannin-dereplicated root extract of *C. sieberiana* exhibited concentration-dependent blockage of virions-PBMCs binding with an EC_{50} value of 70.2 ± 3.9 μg/ml. The bark extracts bereft of tannins also achieved the same inhibition but with an EC_{50} value 90.8 ± 4.1 μg/ml.

C. sieberiana ethanolic root extracts without tannins, blocked the entry of HIV-1c (MJ4) in PBMCs recording an EC_{50} value of 88.9 ± 2.1 µg/ml whereas tannin-free bark extracts yielded an inhibition of HIV-1c (MJ4) entry with an EC_{50} value of 100.5 ± 4.5 µg/ml (56). The phytochemical characterization of *C. sieberiana* roots extract signaled the presence of polyphenols, anthocyanins, anthraquinones, polyphenols tannins and anthranoids.

2.6.5. Combretum molle R.Br. ex G. Don (Combretaceae)

Combretum molle is an African medicinal plant that is used to treat a variety of health diseases. In Ethiopia, a country situated in the Horn of Africa it is traditionally used to treat ailments such as liver diseases, malaria and tuberculosis. This plant has been shown to possess anti-HIV properties (59). *C. molle* plant extracts prepared using methanol, petroleum ether, chloroform and acetone were tested for HIV-1 and HIV-2 replication inhibition. Asres (2005) reports that the acetone fraction displayed the greatest selective inhibition of HIV-1 replication against MT-4 cells *in vitro*. The structural elucidation of the plant's extract using solid phase extraction (SPE) and spectrometry led to isolation of two triterpene glycosides and two tannins. The two tannins, being and CM-A and puncalagin were responsible for the inhibition HIV-1 in this medicinal plant extract, but showed no inhibition on HIV-2 (59).

In a different study, the methanolic extract of the roots of *Combretum molle* R.Br. ex G. Don (Combretaceae), which is locally called Mugwiti in Venda (South Africa) was found to be inhibitory on the HIV-1 RT ribonuclease H (RNase H) activity with an IC_{50} of 9.7 µg/ml (60). This study was carried out in South Africa where the plant is used by traditional healers to treat abdominal pains, fever and convulsions.

2.6.6. Croton megalobotrys Müll.Arg. (Euphorbiaceae)

C. megalobotrys Müll Arg. locally known as Mukungulu in Northern Botswana, belongs to a family known as Euphorbiaceae. Its ethnomedical use in Northern Botswana include HIV treatment, diarrhoea of HIV patients, loss of appetite, stomach problems (61).

Using a combination of community and laboratory-based approaches Tietjen *et. al* (2018) identified a potent HIV-latency modulating traditional medicinal plant, *Croton megalobotrys Müll Arg.* ("Mukungulu"), used by HIV-patients in Northern Botswana, Africa.

This was part of a 3-step medicinal plant regimen used for HIV/AIDS management in the said district as administered by a traditional health practitioner. Qualitative data was collected using interviews and focus group discussions with study participants (including two HIV-positive traditional medicine users) on medicinal plants to treat HIV/AIDS (61).

Laboratory techniques then followed to generate quantitative data. The researchers used flow cytometry-based reporter cell assays (J-Lat, clone 9.2) to assess the plant extracts for the possible latency reversal activity. *C. megalobotrys Müll Arg.* extracts, the third and last component of the 3-step medicinal plant regimen, exhibited latency reversal activity *in vitro* as it activated latent HIV-1 expression in J-Lat 9.2, a Jurkat-derived clone that contains a transcriptionally-silent HIV provirus. The latency reversal activity of *C. megalobotrys Müll Arg.* was comparable to prostratin, a known LRA, a protein kinase C agonist. “Mukungulu” at a concentration as low as 0.5 µg/mL induced 1.3 ± 0.2% GFP expression in J-Lat 9.2 cells while 3µg/mL Prostratin activated 5.5 ± 0.5%. Apoptosis in Jurkat cell cultures which was measured by annexin V staining showed that cells tolerated “Mukungulu” and prostratin (59). Synergistic experiments suggested *C. megalobotrys* is also a PKC activator as it synergized with multiple LRAs, but not with Prostratin suggesting a similar mechanism. Using open column chromatography, reverse-phase HPLC, NMR spectroscopy and mass spectrometry novel phorbol esters (Namushen 1 and 2) were isolated, elucidated and identified. Interestingly, these new phorbol esters (Namushen 1 and 2) were shown to induce latent HIV-1 expression (61).

C. megalobotrys Müll Arg. extract was also found to inhibit HIV-1NL4.3 replication in a dose-dependent by 45% (the highest) at concentrations as low as 0.05µg/mL, and displayed efficacies similar to licensed ARVs (62). So, this medicinal plant block HIV replication and cause latency reversal at the same time. The explanation to this could be that, as a PKC activator containing compounds similar to PMA and prostratin, it’s probably downregulating CD4 entry receptors and co-receptor used by HIV (CD4, CCR5, CXCR4) as well as inducing NF-kB signaling (62).

2.6.7. *Croton tiglium L.* (Euphorbiaceae)

Croton tiglium, a plant used in the Egyptian traditional medicine was assessed for anti-HIV properties and found to block the HIV-elicited cytopathogenicity and infectivity of HTLV-I-containing MT-4 cells (63). This plant belongs to the *Euphorbiaceae*. Using *C.*

tiglium seeds, El-Mekkawy (2000) prepared MeOH extract and isolated several phorbol diesters using bioassay guided fractionation, a methodological approach that involved mass spectrometry, column chromatography and selective hydrolysis of acyl groups for structural elucidations.

In this study, HIV-1 infected MT-4 cells (TCID50 of 0.001/cell) were exposed to test compounds isolated from the seeds of *C. tiglium alongside controls (without test agents). The IC of test agents were viewed and determined on the 5th day on an optical microscope. The growth rate of the cells was assessed, to determine the* CC_0 *that lowered the* MT-4 cells viability. Compounds were also evaluated for the pro-tumor activation of the protein kinase C (PKC) using a Biotrak PKC enzyme assay system code RPN 77 kit.

C. tiglium seed extract were found to contain Eight (8) phorbol diesters namely: 13-O-Acetylphorbol-20-linoleate, 13-O-Tigloylphorbol-20-linoleate; 12-O-Acetylphorbol-13-tigliate; 12-O-Decanoylphorbol-13-(2-methylbutyrate); 12-O-Tigloylphorbol-13-(2-methylbutyrate); 12-O-Acetylphorbol-13-decanoate; 12-O-(2-Methylbutyroyl)phorbol-13-dodecanoate and 12-O-Tetradecanoylphorbol-13-acetate.

Many of these phorbol diesters were found to have an anti-HIV biological activity. For example,12-O-Tetradecanoylphorbol-13-acetate (TPA) exhibited the strongest HIV-1-induced cytopathic effect (CPE) inhibition with an IC_{100} [complete inhibitory concentration] value of 0.48 ng/ml. This compound also showed the highest activation of PKC, whereat 10 ng/ml achieved maximum activation (96%). The minimum cytotoxic concentration (CC_0) value for 12-O-Tetradecanoylphorbol-13-acetate (TPA) was 31.3 mg/ml. The IC_{100} and CC_0 values of 12-O-Acetylphorbol-13-decanoate were 7.6 ng/ml and 62.5 mg/ml respectively, however this compound could not activate protein kinase C even at high concentrations of 100 ng/ml. 15.6 mg/ml of 13-O-Acetylphorbol-20-linoleate achieved complete inhibition of HIV-elicited cytopathogenicity with CC_0 value of 62.5 mg/ml; while 12-O-Tigloylphorbol-13-(2-methylbutyrate) recorded complete inhibition of CPE at a 31.3 mg/ml (CC_0 62.5 mg/ml) (63).

2.6.8. *Euphorbia granulata* Forssk. (Euphorbiaceae)

Hussein *et. al* (1999) performed an anti-HIV screen of multiple native plants that are used in traditional medicine in Sudan (Africa) and found out *Euphorbia granulata* was

significantly inhibitory to the HIV protease (PR) enzyme, which is involved in a crucial biological process of hydrolysis of the peptide bond in retroviruses. *Euphorbia granulata* Forsk. (Euphorbiaceae) is part of the diverse flora of Sudan and it features prominently in the Sudanese traditional medicine (64).

The researchers used HIV-1 (HTLV-IIIB) infected MT-4 cells to assess the *E. granulata* plant's extract effects on viral replication, including the determination of HIV-induced cytopathic effect (CPE). For the anti-HIV protease biological activity, an HIV-1 PR assay and liquid chromatograph (HPLC) were adopted. Tannin content was also investigated. The leaf extracts of *E. granulata* were prepared using water and methanol, whose yield was 37.1 and 21.0% respectively. The minimum concentration (CC) for appearance of MT-4 cell toxicity as viewed in a microscope was 62.50 µg/ml (64).

E. granulata's leaf water extract showed inhibitory activity on HIV-induced CPE at 62.5 µg/ml, which was the minimum concentration (IC) required for complete inhibition of HIV-1 induced CPE in MT-4 cells by way of microscopic observation. The methanolic extract of *E. granulata*'s yielded 48.5% of HIV-1 PR inhibition while the water extract resulted in a weak PR enzymatic inhibition, 13.1%. Thus, the methanol extract of this herb possesses some *inhibitory properties against HIV protease.*

2.6.9. *Euphorbia usambarica* Pax. (Euphorbiaceae)

Euphorbia usambarica, an ethnomedicine for urogenital, gynecologic and endocrine health disorders in East Africa was found to display potent HIV-1 latency reversal activities *in-vitro* (65, 66,67). The methanolic extract of *E. usambaric* which was subjected to a bioactivity-guided isolation and phytochemical analysis, yielded 15 novel diterpenoids, in addition to the previously discovered 16 compounds. Some of the new compounds included Euphordraculoate C, 4b-Crotignoid K, Usambariphane B, Usambaricinophane E, Isoterracinolides C, whereas 12-O-benzoyl-13-acetoxy-4,20-dideoxyphorbol-4-ene, crotignoid K and isoterracinolide A represented some of the *E. usambaric*'s known chemical compounds (67).

The resulting small molecules from the methanolic extract were investigated for HIV-1 latency reversal properties. To this end, J-Lat assay using the J-Lat 10.6 cell line, which

contain an integrated HIV-1 proviral DNA that lacks nef and env, but GFP instead was used assess viral latency reactivation, by way of quantifying GFP-expression in flowcytometry. This is hinged on the fact that Jurkat 10.6 contain an integrated HIV-1 proviral DNA that lacks nef and env, instead has GFP — thus GFP expression signals the proviral reactivation. Basically, 2.5 X 10^5 J-lat 10.6 cells were stimulated with multiple concentrations of the isolated compounds (100, 10, and 1µM) in replicates and before quantifying their GFP expressions. 1% DMSO was used as the negative control for the assay. For example, 4b-Crotignoid K showed latent HIV-1 induction had an EC_{50} of 0.015 µM and CC_{50} of more than 160µM, in juxtaposition with the stereoisomer, crotignoid K., was a 250-fold increase viral latency reactivation. The compounds induced HIV-1 latency reversal via PKC signaling as their latency reversal could be antagonized by pan-PKC inhibitor GÖ-6983, suggesting that they are PKC activators (67).

2.6.10. *Lobostemon trigonus* (Thunb.) H.Buek (Boraginaceae)

Lobostemon trigonus (Boraginaceae) is a plant used in South Africa (SA) in traditional medicine as a decoction for various health ailments that include HIV, ringworm, ulcers or as an infusion for blood purification. Harnett et al, (2005) screened this medicinal plant for anti-HIV activities to ascertain if it possessed any, and the results were in the affirmative. The plant used in this study was collected from the Eastern Cape region in SA.

In this study, the methodology for assessing anti-HIV activities of *L. trigonus* included reverse transcriptase (RT) assay, protease (PR) assay and glycohydrolase enzyme assays. The researchers performed assays which removed sulphated polysaccharides and tannins whose activity against HIV is well researched and documented. Tannins have been found to inhibit HIV-RT but are non-specific as they target a wide range of enzymes of the host.

Inhibitory effects were observed in all crude extracts of *L. trigonus* but acetone extracts. When these extracts were assayed in the presence of 0.2% (w/v) BSA to remove the possible effects of tannins, the researchers observed a loss of the RT inhibition across the different extracts, which suggested the inhibition was due to tannins. However, significant RT inhibition was retained in the *L. trigonus* leaf extracts that had polysaccharides (96.5 %) and those without polysaccharides (63.5%) (68). An IC_{50} value of 49 µg/ml was

recorded for the aqueous Lobostemon leaf extract without sulphated polysaccharides in the RT assay, suggestive of a strong HIV-I RT inhibition of this *L. trigonus* plant species.

For glycohydrolase enzyme assays, the researchers observed inhibition of the α and β-glucosidase enzymes in all 0.2 mg/mL *L. trigonus* leaf extracts. The addition of BSA to the plant extracts resulted is some loss of inhibition, but the retained and/or remaining inhibition of the enzymes was significant except for *L. trigonus* leave extract (68).

When the extracts were tested using the protease (PR) assay at 0.2 mg/ml, no significant inhibition of the HIV-II PR (≥50%) was observed. That said, some insignificant inhibition of PR was recorded in the *L. trigonus* aqueous and methanol extracts, at values 29.4 and 30%, respectively.

In a separate study, *L. trigonus* was evaluated and explored for its ability to inhibit HIV-1 by Chawuke *et al.* (2021). The methodological approaches in this study included Fusion arrest assay in TZM-bl cells, XTT assay to assess the cytotoxicity effects of *L. trigonus* as a natural microbicide; ultra-performance liquid chromatography/quadrupole time-of flight mass spectrometry (UPLC-qTOF-MS) to profile potential active ingredients.

L. trigonus was found to inhibit multiple HIV-1 subtypes of A, B and C using TZM-bl cell model with a range of IC_{50} values between 0.10 to 7.21 µg/mL (69.) The assessment of *L. trigonus*'s activity against virus induced cytopathic effects in CEM-SS cells showed that the plant had an inhibitory activity with EC_{50} value of 8.9µg/mL. When investigated for the anti-HIV activity *ex vivo* using macrophages and peripheral blood mononuclear cells (PBMC), *L. trigonus* aqueous extract yielded an inhibition with IC_{50} values of 4.4 and 0.97 µg/mL respectively.

The UPLC-qTOF-MS analysis revealed that *L. trigonus* contained salvianolic acids B and C, lithospermic acid and rosmarinic acid as its chemical constituents. With respect to the mechanism of action, *L. trigonus* was found to be target the entry and fusion stage, where it interfered with HIV-1 attachment to the target cells. Molecular dynamic simulations suggested that the HIV-1 inhibition was due to one of the *L. trigonus* phtoconstituents, salvianolic acid B which binds to gp120, a protein which is crucial for HIV entry into host cells (69).

2.6.11. *Maytenus senegalensis* (Lam.) Exell (Celastraceae)

A plant screen conducted in Sudanese traditional medicine by Hussein *et. al*, (1999) identified *Maytenus senegalensis* as an anti-HIV plant-based medicine. The plants were screened for anti-HIV activity using MT-4 cells which were infected for with HIV-1 (HTLV-IIIB) at $TCID_{50}$ of 0.001/cell (64). *M. senegalensis* stem and bark extracts, prepared using water and methanol were tested for HIV-1-induced cytopathic effect (CPE) in MT-4 cells and assessed through optical microscopy. The methodological design and approach of this study included the determination of tannin content. To investigate anti-HIV activity, researchers used HIV-1 PR assay and HPLC to determine if *M. senegalensis* plant extracts were inhibitory to the protease enzymatic function. Hussein et. al (1999) also took keen interest in isolating of principles from the bark of *M. senegalensis* to profile its chemical composition. This was achieved through defatting methanol extract with hexane and then partitioning it between ethyl acetate and water, which were then further fractionated in a column of Sephadex LH-20 eluted with (EtOH, H_2O to MeOH), and finally through chromatography on silica gel.

M. senegalensis methanolic extract was found to contain 11.13% tannins while the water extract contained 12.5% (64). The minimum concentration for complete inhibition (IC) of HIV-1 induced CPE in MT-4 cells was 125 µg/ml. Significant HIV-1 PR inhibition was observed in the water and methanol extracts of the *M. senegalensis*, 56.8% and 48% with IC_{50} of 88 and 105 µg/ml, respectively.

Chromatographic isolates of *M. senegalensis* which were water-soluble, when juxtaposed with ethyl acetate-soluble isolates, showed higher HIV-1 PR inhibition at 100 µg/ml. To be precise, 42.5 ± 3.4%–68.9 ± 4.5% was observed with the phenolic fractions which were soluble in water, whereas the ethyl acetate-soluble fractions recorded 25.3 ± 3.7% HIV PR inhibition.

The phenolic compounds isolated from the stem-bark of *Maytenus senegalensis* included among others, (—)-4'-Methylepigallocatechin 5-O- ß -glucopyranoside (II), (+)-4'-Methylgallocatechin 3'-O-ß-glucopyranoside (IV), Phloroglucinol-1-O- ß -D-glucopyranoside (VIII), whose anti-HIV-1-protease activity stood at 72.9 ± 4.5%, 68.2 ± 5.3% and 68.2 ± 4.2% respectively (64).

2.6.12. *Moringa oleifera* Lam (Monringaceae)

One of the plants widely used in African traditional medicine (ATM) to treat HIV/AIDS patients is *Moringa oleifera* Lam (Monringaceae), which is commonly known as drumstick tree or horseradish tree. It is a reportedly a versatile plant that has multiple medicinal purposes whose medical profile include anti-viral, cardiac and circulatory stimulation, antitumor properties (70).

Given the claims of effectiveness of Moringa in improving the quality of life of people living with HIV (PLWH), Nworu (2013) set out to investigate antiretroviral bioactivities of three different extracts of *M. oleifera* on lentiviral vector infectivity using HeLa cells which they quantified through green fluorescent protein (GFP) expression using flow cytometry. The researchers collected the *M. oleifera* plant leaves in Nigeria (Anambra State) and prepared three plant extracts; methanol extract (MM), petroleum ether extracts (EM) and the aqueous extract (AM) which they used to assess this plant's antiviral properties. The phytochemical analysis of *M. oleifera* extracts revealed the presence of multiple phytoconstituents: saponins, alkaloids, proteins, glycosides, resins, tannins, carbohydrates, flavonoids and acidic compounds — with the methanol extract showing that it has more phytoconstituents in juxtaposition with other solvent extracts.

Through the said viral vector-based assay technique on HeLa cells and using flow cytometry and FACS analysis of viral infectivity rate, Nworu (2013) showed that the aqueous extract of *M. oleifera* inhibits the HIV-1 in a concentration-dependent manner (5 to 62.5 µg/ml). The HeLa cells exposed to 62.5 µg/mL *M. oleifera* aqueous extract showed an inhibition of approximately 77.51% compared to untreated cells. 7.17 µg/ml was the recorded IC_{50} of the viral vector infectivity. The researchers also reported that this aqueous extract of *M. oleifera* targets the early stages of the viral replication, but didn't specify which one concretely. In this study, Nworu (2013) showed that cells tolerated *M. oleifera* aqueous extract using MTT assay, a 50% cytotoxic concentration (TC_{50}) was recorded at 41.58 µg/ml. The inhibition selectivity index (SI) stood at 5.80 (70).

Both methanol and ether extracts showed some antiviral activity, blocking 50% of viral infectivity (IC_{50}) of HeLa cells at a concentration of 7.72 µg/ml and 7.59 µg/ml respectively.

2.6.13. *Mucuna coriacea* Baker (Leguminosae)

Mucuna coriacea Baker grows naturally in South African Limpopo province, and is locally called Vhaulada which is a Tshivenda. The English call it the buffalo-bean. The roots of this plant have important medicinal purposes to the Venda people who use them to treat various health ailments such as fever, diarrhoea (60).

Bessong *et al*, (2015), after engaging traditional health practitioners, in the Limpopo province identified *Mucuna coriacea Baker* as anti-HIV-1 medicinal plant. Through assays such as HIV-1 RT RNA-dependent-DNA polymerase (RDDP) activity quantification, HIV-1 RT RNase H activity assay, Bessong *et al*, (2015) found out that *Mucuna coriacea Baker* had strong activity against HIV-1 Reverse Transcriptase activities The IC_{50} values for Mucuna *coriacea Baker* methanolic extract on HIV-1 RT RNA-dependent-DNA polymerase (RDDP) activity and HIV-1 RT RNase H activity were recorded as 10.5µg/ml and 17.5 µg/ml respectively. The cell viability tests done suing HeLaP4 cell line, did not indicate any significant cytotoxicity associated with this plant. DMSO was the negative control in this cytotoxicity evaluation.

2.6.14. *Pelaragonium sidoides* DC. (Geraniaceae)

Pelargonium sidoides also known as African geranium, is a traditional medicinal plant that grows naturally in South Africa and used by the Zulu community to treat upper respiratory ailments. This herb is reported to exhibit a potent anti-HIV-1 profile, with a distinct mechanism of action (71). The root extract of this plant called EPs®7630 or Umckaloabo®, which has undergone human clinical trials and proven safe, is officially sanctioned (licensed) used in Europe (Germany) to treat health conditions such as acute bronchitis.

Helfer *et. al* (2014), inspired by the proven safety profile of this herbal medicine and its traditional use, set to investigate its anti-HIV activity. The researchers, report that *P. sidoides* extract strongly inhibited infection of PBMCs and macrophages by HIV-1 strains (X4, R5 tropic and clinical isolates) at concentrations ≥25µg/ml and ≥50µg/ml respectively. The anti-HIV activity tests were done using an Easy-Hit technology or assay where a dose-dependent inhibition was observed. The *P. sidoides* extract blocks HIV-1 replication at early stages of the cycle with EC_{50} values below 6µg/ml in clinical isolates, without killing cells as 80% of them were viable as detected by the MTT assay (71). Using Time of

Addition (TOA) assay, with entry inhibitors (Griffithsin and T-20) as positive controls and AZT (reverse transcription inhibitor) as a reference drug, Helfer *et. al* (2014) also showed that *P. sidoides* root extract inhibition occurs before reverse transcription takes place, it interferes with HIV-1 entry into the target cells. The herb's extract also prevented cellular attachment of HIV-1 to host cells. The separation of *P. sidoides* extract and chemical characterization was done using Ultra Performance Liquid Chromatography and Ultrahigh Resolution Mass Spectrometry. Polyphenols that are richly contained in this herb were shown to mediate the observed anti-HIV-1 bioactivities.

2.6.15. *Peltophorum africanum* Sond. (Leguminosae)

An ethnobotanical study carried out by Bessong et al. (2005) that involved seventeen South-African medicinal plants revealed that *Peltophorum africanum Sond.* (Fabaceae) has a potent activity against Reverse transcriptase (RT), an HIV enzyme responsible for the conversion of the virus RNA genetic material to cDNA that is later integrated in the host DNA. In this study, the activities measured were the inhibition against the RNA-dependent-DNA polymerase (RDDP) activity of RT and the ribonuclease H (RNase H) activity (60).

The identification of *P. africanum* as an anti-HIV medicinal plant came from two healers who were interviewed about the medications, they offer to HIV patients who consult them. After collection of the plant material in Limpopo Province of South Africa, aqueous and methanol extracts of these plant were prepared. In this study, the researchers employed assays such as cytotoxicity test using HeLaP4 cell line; RNase H assays where the inhibition of RNase H was quantified by 3H-labelled RNA degradation hybrid in the presence or absence of test agent; HIV-1 RT RDDP activity evaluation; bioassay guided fractionation and extraction of active phytoconstituents using liquid column chromatographic techniques; assessment of test agents against the 3 -end processing activity of HIV-1 integrase.

Bessong et al. (2005) found out that *P. africanum* aqueous and methanolic extracts inhibit both RNA-dependent-DNA polymerase and ribonuclease H activities of HIV-1 RT. For example, the roots methanolic extract of this plant interfered with the RDDP activity of RT inhibition, recording an IC_{50} value was 8.0 µg/ml. The IC_{50} value for the inhibition of ribonuclease H activities of HIV-1 RT by was *P. africanum* (bark) was 10.6 µg/ml. The

general pattern was such that the methanol extracts, when compared to aqueous extracts, had higher inhibitory effects.

Photochemical analysis bioassay-guided fractionation revealed that this medicinal plant contains compounds such as bergenin, catechin and red coloured gallotannin. One of the isolated fractions, gallotannin, had activity against HIV Reverse transcriptase, where it displayed complete HIV-1 RT RDDP activity inhibition with an IC_{50} value of 6.0 μM, in juxtaposition with 0.048 μM for AZT-TP (positive control). A dose-dependent inhibition of HIV-1 RT RNase H activity was also observed in gallotannin photochemical (IC_{50}: 5.0 μM). However, catechin and bergenin had no activity against RNase and RDDP functions of HIV-1 RT even at a high concentration of 100 μM. Furthermore, gallotannin (IC_{50}: 6.0 μM) and catechin both had activities against HIV-1 integrase, while bergenin showed no activity. 100 μM of catechin yielded about 65% HIV integrase inhibition (60).

Peltophorum africanum, in a different study was also found to contain an anti-HIV-1 constituent, betulinic acid, which inhibits HIV-1. Theo *et al*. (2009) investigated betulinic acid derived from *P. africanum* to characterize its anti-HIV activities. This test was performed against HIV-1JRCSF (R5 HIV-1) and HIV-1NL4-3(X4 HIV-1) using MAGI-CCR5 cells. The *P. africanum*-derived betulinic acid yielded inhibition with an IC50 of 0.04μg/ml (using HIV-1NL4-3), while glycyrrhizin recorded an IC_{50} of 0.04 μg/ml. The same MAGI assay, now with HIV-1JRCSF gave anti-viral blockage whose IC_{50} value was 0.002 μg/ml for betulinic acid compared to glycyrrhizin, whose IC50 was 0.58 μg/ml. In this study, > 0.09 μg/ml was CC_{50} for betulinic acid and 1.65 μg/ml for glycyrrhizin (72). These findings justify the use of this plant by traditional health practitioners in South Africa.

2.6.16. *Sutherlandia frutescens* (L.) R.Br. (Leguminosae)

The anti-HIV activities of organic and aqueous extracts of *Sutherlandia frutescens,* a medicinal plant indigenous to Africa, were tested using the reverse transcriptase (RT) assay, protease (PR) assay and glycohydrolase enzyme assays. This medicinal plant is used in South-Africa as an over-the-counter complimentary medication to treat several ailments including HIV infection and its opportunistic diseases.

Plant extracts used were acetone, methanol and ethanol, methylene dichloride and aqueous extracts. The researchers removed both tannins and sulphated polysaccharides

from the plant extracts as they have been previously shown to possess some inhibitory HIV activity. From all the extracts tested, only the aqueous extract of *S. frutescens* leaves (0.2 mg/ml) showed significant HIV-RT inhibitory effects (68).

The aqueous extract of *S. frutescens* leaves retained its HIV-RT inhibitory properties even after the removal of sulphated polysaccharides using ethanol precipitation. This was indicative that the HIV inhibitory effects were not due to sulphated polysaccharides. The removal of tannins in most plant extracts (using 0.2% (w/v) BSA) resulted in loss of reverse transcriptase inhibition, an indication that tannins could be responsible for *S. frutescens* anti-HIV activity (68). That said, only *S. frutescens* leaf aqueous extract retained HIV inhibition activity (~30%) even after tannin dereplication. No significant glycohydrolase enzymes and HIV-II PR inhibition (≥ 50%) was observed in *S. frutescens* various extracts when assayed at 0.2 mg/ml.

In a separate study by Wilson et. al, (2015), *S. frutescens* was tested for safety in a clinical set-up in South Africa. In this study, HIV-positive individuals, whose CD4 T-lymphocyte count was above >350 cells/μL, were put into a randomized double-blind human clinical trial designed in way that one group consumed *S. frutescens* and the other took a placebo as the vehicle control. The safety of this traditional medicinal plant was assessed for 6 months. Wilson *et al*, (2015) found out that *S. frutescens* did not alter the count of CD4 T-lymphocytes, neither did it cause a change in HIV viral load over the course of the study. The safety evaluations of *S. frutescens* also involved measuring a variety of biochemical and haematological parameters in the two cohorts, all which did not have any significant difference when juxtaposed with the demographic and clinical characteristics or values recorded at baseline (73). The biochemical and haematological tests which were performed in intervals (week 12 and week 24) included sodium, urea, creatine kinase, calcium, total bilirubin, inorganic phosphate, random glucose evaluations in both arms — those who took *S. frutescens* and a placebo. Wilson *et al*, 2015 however report that, the burden of infection (BOI) was higher in *S. frutescens*-treated group compared to the placebo-taking arm. The figures recorded for *S. frutescens arm was* 5.0 (5.5) [mean (SD)], while the placebo-taking arm stood at 9.0 (12.7) days with the statistical significance value of ($p = 0.045$). The BOI included viral, bacterial, fungal, protozoal infections. This difference was attributed to two study participants who were diagnosed with TB cases, who were simultaneously enrolled in isoniazid preventive therapy (IPT), an occurrence

which researchers said needed further investigations to establish the possible interactions between *S. frutescens* and IPT (73).

2.6.17. *Terminalia sericea* Burch. ex DC. (Combretaceae)

Terminalia sericea is a plant used in South Africa (Limpopo province) by traditional health practitioners as traditional medicine. It is locally known as Mususu in Tshivenda vernacular and is commonly called Silver cluster leaf in English. It also grows in other parts of Southern-Africa including Botswana. Its ethno-medical profile includes its known use for cough, skin infections, diarrhoea treatment. It is also used to treat HIV/AIDS in this province (74). In this study, the *T. sericea*, which was ethnobotanically selected alongside other two South-African medicinal plants was assessed for its bioactivity against HIV-1 Reverse transcriptase (RT) RNA-dependent DNA polymerase (RDDP) and RNase activities.

Bessong (2004) consulted and enquired from healers (4) which plants they used treat HIV patients and the interviews pointed to 3 medicinal plants: *Terminalia sericea* (leaves), *Bridelia micrantha* and *Combretum molle* (roots & stem-bark). Aqueous and methanol crude extracts of these plants were prepared and evaluated for anti-HIV activity.

Using HIV-1 Reverse transcriptase assay, the crude extracts were evaluated for their RNA dependent-DNA polymerase (RDDP) and the ribonuclease H (RNase) HIV-1 RT inhibition. The researchers found out that the methanol extract of the leaves of T. sericea was inhibitory against HIV-1 RT as measured by its RDDP and RNase H activities *in vitro*. This inhibition was dose-dependent with an IC_{50} of 7.2 µg/ml for RDDP and 8.1 µg/ml RNase H. The general pattern was that methanol extracts of T. sericea blocked RT activities better than the aqueous extracts. Cell Proliferation Assay was used to measure cytotoxicity of the crude extracts on HeLaP4 cell line and all the extracts were found to be non-toxic to the said cells (74). This study shows that it important to screen herbal medicine which healers claim has some anti-HIV activity and subject them to laboratory tests.

2.6.18. *Vernonia amygdalina* Delile (Compositae)

Vernonia amygdalina, a shrub that is grows naturally in the tropical region of Africa, was evaluated for immunological effect on HIV sero-positive HIV patients enrolled in the

antiretroviral therapy. (ART). In this study, the plant (fresh leaves) was collected in Nigeria (Nsukka, Enugu State). This small shrub, *V. amygdalina* is used in Nigeria traditional medicine for several purposes; it is known to be anthelmintic and antiprotozoal on top of being an antioxidant (76).

Several compounds of medicinal value have been isolated, including saponins e.g., vernoniosides A1, D3 and C. It has been reported that the leaves of *V. amygdalina* contain flavonoids such as luteolin, luteolin 7-O, ß—glucuronoside; it is these compounds that confers this plant an antioxidant profile and characteristic. Hence in this study Momoh *et al*, (2011) set out to evaluate the *V. amygdalina*'s antioxidant activity on virally suppressed patients taking HIV antiretroviral medication. This effect was compared to immunace, a known immune booster (76).

This study was a clinical trial which was randomized, double-blinded and placebo-controlled. It had 40 participants, whose CD4 baseline ranged between 120 to 212. The study participants were divided into 4 groups comprising of 10 patients each. Group A took the immunace capsule alone while group B took *V. amygdalina* water extract alone. Group C were administered with both *V. amygdalina* aqueous extract and the immunace medication. Group D was the control cohort. All the treatments were taken twice a day alongside antiretroviral drug. Clinical and laboratory parameters were evaluated on monthly basis for consecutive four months (76). $CD4^+$ cells were counted and plasma cell volume (PCV) was evaluated.

When researchers carried-out a phytochemical screen of the *V. amygdalina*'s ethanolic leave extracts they found it to contain a variety of compounds including tannins, saponins, cardiac glycosides, proteins, flavonoids, which the Momoh et al, (2011) claims could be responsible for the changes they saw regarding $CD4^+$ cells count and other measurable parameters.

An average increase of 12% $CD4^+$ cells count was recorded in patients who simultaneously took *V. amygdalina* aqueous extract and the immunace capsules (group C) while Group B, who took *V. amygdalina* water extract alone registered 4.0% in $CD4^+$ cells count. This was statistically significant ($P < 0.05$) when compared with those that were the controlled group whose change in this parameter was a paltry 1.0 % (76). An

increment was observed in PCV with the most effect seen in those patients that took both *V. amygdalina* extract and immunace, most probably due to a synergetic effect.

The exact mechanism by which *V. amygdalina* extract and immunace increase $CD4^+$ cells count is unknown, but the researchers claimed that the theory that antioxidants seems to lower apoptosis of $CD4^+$ cells in immunocompromised patients could be the reason behind this pharmacological effect — since *V. amygdalina* contains oxidants in its chemical profile. This study revealed that *V. amygdalina* is an immunostimulant that could enhance the conditions of people living with HIV.

2.6.19. *Vitex doniana* Sweet (Lamiaceae)

Tietjen *et. al* (2018) when characterizing a three-step traditional HIV/AIDS treatment regimen in Botswana identified *Vitex doniana* as a medicinal plant inhibitory to HIV replication (61). *Vitex doniana* grows naturally in the Northern parts of Botswana. Traditional healers in the Kanzungula (Northern Botswana) communicated information to the effect that the roots of *V. doniana* are used traditionally to treat HIV/AIDS, non-stop menstruation.

Crude extracts of *V. doniana* was investigated for in vitro cytotoxicity and inhibition of HIV-1 replication using CEM-GXR cells which were infected with NL4.3 (HIV- 1NL4.3) strain. CEM-GXR cells are a $CD4^+$ T-cell line that has been engineered to contain an HIV-1 LTR-driven GFP reporter which is quantifiable by flow cytometry. Tietjen *et. al* (2016) reported that *V. doniana* extract was tolerated by cells and significantly blocked HIV-1NL4.3 replication, 25 µg/ml yielded 50 ± 8% inhibition. *V. doniana* root extracts did not interfere with the ARV-mediated inhibition of viral replication (61).

Previously, another plant species from the genus Vitex known as *Vitex leptobotrys*, through bioassay-guided fractionation has been shown to contain some chemical compounds that had anti-HIV properties, including lignan (Ivitexkarinol and chalcone), -(4-hydroxyphenyl)-1-(2,4,6-trimethoxyphenyl)-2-propen-1-one and dipeptides (aurantiamide and aurantiamide acetate). For instance, the novel chalcone was shown to block HIV-1 replication by 77% at 15.9 µM (76). This demonstrates that vitex genus possesses some anti-HIV pharmacologic properties, hence *Vitex doniana* was found to be inhibitory to HIV -1 (61, 76).

Table 2.1: African Medicinal Plants, their active ingredients and mechanism against HIV-1 infection

Medicinal Plant	Family	Plant part	Assays	Concentration	Active ingredients	Mode of action	*In vivo*/Clinical studies
***Azadirachta indica* A.Juss.**	Meliaceae	leaf	BD FACS Canto II methods; surface staining and flow cytometry; Chembio HIV 1/2 STAT-PAK® Assay	0.5 µg/mL	Not determined	HIV-1 reverse transcriptase inhibitor; HIV-1 fusion inhibitor; downregulated CD4+ T cell activation; Block the attack of lymphocytes by HIV-1 (48,53)	Done
Bridelia micrantha (Hochst) Baill.	Phyllanthaceae	root	HIV-1 Integrase assay; RNA-dependent-DNA Polymerization (RDDP) activity of HIV-1 Reverse Transcriptase (RT) assay; thin layer chromatography (TLC) and mass spectrometry	7.3 µg/mL. (IC_{50})	Flavonoids and tannins	Inhibition of RNA-dependent-DNA Polymerization (RDDP) activity of HIV-1 Reverse Transcriptase (54)	Not done
Cassia abbreviata Oliv. Oliv.	Leguminosae	root	Tannin removal; HIV-1c binding and entry assay; HIV-1c p24 antigen detection assay; Thin layer chromatography; MTT viability assay	102.7 µg/ml (EC_{50})	anthocyanins, anthranoids, anthraquinones, polyphenols and tannins	Suppression of the functional glycosylation of HIV gp120 (56,57,58)	Not done

Cassia sieberiana D.C	Leguminosae	root	Tannin removal; HIV-1c binding and entry assay; HIV-1c p24 antigen detection assay; Thin layer chromatography; MTT viability assay	65.1 µg/ml (EC_{50})	polyphenols, anthocyanins, anthraquinones, polyphenols tannins and anthranoids	Inhibition of HIV-1 at binding and entry stage (56)	Not done
***Combretum molle* R.Br. ex G. Don**	Combretaceae	Root, stem-bark	HIV-1 RT ribonuclease H (RNase H) assay;	9.7 µg/ml (IC_{50})	CM-A, puncalagin pentacyclic triterpene glucosides arjunglucoside and sericoside	Inhibition of HIV-1 Reverse transcriptase (RT) RNA-dependent DNA polymerase (RDDP) and RNase activities (59)	Not done
Croton megalobotrys Müll Arg.	Euphorbiaceae	bark	Latency reversal through flow cytometry-based reporter cell assays; column chromatography, reverse-phase HPLC, NMR spectroscopy and mass spectrometry	0.5 µg/mL	novel phorbol esters (Namushen 1 and 2)	HIV-latency modulator through Protein Kinase C activation (61)	Not done
Croton tiglium L.	Euphorbiaceae	seeds	Protein kinase enzyme assay; column chromatography; Assay of HIV-1 induced cytopathic effect (CPE) using MT-4 cell line	0.48 ng/ml (IC_{100})	phorbol diesters (13-O-Acetylphorbol-20-linoleate, 13-O-Tigloylphorbol-20-linoleate; 12-O-	Protein Kinase C activation (63)	Not done

					Acetylphorbol-13-tigliate; 12-O-Decanoylphorbol-13-(2-methylbutyrate); 12-O-Tigloylphorbol-13-(2-methylbutyrate); 12-O-Acetylphorbol-13-decanoate; 12-O-(2-Methylbutyroyl)phorbol-13-dodecanoate and 12-O-Tetradecanoylphorbol-13-acetate)		
***Euphorbia granulata* Forssk.**	Euphorbiaceae	leaf	Anti-HIV-1 Protease assay; liquid chromatograph (HPLC)	62.5 µg/ml (IC_{100})	Not determined	HIV protease (PR) enzyme inhibition (64)	Not done
***Euphorbia usambarica* Pax.**	Euphorbiaceae	whole plant	HIV-1 Latency Reversal assay (J-lat 9.2); column chromatography, reverse-phase HPLC, NMR spectroscopy and mass spectrometry; J-Lat assay 9.2	0.015 µM (EC_{50})	Euphordraculoate C, 4b-Crotignoid K, Usambariphane B, Usambaricinophane E, Isoterracinolides C,	HIV-latency modulator through Protein Kinase C activation (67)	Not done

			cells inhibition by pan-PKC inhibitor GÖ-6983, Flow cytometry;		whereas 12-O-benzoyl-13-acetoxy-4,20-dideoxyphorbol-4-ene, crotignoid K and isoterracinolide A		
***Lobostemon trigonus* (Thunb.) H.Buek**	Boraginaceae	leaf	Reverse transcriptase (RT) assay; Protease (PR) assay; Glycohydrolase enzyme assays Fusion arrest assay in TZM-bl cells, XTT assay; ultra-performance liquid chromatography/quadrupole time-of flight mass spectrometry (UPLC-qTOF-MS)	49 µg/ml 0.97 (IC_{50})	salvianolic acids B and C, lithospermic acid and rosmarinic acid	Reverse Transcriptase inhibition HIV attachment inhibitor (68)	Not done
Maytenus senegalensis (Lam.) Exell	Celastraceae	stem-bark	HIV-1 PR assay and HPLC	88 µg/ml (IC_{50})	(—)-4'-Methylepigallocatechin 5-O- ß -glucopyranoside (II), (+)-4'-Methylgallocatechin 3'-O-ß-	HIV-1 PR inhibition (64)	Not done

					glucopyranoside (IV), Phloroglucinol-1-O- ß -D-glucopyranoside (VIII)		
***Moringa oleifera* Lam**	Monringacea e	leaf	MTT assay; Vector-based antiviral assay quantified through flow cytometry;	7.17 µg/ml (IC_{50})	saponins, alkaloids, proteins, glycosides, resins, tannins, carbohydrates, flavonoids	Targets the early stages of the viral replication (70)	Not done
Mucuna coriacea Baker	Leguminosae	root	HIV-1 RT RNA-dependent-DNA polymerase (RDDP) assay; HIV-1 RT RNase H activity assay	10.5µg/ml (IC_{50})	Not determined	Inhibition of HIV-1 RT RNA-dependent-DNA polymerase (RDDP) and HIV-1 RT RNase H activities (60)	Not done
***Pelargonium sidoides* DC.**	Geraniaceae	root	Easy-Hit technology or assay; MTT assay; Ultra Performance Liquid Chromatography; Ultrahigh Resolution Mass Spectrometry	6µg/ml (EC_{50})	Polyphenols	blocks HIV-1 replication at early stages (71)	Not done
Peltophorum africanum Sond.	Leguminosae	bark, root	quantitative PCR, Ultra Performance Liquid Chromatography, Mass Spectrometry	8.0 µg/ml (IC_{50})	bergenin, catechin and red coloured gallotannin, betulinic acid	HIV-1 RT RDDP activity inhibition, HIV-1 integrase inhibition (60)	Not done

***Sutherlandia frutescens* (L.) R.Br.**	Leguminosae	leaf	Reverse transcriptase (RT) assay; protease (PR) assay; Glycohydrolase enzyme assays	0.2 mg/ml	Not determined	HIV-RT inhibition (68, 73)	Done
***Terminalia sericea* Burch. ex DC.**	Combretaceae	leaf	RNA dependent-DNA polymerase (RDDP) & Ribonuclease H (RNase) HIV-1 Reverse transcriptase inhibition assay	7.2 µg/ml (IC_{50})	Not determined	Inhibition of HIV-1 Reverse transcriptase (RT) RNA-dependent DNA polymerase (RDDP) and RNase activities (74)	Not done
***Vernonia amygdalina* Delile**	Compositae	leaf	plasma cell volume (PCV) analysis using Abacus junior machine; $CD4^+$ cells count	2 handful of cleaned fresh leaves soaked in 200 mL water	tannins, saponins, cardiac glycosides, proteins, flavonoids	Not determined (76)	Not done
***Vitex doniana* Sweet**	(Lamiaceae)	root	HIV inhibition using HIV-1 LTR-driven GFP reporter-containing CEM-GXR cell line, quantified through flow cytometry	25 µg/ml	Not determined	Not determined (61)	Not done

2.7. Discussion

Phytomedicine, which has been an integral component of the primary health care of African societies for ages, remains a rich repository for drug discovery that should continue to be relentlessly explored. It is a reservoir for novel antivirals and HIV latency modulating agents. Clearly considerable work has been done to document and investigate African plant-based medicines as revealed by our review in which we were able to identify 18 anti-HIV African medicinal plants, whose antiviral properties are supported by laboratory studies and/or data (53-76). However, more detailed work remains to be done to harvest their full potential.

Maximizing the benefits of phytomedicine and developing it such that its contribution to the people-centred health care in local communities is fully harnessed, would require productive engagement of medicinal plant use. Reverse pharmacology model offers a practical framework and opportunity to investigate and validate phytomedicine and ensure that it is used productively, more so that it is a model that has been inspired by traditional medicines as they are already in use even without biomedical laboratory tests. Reverse Pharmacology is basically the science of developing existing clinical and observational experiences, of a medicinal herb already in use (TCM), into a medical drug or lead by subjecting it to rigorous exploratory clinical and experimental laboratory studies (5). Productive engagement of medicinal plant use would also mean working with local communities, to establish dialogue and collaborative engagements between researchers, THPs, and other community members to characterize medicinal plants in ways that clearly benefit local communities. Through Reverse Pharmacology, a testable hypothesis about the potential of medicinal plants could be formulated leading to isolation and mechanistic characterization of chemical compounds that could target HIV-1 latency. Many of these plants may not work and this should be communicated to users and healers, but several have useful leads as unearthed by our review. Most importantly, productive engagement of medicinal plant use should aim to ascertain whether individuals using TCMs associated with the management of HIV/AIDS-related illnesses are still infectious. Furthermore, there is still need to determine whether these plants could impact ARV use. cART is the gold standard, but in its absence productive of medicinal plant use could be helpful.

In order to achieve productive of use of anti-HIV medicinal plants studies that combine both community-based approaches and laboratory biomedical approaches are required. Such a study would see the researcher utilize both qualitative data and quantitative data to explore and answer a given research question. The kind of work under discussion, could be executed within the framework of 'Exploratory Sequential Mixed Methods' where two sets of data (qualitative and quantitative data) which are usually viewed from a binary lens, are collected at different stages in the research pipeline but are then combined to cooperatively inform one another (76). The nature of such a study is collaborative; the study participants (traditional healers or community members) inform and influence the structural anatomy of the research process and thought. Study participants being THPs and indigenous knowledge keepers consistently co-analyze data through an agreed continual feedback process within a defined report-back mechanism as described by Andrae-Marobela *et al*., 2012 (4). An added advantage of mixed methods approach is that it also helps bridge the gap between two knowledge systems of western biomedicine and traditional medicine — on top of achieving the agenda of decolonization of knowledge.

In generating qualitative data, conduction of a consultation process that include holding in-depth interviews with THPs and other knowledge keepers to establish and accumulate comprehensive qualitative data is imperative. The qualitative data collected from the interviews would include medicinal plant uses, health outcomes, and conservation statuses of medicinal plants. Just to enlighten and elucidate more on this approach, these interviews could be conducted within a defined theoretical framework. For example, the Rapid Participatory Rural Appraisal (rPRA) methodological framework, which uses a variety of research tools and platforms that will allow researchers to intermingle with study participants e.g., community meetings and focused group conversations, semi-structured interviews, pictorial diagrams etc. (4). rPRA methods are reputable as they have been successfully tested and used in a variety of disciplines, including more complex research areas such as biodiversity, because they engage local populations and allow for community input into study design, data analysis and interpretation (78). Through this approach, community members and THPs themselves direct the generation of data.

Furthermore, there is still paucity of information with regards to TCM mechanisms. Thus, there is need to continue elucidating mechanisms of African plant-derived antivirals and latency modulators in cell-based assays to close the existing gap.

Additionally, there is need for new distinct mechanisms for HIV latency modulation, and indeed for new targets on HIV replication (34). The current library of the latency reversing agents which function through various molecular pathways have failed to bear any fruits at clinical stage. These LRAs act through pathways such as PKC activation, histone methylation inhibition, bromodomain inhibition and histone deacetylases (HDACs) inhibition (34-36). Medicinal plants-derivatives with their diverse structural make-up offer an avenue to discover unique mechanisms for proviral reactivation and transcription. This could boost the 'shock and kill' intuitive strategy and make it feasible. Similarly, owing to their unique chemical compositions, herbal medicines could be a reservoir for 'block and lock' hypothetical therapy. Also, drug discovery research on HIV chemotherapy in Africa must shift to prioritizing *in vivo* and human clinical studies of the isolated natural products. This could improve 'Reverse pharmacology and find regional-specific ways to manage HIV-1, especially in resource limited settings which are overburdened with HIV/AIDS.

The next chapter is a typical example of an ethnomedical survey aimed at documenting medicinal plants used for HIV/AIDS management within the reverse pharmacology model.

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Chapter 3.

Ethnopharmacological survey of medicinal plants used for the management of HIV-related diseases and other health conditions by the BaKalanga people of Tutume subdistrict in Central Botswana

3.1. Abstract

Traditional and complementary medicines (TCM), as part of indigenous and pluralistic medical systems worldwide, are important contributors to primary health care and ample reservoirs for drug discovery. To explore the extent to which TCM is used for HIV/AIDS and related disease management by the BaKalanga Peoples of the Tutume subdistrict in Central Botswana, we designed an exploratory community-based project to document the medicinal plants of this region. Using the snowball sampling technique, we recruited 13 Traditional Health Practitioners (THPs) and conducted in-depth interviews to establish medicinal plant uses and TCM-related uses. From these interviews, we documented 83 medicinal plants, representing 39 families, which are traditionally used for HIV/AIDS management or associated conditions. Plants from the family Fabaceae were most commonly documented, making up 25.0% of all plants, followed by Euphorbiaceae at 6.0%. Notably, 38 (45.8%) of the identified medicinal plants have not been previously reported or assessed for HIV-1 specific or related bioactivities and/or ethnomedical uses. This study is the first detailed ethnobotanical survey of plants used for HIV/AIDS and related diseases management by the BaKalanga Peoples of the Tutume-sub district in Central Botswana.

Key words: HIV/AIDS; Traditional and complementary medicines (TCM); medicinal plants; Tutume subdistrict; reverse-pharmacology;

3.2. Introduction

Despite the increasing accessibility of antiretroviral therapy (ART), HIV and AIDS continue to persist as a serious health challenge, particularly in sub-Saharan Africa. This is a disease that has killed over 36.3 million people globally, by the end of 2020 (1). Among Sub-Saharan African countries, Botswana continues to exhibit a high prevalence of HIV-1 infection in adults aged 15 to 49, ranking amongst the top four heavily burdened countries worldwide with a prevalence rate of 19.9% (2). Although the country has over 87% coverage of people receiving antiretroviral therapy (ART), Botswana recorded approximately 8900 new HIV-1 infections and about 5100 AIDS-related deaths in the year 2020 (2). Furthermore, the ever-rising rate of antiretroviral drug resistance in HIV-infected individuals in Botswana, especially with the advent of 'treat all' ART initiatives, represents an existential challenge that could undermine the HIV cascade of care and efforts to eradicate the epidemic. Rowley, *et al*., (2016) reported that transmitted drug resistance (TDR) in Botswana sharply shoot-up from 2.9% in 2012/2014 to 9.7% in 2014/15 (3). The prevalence of drug resistance mutations (DRM) in Botswana continues to grow and has been observed in relatively new ARV regimen, including dolutegravir (Integrase Strand Transfer Inhibitor) whose DRM in 2021 has been estimated to be 32% (4). Pretreatment drug resistance mutations in treated infants in Botswana has also been reported recently, in 2021 (5). Thus, antiretroviral drug resistance further highlights existing gaps where additional antiretrovirals may fit.

Botswana, a landlocked country in Southern Africa (Figure 3.1), is made up of 10 administrative districts, which are split into 28 sub-districts (6). The disease burden is — as expected — not evenly distributed across the sub-districts. For example, the Tutume sub-district, which is one of the administrative sub-districts of the Central District in Botswana (Figure 1), has an HIV prevalence rate of 18.2% according to Botswana AIDS Impact Survey IV (7). Tutume village, with a population of 18 295 (as of 2011 Census) is the subdistrict's headquarters (8). The Tutume sub-district is a multiethnic territory comprising of various populations including IKalanga and Sekhurutshe speaking people and with BaKalanga being the predominant ethnic group (9-10). This district shares a

border with Francistown (Figure 1), the second largest city in Botswana, which is also characterized with an HIV prevalence of 24.3% (2nd highest in the country) (6-7).

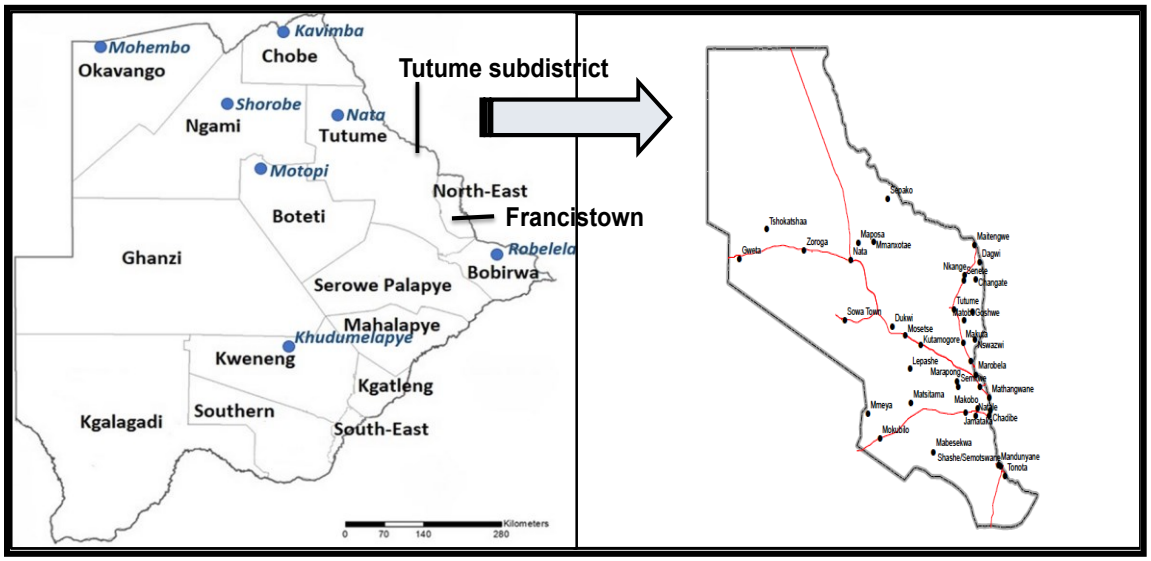

Figure 3.1: Map showing Tutume subdistrict in and Francistown city in Botswana, Africa; (adapted and modified from [8, 20])

While access to ART has greatly improved over the last decade in Sub-Saharan Africa, people enrolled in an ART regimen frequently continue to use TCM as part of their primary health care (11-14), with the World Health Organization (WHO) estimating that 80% of people worldwide depend on traditional medicines (15-16). In the Tutume subdistrict, the vegetation is generally classified as Mopane woodland (due to the predominance of *Colophospermum mopane)* which represents a rich socio-cultural resource for food and ethnomedicinal use (17-19). However, despite the distinct floral biota and ecosystems of this region, much of this local knowledge has yet to be documented and/or investigated for bioactivities. Thus, we designed an exploratory community-based project to engage and work with TCMs to document medicinal plants used for HIV/AIDS management and comorbidities by the BaKalanga peoples of Tutume subdistrict in Central Botswana.

3.3. Methodology

3.3.1. Study site and study population

An ethnomedical survey was undertaken primarily in Tutume Central District in Central Botswana. **(Fig. 3.1)** The Tutume Central District comprises of 41 villages that include Tutume village along with Maitengwe, Goshwe, Mafhungo-Hubona, Sebina, Mathangwane, Goshwe, Nshakashogwe, Nkange, Tonota, Borotsi, Mosetse and Marobela, among others, and collectively constitute a population of about 147,377 as of the 2011 census (8). The 13 THPs recruited for this study all originate from the Tutume subdistrict although two have since relocated to Francistown. In terms of ethnic composition, BaKalanga is the predominant tribe in the Tutume subdistrict, although others such as Bakhurutshe also reside here, particularly in and around Tonota village.

Following consultation with and approval from the traditional Tutume village leader (*Kgosi* (Setswana) or *She* (Ikalanga)), the *She* and his deputies organized and facilitated a forum to meet with the village wards headmen (*Dikgosana*) and the Village Development Committee (VDC) who represent critical stakeholders in the management of village affairs and development. At these meetings the project was introduced to solicit community approval and consent. The traditional leaders and VDC then facilitated discussions within their community to identify local THPs. In some cases, THPs voluntarily introduced additional healers within their catchment area and network to their study.

In terms of research positioning: the researcher is a native Kalanga, who proficiently and fluently speak the IKalanga language. However, I do not come from the research site (Tutume subdistrict, Central District), instead I come from Gambule village and now resides in Masunga village which are both in the North-East District. I also mention that I have never had contact with the indigenous knowledge keepers in Central district and/or any of its village leadership prior to this study, as such it can't be argued that I interfered with free consent in this research work. Be that as it may, being a native Kalanga, conducting an ethnobotanical study in the predominantly-Kalanga area, was an added advantage which allowed me to easily interact with the study participants because I am well conversant with their cultural nuances, norms, value system and beliefs. That is, it helped me connect and relate well with study participants — who readily viewed me as part of their community by virtue of being a Kalanga tribesman. Essentially, being Kalanga

broke the boundaries and closed the distance between the researcher and study participants; traditional healers and the community leadership were readily willing to share more useful information as they trusted their fellow tribesman-cum-researcher.

3.3.2. Study design, sampling and ethical considerations

This study is exploratory, there is no fixed outcome prescribed or anticipated, as such this is a sequentially-explorative, qualitative research (21). A qualitative non-probability sampling method known as snowballing technique was used to recruit THPs, whose prior informed consent was sought before their participation in the survey (22-25). In this sampling technique, suitable study partcipants (THPs) who have been recruited assist in the identification of other potential study partcipants who practice plant-based traditional medicine by making suggestions to the researcher (22-25). Study protocols were approved by the Human Research and Development Committee of the Ministry of Health of Botswana (Study number HPDME-13/18/1), and the Research Ethics Board of Simon Fraser University (Study number 20190227). Additionally, in order to protect their knowledge and intellectual property, material transfer agreements were signed with each traditional health practitioner (THP) before they could each submit medicinal plant samples to the researchers for further investigations and testing.

Individual face-to-face in-depth interviews, guided and supplemented by semi-structured questionnaires, were conducted with THPs to establish medicinal plant traditionally used to manage HIV/AIDS and comorbidities. The questionnaire was structured such that it had 6 main sections (A-F). Section A focused on the background and demographic profile of the respondent, to intentionally establish whether the person meet the participation criteria, including being a medicinal knowledge holder and/or practitioner. Section B probed the healers about different health conditions that patients bring to their attention; this section also sort to understand how consultation is conducted by the THP. Section C enquired about TCMs used to treat the health conditions brought to their attention — this section was important because it aimed to generate the specific list of ethnomedicinal plant species used in Tutume-subdistrict. In section D, THPs were probed on how the medicine administered works to achieve healing of the patient. Section E aimed to find whether the study participant has a working relationship with other THPs, health professionals in hospital settings, and whether they believed if cooperation and collaboration with biomedical scientists could help in addressing some of the health

challenges faced by local THPs. Section F inquired about medicinal plants used to treat specific conditions and also involved the collection of plant species and voucher specimens for further analysis.

In the questionnaire, we probed for the following HIV-associated signs: persistent cough, cough and chest pains, frequent fevers, diarrhoea, and/or weight loss and skin rashes. We also asked study participants to specifically list medicinal plants used to treat pulmonary tuberculosis, skin conditions such as thrush and herpes, herpes simplex virus (HSV) and other sexually-transmitted infections (STIs), and cancers, as these can be disproportionately found in people living with HIV in the absence of effective ART (26-29). We note that, while this study emphasized TCMs used for management of HIV/AIDS and associated conditions, THPs were permitted to share information about TCMs used to treat other conditions unrelated to HIV/AIDS.

Data collected from study participants included local names of medicinal plants, medicinal plant uses, plant parts used, modes of preparation, methods of administration and dosages. All interviews were conducted in the local vernacular (IKalanga). Following optional consent from study participants, interviews were audio recorded and transcribed and translated to English. THPs largely collected the plant voucher specimens by themselves, although in some instances THPs invited researchers on field trips to identify and collect medicinal plant voucher specimens. All plant voucher specimens were taxonomically authenticated by a trained botanist and deposited at the University of Botswana Herbarium (Gaborone, Botswana).

3.3.3. Data generation and data analysis

The questionnaire used in the Tutume-subdistrict ethnomedical survey comprised of semi-structured and nonstructured open-ended questions. As per the dictates of thematic analysis, analytic themes were generated from these open-ended questions; they were then coded and counted by way of descriptive statistics to determine how often these identified themes were overarchingly used by the study participants. Descriptive statistics were also used to establish the frequency distributions (percentages) of plant parts used and plant families of the collected plant voucher specimens.

3.4. Results

Using the snowballing sampling technique, we recruited 13 THPs who all belonged to the BaKalanga tribe (Table 3.1). Of the 13 THPs, 6 (46.2%) were female and 7 (53.8%) were male. The average age of study participants was 51.8 years, with a range of 33—69 years (Table 3.1). All THPs reported that their practice was a spiritual gift which had become a family tradition. Furthermore, all THPs (100%) mentioned that their knowledge of medicinal plants was passed to them by elders within the family (i.e., parents and grandparents) who had previously practiced traditional healing but were also trained by a knowledgeable THP (a process known as '*ku thwasa'* in Ikalanga culture) (Table 3.1).

Table 3.1: Sociodemographic characterization of the study participants

Healer	Gender	Age	Place of birth	Ethnicity	Village (Ward)	Practicing experience (Years)	Years of training	Source of medicinal knowledge	Trained others (No. of apprentices)	No. of patients consulted on daily basis	Kind of patients consulted	Patients consulted come from
	Male	46	Tutume	Nkalanga	Currently Francistown (Blue town) – but from Tutume (Magapatona)	16	5	Grandfather and the trainer	No (0)	10	Adults & children	All over Botswana
2	Male	39	Francistown	Nkalanga	Mafhungo-Hubona (Mafungo)	13	1	Father and the trainer	Yes (1)	10	Adults & children	In and outside Botswana
3	Male	59	Tutume	Nkalanga	Tutume (Magapatona)	39	4	Father, grandfather and the trainer	Yes (90)	10	Adults & children	In and outside Botswana

	Male	47	Maitengwe	Nkalanga	Maitengwe (Mazuwa)	23	6	Father and the trainer	Yes (5)	3- 4	Adults & children	All over Botswana
5	Female	50	Francistown	Nkalanga	Nshakashogwe (Sukudza)	3	5.5 (5 years, 6months)	Father and the trainer	No (0)	3-4	Adults & children	All over Botswana
6	Male	33	Maitengwe	Nkalanga	Maitengwe (Mpapho)	12	1.4 (1 year, 5 months)	Father and the trainer	Yes (10)	5-6	Adults & children	In and outside Botswana
7	Male	57	Masunga	Nkalanga	Mosetse (Bontleng)	16	2	*Father and the trainer	Yes (4)	6	Adults & children	In and outside Botswana
8	Female	52	Nkange	Nkalanga	Nkange (Mabuwe)	1	2	Father and the trainer	No (0)	4	Adults only	Central Botswana
9	Female	69	Maitengwe	Nkalanga	Maitengwe (Mazuwa)	22	2	Grandfather and the trainer	Yes (10)	0.14 (1 per week)	Adults & children	All over Botswana

10	Female	62	Goshwe	Nkalanga	Goshwe (Mangole)	39	4	Grandfather and the trainer	Yes (10)	1 (6-8 per week)	Adults & children	All over Botswana
11	Female	61	Tutume	Nkalanga	Maitengwe (Mpapho)	38	0.4 (5months)	Grandfather and the trainer	Yes (12)	3-4	Adults & children	All over Botswana
12	Female	64	Senete	Nkalanga	Goshwe (Mabunde)	8	2	Grandfather and the trainer	No (0)	0.3 (2 per week)	Adults & children	Central Botswana and Francistown
13	Female	34	Francistown	Nkalanga	Currently Francistown (Blue town) – but from Nswazwi village in Tutume subdistrict	1	1.5 (1 year, 6 months)	Grandfather and the trainer	Yes (1)	5	Adults & children	In and outside Botswana

Table 3.2: Medicinal plants used for management of HIV/AIDS-related diseases and other health conditions in Tutume subdistrict in Central Botswana.
N/A, not reported in supporting literature.

Family name	Scientific name	Local name	Part(s) used	Health condition Treated	Mode of preparation	Method of administration & Dosage	Supporting Literature
Acanthaceae	*Justicia od ora* (Forssk.) Lam.	Pesu-tje-mbudzi	Root	STI	Sun-dried and pounded to powder. Add 2 spoons of the powder to 2L cold water.	Drink the decoction; ½ a cup, 3 times a day	N/A

Amaranthaceae	*Chenopodium album* L.	Ntatabadzimu-we-kulagula	Leaf	Malaria	Cut into small pieces when fresh. Mix with other prescribed medicines; Boil and give the patient to drink when steaming	Steaming with the hot medicated water; 3 times a day. Also drink the decoction; ½ a cup, 3 times a day for 2 consecutive days.	N/A
Anacardiaceae	*Lannea discolor* (Sond.) Engl.	Sefighle	Root	Diarrhoea	Pounded into powder; add ½ teaspoon of powder to soft porridge and eat the meal	Taken with prepared soft-porridge meal; twice a day	Ethnomedicine for diarrhoea in Rundu, East Region, Namibia [30]
Anacardiaceae	*Lannea edulis* (Sond.) Engl.	Nkwidzi	Root	Skin rashes	Pounded fresh into paste and mixed with other measured medicinal plants in bathing water	Bathing; a single tablespoon of the paste in 5L of water	Ethnomedicine for gonorrhoea and syphilis in Rundu, East Region, Namibia [30]
		Nkwidzi	Root	HIV	Cooked to prepare in a thin meat soup or boiled to prepare medicated water	Drink the decoction; 3 times a day	*in vitro* inhibition of HIV reverse transcriptase; 100µg/ml aqueous extract exhibited just above 20% HIV-1 RT inhibition [31]

Anacardiaceae	*Ozoroa insignis* Delile	Nlungu	Root	Skin rashes	Sun-dried and pounded into powder. Mix ½ teaspoon of the powder of this plant with other measured medicinal plants in 2L of water.	Drink the decoction	Ethnomedicine for Tuberculosis, skin rashes and oral candidiasis in Rundu, East Region, Namibia [30]
		Nlungu	Root	Diarrhoea	Pounded to powder and added to cold water	Drink the decoction; a full cup	Ethnomedicine for Cough, diarrhea, genital warts, vaginal candidiasis, cancer, boils, gonorrhea, syphilis in Mbulu District [32]
Anacardiaceae	*Sclerocarya birrea* (A.Rich.) Hochst.	Nthula	Bark	Heart disease	Pounded into powder. Mix with other prescribed medicines and add boiling water.	Drink 2L in 4 days and go for another 2L	Ethnomedicine for diarrhoea and oral candidiasis in Rundu, East Region, Namibia [30]
		Nthula	Leaf	Skin rashes	Fresh leaves pounded and soaked in cold water alongside another prescribed medicinal plant. Add 1 tablespoon to 5 L of water and bath.	Bathe using the medicated water; once a day for 3 consecutive days.	

Apocynaceae	*Pergularia daemia* (Forssk.) Chiov.	Kgaba	Leaf	Skin rash	Sun-dried and pounded into powder. Add to bathing water.	Bathe using the medicated water; once a day	N/A
Asparagaceae	*Asparagus africanus* Lam.	Mohalatsha-maru	Root	STIs	Boiled while fresh or sun-dried then pounded into powder alongside other prescribed medicinal plants; add water.	Drink the decoction; 1 full cup; 3 times a day until the rash, or discharge come out and is cleared.	Antimicrobial activity against an array of strains; *Staphylococcus aureus, Salmonella gallinarum, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Candid a albicans*. [33-34]
		Liwulula	Root	STIs	Pound while fresh alongside other prescribed medicinal plants. Add burnt grass powder and mix in a 2L bottle of warm water.	Drink the decoction; ½ a cup; 3 times a day.	
		Liwulula	Root	Weight loss	Sun-dried and pounded to powder separately and then mixed with other medicinal plants in cold water.	Drink the decoction; ½ a cup	
		Liwulula	Root	STIs	Pounded and mixed with other prescribed medicines.	Drink the decoction; 3 times a day	

					Add ½ teaspoon of the powder to 2L of warm water.		
Asphodelaceae	*Aloe marlothii* A.Berger	Gonde	Leaf	STIs	Pounded fresh	Drink the sap	Ethnomedicine for sexually transmitted infections in South Africa [35]
		Gonde	Root	STIs	Boil the leaves while fresh or sun-dry and pound into powder. Add 1 teaspoon into 2L water. Roots: When fresh, boil the bundled roots in milk. When dry, boil the bundled roots in water.	Drink the decoction; one full cup; 3 times a day until the rash, or discharge come out and is cleared	
		Gonde	Leaf	Herpes	Cut fresh leaves into small pieces. Measure the pieces in a full teaspoon and soak in 2L cold water 2L	Drink the decoction; ½ a cup, 3 times a day	
		Gonde	Root and leaf	STIs	Leaf: Boil the leaves while fresh or sun-dry and pound into powder. Add 1 teaspoon into 2L water. Root: When fresh, boil the bundled roots in milk. When dry, boil the bundled roots in water.	Drink the decoction; 1 cup, 3 times a day	

Asphodelaceae	*Aloe zebrina* Baker	Gonde	Root	HIV	Cut the roots; boil and administer the medicated water to the patient	Drink the decoction; twice a day	Ethnomedicine for sexually transmitted infections in Limpopo, South Africa [36]
Asteraceae	*Laphangium luteoalbum* (L.) Tzvelev	Thitha	Root	Skin rashes	Sun-dried and pounded into powder. Add cold water	Drink the decoction; ½ a cup	N/A
		Thitha	Leaf	Malaria	Sun-dried and pounded into powder. Add cold water	Drink the decoction; ½ a cup	
		Thitha	Leaf	Covid-19	Sun-dried and pounded into powder. Add cold water	Drink the decoction; ½ a cup	
Boraginaceae	*Cordia monoica* Roxb.	Moarasupi/ntatabadzimu	Root	Cancer	Sun-dried and pounded into powder. Take ¼ teaspoon of the powder and add water and give to the patient	Drink the decoction; twice a day	N/A
Burseraceae	*Commiphora pyracantho*ides Engl.	Nsapo	Bark	Skin rashes	Pounded into powder. Add boiling water so as to steam and drink.	Steaming with the hot medicated water; Drink the decoction	N/A

Capparaceae	*Boscia albitrunca* (Burch.) Gilg & Benedict	Motlopi	Root	Diarrhoea; STIs; Kidney disorder	For Diarrhoea: Boiled in water while fresh. Secondly, boil roots and use the medicated water to make soft porridge For STIs & Kidney disorder: Boiled while fresh or sun-dried then pounded into powder and add water.	For Diarrhoea: Drink the decoction; 3 tablespoons, 3 times a day Also, eat the medicated soft porridge, once a day. For STIs: Drink the decoction; one full cup; 3 times a day until the rash, or discharge come out and is cleared. For Kidney disorder: Drink the decoction; one full cup; 3 times a day for 3 consecutive days.	Ethnomedicine for syphilis in Rundu, Kavango East Region, Namibia [30]
Celastraceae	Gymnospo ria	Nzhuzhu	Leaf	Skin rashes	Pound into paste while fresh; add to warm water; soak and	Bathing using the medicated water while	In-vitro HIV-1 PR inhibition using HIV-1

	senegalensis (Lam.) Loes.				use the medicated water to bath.	simultaneously and continuously rubbing the body with leaves; twice a day.	infected MT-4 cells; 88 µg/ml (IC50) [37]
		Nthunu	Root	Diarrhoea	Boil the roots and give the patient to drink or use the resultant medicated water to prepare soft porridge.	Drink the decoction; or taken with the prepared soft-meal; 3 times a day.	
		Sebete	Root	STIs	Boiled while fresh or sun-dried then pounded into powder and add water alongside other prescribed medicinal plants.	Drink the decoction; one full cup; 3 times a day until the rash, or discharge come out and is cleared.	
Combretaceae	*Combretum apiculatum* Sond.	Ntshingitshi	Root	STIs	Cut the fresh roots into small pieces and add cold water, alongside other prescribed medicinal plants.	Drink the decoction alongside other plants; ½ a cup, 3 times a day	N/A

Combretaceae	*Combretum imberbe* Wawra	Ngweti	Root	STIs	Roasted over fire and added to water alongside the powder of other multiple plants	Drink the decoction; ¼ of a cup; twice a day.	Ethnomedicine used to treat general STIs in Rundu, Kavango East Region, Namibia [30]
		Motswere	Root	Diarrhoea	Pounded and roasted into powder. Boil the powder in water and drink.	Drink the decoction; one-off administration.	
Combretaceae	*Terminalia prunioides* M.A.Lawson	Nthari	Root-bark	Diarrhoea	Taken as fresh. Slice the root-bark into pieces and continuously chew.	Chew the root-bark	Ethnomedicine for gonorrhoea, syphilis, HIV/AIDS in Zambia [38]
		Nthari	Fruit	Migraine Headache	Boil the dry or fresh fruit in water and drink as a tea.	Drink the decoction; 1 cup full, once a day until headache stops	
Combretaceae	*Terminalia randii* Baker f.	Bulimbogwenshashagwe	Fruit	Weight loss	Grind the fruits, sun-dry and then pound to powder. Add the powder of this plant alongside other plants to water as and when is needed	Add 2 full teaspoon of the powder to 1L of water and drink the decoction, 3 times a day	N/A

Combretaceae	*Terminalia sericea* Burch. ex DC.	Mogonono	Root	Diarrhoea	Boiled when fresh	Drink the decoction; ½ a cup; twice a day	In vitro inhibition of HIV-1 Reverse transcriptase (RT) activities; 7.2 µg/ml (IC50) [39]
		Nsusu	Root	Skin rashes	Sun-dry the roots and then pound to powder	The powder (¼ of the teaspoon) is added to soft-porridge and taken as a meal	
Crassulaceae	*Kalanchoe brachyloba* Welw. ex Britten	Setotojane	Root	STIs & Kidney disorder	Boiled while fresh or sun-dried then pounded into powder and add water.	For STIs: Drink the decoction; one full cup; 3 times a day until the rash, or discharge come out and is cleared. For Kidney disorder: Drink the decoction; one full cup; 3 times a day for 3 consecutive days.	N/A
Cucurbitaceae	*Coccinia adoensis* (Hochst. ex A. Rich.) Cogn.	Gatigati	Fruit & Root	Colic	Grind into powder; mix with other medicinal plants and add water. Also add a ¼ teaspoon of the powder to soft porridge and give to the patient to eat.	Drink the decoction; also taken with the prepared soft-porridge meal	N/A

Cucurbitaceae	*Kedrostis foetidissima* (Jacq.) Cogn.	Nkakabudze	Root	A combination of HIV-1 diagnostic symptoms	Sun-dried and pounded to powder. Add 2 table spoons of the powder to 2L of water	Drink the decoction; a full cup	Reported to have antimicrobial, antioxidative, anticancer, anti-inflammatory properties [40]
Dracanaceae	*Sansevieria aethiopica* Thunb.	Mosokelatsebe	Root	STIs	Boiled while fresh or sun-dried then pounded into powder and add water alongside other prescribed medicinal plants.	Drink the decoction; one full cup; 3 times a day until the rash, or discharge come out and is cleared.	N/A
Euphorbiaceae	*Croton gratissimus* Burch.	Mbagwe	Root	Weight loss	Grind the root, sun-dry and then pound to powder. Add the powder of this plant alongside other plants to water as and when is needed	Add 2 full teaspoon of the powder to 1L of water and drink the decoction, 3 times a day	Inhibition of HIV-1iib in vitro; 9.6 µg/ml (EC50) [41]

Euphorbiaceae	*Euphorbia schinzii* Pax	Thokabotshwaro	Branch	A combination of HIV-related symptoms and/or conditions	Soak in hot water	Drink the decoction; 1 table spoon, 4 times a day, alongside other prescribed medicinal plants.	N/A
		Gweshulephele	Fruit	STIs	Cut the fresh fruit into small pieces and add cold water, alongside other prescribed medicinal plants	Drink the decoction alongside other plants; ½ a cup, 3 times a day.	
		Tjigala-njiba	Root	STIs	Mash the roots and boil them in water, then use ½ cup of the boiled water to cook soft-porridge	Taken with the prepared soft-porridge meal; ½ a cup	
		Zwifozonke	Root	Paralysis or Arthritis & STIs	For Paralysis or Arthritis: Pounded fresh into powder. Add ½ teaspoon of powder alongside other measured medicinal plants in 2L of warm water and give to the patient to drink.	For Paralysis or Arthritis: Drink the decoction	

					For STIs: Cut and pounded fresh, sun-dried; sift into powder and mix with other medicinal plant in warm water; a full teaspoon	For STIs: Drink the decoction; ¼ of a cup	
		Gushulephele	Root	Persistent Cough	Cooked using milk or water	Drink the decoction; ½ a cup, twice a day	
Euphorbiaceae	*Euphorbia tirucalli* L.	Morwarwane	Root	STIs	Cut the fresh root into small pieces and add cold water, alongside other prescribed medicinal plants	Drink the decoction alongside other plants; ½ a cup, 3 times a day	*Ex vivo* and *in vitro* activation of HIV LTR in latently infected cells; Blocking of HIV replication in MT-4 cells and PBMCs; 0.02 and 0.09μM (EC50) respectively [42]
Euphorbiaceae	*Fluggea virosa* (Roxb. Ex	Nshangoma	Root	Herpes	Boil the roots in water. Let it cool down and give the patient to drink.	Drink the decoction; 2 times a day.	*in vitro* anti-HIV-1 activity using MT-4 cells; isolated alkaloids

	Willd.) Royle	Nshangoma	Root	A combination of HIV-related symptoms and/or conditions	Sun-dried and pounded to powder. Add 2 table spoons of the powder to 2L of water alongside other prescribed medicinal plants powder.	Drink the decoction; a full cup	exhibited EC50 values ranging from 7.8 to 122 μM [43]
Euphorbiaceae	*Tragia oka nyua* Pax	Mbabashulo	Root	STIs	Cut the fresh fruit into small pieces and add cold water.	Drink the decoction alongside other plants; ½ a cup, 3 times a day.	Ethnomedicine for dizziness and STIs in Namibia and Botswana [44-46]
		Mbabashulo	Root	Fontanelle disorder	Sun-dried and pounded to powder, then mixed with other medicines	Smoking around the head and inhaled as it burns in charcoal.	
		Mbabashulo	Root	Fontanelle disorder	Sun-dried and pounded into a powder. Add the powder to goat fat and inhale; also add some of the powder to soft-	Inhalation; also taken with the prepared soft-porridge meal	

					porridge to be eaten by the patient		
Fabaceace	*Phaseolus* vulgaris L.	Ipule	Seed	Sores/herpes (Wounds on sex organs)	Pound while fresh to make a paste of the medicine which would be stuck on the wound	Paste and smear over the sore	The isolated lectin inhibits about 95.4% HIV-1 replication by way of blocking Reverse Transcriptase (RT); 2.19 mg/ml (EC50) [47]
Fabaceae	*Acacia pur pusii* Bran degee	Nlalaanga	Bark	Cough and chest pains	Cut into small pieces and mixed with other medicinal plants. Add warm water as and when needed. Let it soak for 60 mins.	Drink the decoction; ½ a cup; 3 times a day	N/A
Fabaceae	*Albizia amara* (Roxb.) B.Boivin	Mbola	Root & Bark	Fontanelle dysfunction	Pounded and mixed with other prescribed medicines	Rub the medicine on soft fontanelle spot	N/A
Fabaceae	*Albizia ant helmintica* Brongn.	Nkadzibuku	Root	Erectile dysfunction & impotence	Sun-dry the roots and then pound into powder; mix this powder with the other 2 prescribed plants powders. Cook meat, scoop a cup full of	Drink the soup or have it as a normal meal alongside porridge; 3 times a day.	Ethnomedicine for HIV, herpes zoster, malaria in Lusaka, Zambia [48]

					broth/soup and add one teaspoon (1) of the medicinal powder.		
		Nkadzibuku	Bark	Cough; Fontanelle disorder	For Cough: Crush, when dry or fresh. Boil in water for the patient to steam. For Fontanelle disorder: Sun-dried and then burnt in charcoal. Smoke the burning medicine around the head of the patient.	For Cough: Steaming using the medicated water; 2 times a day. For Fontanelle disorder Inhalation as the medicine burns in charcoal; one-off administration	
		Nkadzibuku	Bark	Cough and chest pains	Sun-dried and pounded to powder separately and mixed with other medicinal plants. Add warm water as and when needed. Let it soak for 60mins.	Drink the decoction; ½ a cup, three times a day.	
		Nkadzibuku	Bark	Cough; Fontanelle disorder	For Fontanelle disorder: Sun-dried and then burnt in charcoal. Smoke the burning medicine around the head of	For Fontanelle disorder Inhalation as the medicine burns in	

					the patient. For Cough: Crush, when dry or fresh. Boil in water for the patient to steam.	charcoal; one-off administration. For Cough: Steaming using the medicated water; 2 times a day	
Fabaceae	*Arachis hypogaea* L.	Manongo	Seed	Colic	Sun-dry the roots and then pound into powder. Mix with other 2 prescribed medicinal plants	The powder mixture is added (½ a teaspoon) to soft-porridge and taken as a meal	N/A
Fabaceae	*Bauhinia petersiana subsp. macrantha* (Oliv.) Brummitt & J.H.Ross	Motjantje	Root	STIs	Cut the fresh root into small pieces and add cold water, alongside other prescribed medicinal plants.	Drink the decoction alongside other plants; ½ a cup, 3 times a day	N/A
Fabaceae	*Cassia abbreviata* Oliv.	Nshashanyama	Leaf	Frequent fevers	Pounded to powder together with the mixture of another plant; ¼ of the teaspoon is dissolved in 2L of water.	Drink the decoction; ½ a cup; twice a day	inhibition of HIV-1c (MJ4) replication;102.7 µg/ml (EC50) [49]

		Nshashanyama	Root	Back and waist pain	Sun-dried and pounded into powder. Mixed with another medicinal plants; boiled and the medicated water used to prepare soft-porridge.	Taken with the prepared soft-porridge meal; once daily for consecutive 2 days.	
		Nshashanyama	Root	Weight loss	Grind the fruits, sun-dry and then pound to powder. Add 2 full teaspoon of the powder of this plant to 1L of water alongside other plants to water as and when is needed.	Drink the decoction, 3 times a day.	
		Nshashanyama	Bark	Skin rashes & STIs	Sun-dried and pounded into powder. Mix the powder of this plant (a quarter of a teaspoon) with other measured medicinal plants in 2L of water.	Drink the decoction; twice a day.	
		Nshashanyama	Root	Blood cleansing; High	Used when both fresh or dry. Boil the fresh roots in water and give to the patient to drink. Alternatively, sun-dry the roots and pound into	Drink the decoction; ¼ a cup; 3 times a day	

				Blood Pressure	powder. Add 1 teaspoon of the powder into 1L of water.		
		Nshashanyama	Bark	STIs	Pound while fresh alongside other prescribed medicinal plants. Add burnt grass powder and mix in a 2L bottle of warm water.	Drink the decoction; ½ a cup; 3 times a day	
Fabaceae	*Colophospermum mopane* (Benth.) Leonard	Mpani	Root	Diarrhoea	Boiled when fresh	Drink the decoction; ½ a cup; twice a day.	Ethnomedicine for Chlamydia in Blouberg, South Africa [50]
		Mpani	Root	Diarrhoea	Boil the roots alongside a bundle of other prescribed medicinal plants.	Drink the decoction; ½ a cup, 2 times a day	
		Mophane	Root	Diarrhoea; STIs	For Diarrhoea: Boiled in water while fresh. Secondly, boil roots and use the medicated water to make soft porridge.	For Diarrhoea: Drink the decoction; 3	

					For STIs: Boiled while fresh or sun-dried then pounded into powder and add water	tablespoons, 3 times a day Also, eat the medicated soft porridge, once a day. For STIs: Drink the decoction; one full cup; 3 times a day until the rash, or discharge come out and is cleared.	
Fabaceae	*Dalbergia melanoxylon* Guill. & Perr.	Mbezhela-phanga	Root	A combination of HIV-related symptoms and/or conditions	Sun-dried and pounded to powder. Add 2 table spoons of the powder to 2L of water alongside other prescribed medicinal plants powder.	Drink the decoction; a full cup	Reported to have antimicrobial activities against different strains including *E. coli, S. aureus, S. typhimurium, K. pneumoniae, B. subtilis*. For example, the ethanolic extract showed MIC of 48 µg/ml and 100 µg/ml against *B. subtilis* and *E. coli* respectively [51]

Fabaceae	*Dichrostachys cineria* (L.) Wight & Arn.	Moselesele o monnye	Root	STIs	Boiled while fresh or sun-dried then pounded into powder and add water alongside other prescribed medicinal plants.	Drink the decoction; one full cup; 3 times a day until the rash, or discharge come out and is cleared.	Ethnomedicine for general STI syndromes especially syphilis, in Zambia [38]
		Mpangale	Seed	Persistent cough	Roasted and then pounded into powder alongside another prescribed herbal plant. Add coarse salt to some powder; alternatively, add honey.	Lick the powder mixture on a base of a teaspoon	
Fabaceae	*Elephantorrhiza elephantina* (Burch.) Skeels	Tjizezana	Root	Uterus cleansing	Pounded then sun-dried. Add the measured powder to soft-porridge and take the meal. Also, add the powder to tea without milk and drunk.	Taken with a meal in soft-porridge; ½ a teaspoon; once a day in the morning. Also, drink the decoction; ½ a teaspoon; once a day at the evening.	*In vitro* inhibition of Reverse Transcriptase (RT) enzyme [31]
		Ndamba	Root	Colic	Sun-dry the roots and then pound into powder. Mix with	The powder mixture is added (½ a teaspoon) to	

					other 2 prescribed medicinal plants.	soft-porridge and taken as a meal.	
		Tjizezana	Root	Diarrhoea	Pounded to powder and add cold water.	Drink the decoction; a full cup.	
		Tjizezana	Root	Frequent fevers	Pound the roots alongside other medicinal plants. Then cook the plant's mixture with animal's spleen while it is still dripping blood. Give the patient.	Drink the medicine; twice a day.	
Fabaceae	*Erythrina lysistemon* Hutch.	Mpiti	Bark	Persistent cough	Sun-dry the bark and then pound to powder. Then boil as and when is needed	Drink the decoction; ½ a cup 3 times a day.	N/A
Fabaceae	*Indigofera tinctoria* L.	Nkandilo	Root	Stomach pains	Roots are tied and boiled in water and poured into 2L bottle.	Drink the 2L decoction; one full cup, once a day.	Blocked HIV-1 (III B) and HIV-2 (ROD) replication in MT-4 cells, with EC50 values of 113 and 125 µg/ml, respectively [52]
		Nkandilo	Root	Fontanelle disorder	Sun-dried and pounded into a powder. Add the powder to goat fat and inhale; also add some of the powder to soft-	Inhalation; also taken with the prepared soft-porridge meal.	

					porridge to be eaten by the patient.		
		Nyadza	Root	Skin rashes; STIs & a combination of HIV-related symptoms and/or conditions	Pounded to powder. For skin rashes: Add the powder (a quarter of a teaspoon) to the prepared soft-porridge; For STIs: Add 2 spoons of the powder to 2L cold water; For combined symptoms: Add 2 table spoons to 2L of water.	For skin rashes: Eat the medicated soft porridge; For STIs: Drink the decoction; half a cup, three times a day; For combined symptoms: Drink the decoction; a full cup.	
Fabaceae	*Otoptera burchellii* DC	Mpingambizi	Root	Erectile dysfunction	Fresh roots are boiled alongside other plants.	Drink the decoction; ½ a cup, 3 times a day.	N/A
		Mpingambizi	Root	Diarrhoea; STIs	Sun-dried or used as fresh. When fresh; the roots are boiled and given to the patient. When sun-dried; pound into powder, take ¼	For Diarrhoea: Drink the decoction; ½ a cup, 3 times a day. For STIs: Drink the decoction; a full cup	

					teaspoon of the powder and add to warm water.		
		Tshikadithata/Mosegawapoo	Root	Diarrhoea	Boil roots in water to drink. Also, use the medicated water to make porridge	Drink the medicated water; 3 tablespoons, 3 times a day. Also, eat porridge once a day	
		Mosegawapoo	Root	Prostate cancer	Pounded to powder together with another plant. Take the powder (1 full teaspoon) and add to water in full a cup.	Drink the decoction; one cup daily; for 5 consecutive days	
Fabaceae	*Peltophorum africanum* Sond.	Nzeze	Root & Bark	Paralysis & Arthritis	Pounded fresh into powder. Add ½ teaspoon of powder alongside other measured medicinal plants in 2L of warm water and give to the patient to drink.	Drink the decoction	In vitro activity against enzymatic functions of HIV-1 Reverse transcriptase (RT); anti-HIV-1NL4-3 and HIV-1JRCSF replication; 8.0 µg/ml (IC50) [53, 54,55] Ethnomedicine for gonorrhea, HIV,

							tuberculosis, diarrhoea in Lusaka, Zambia [48]
Fabaceae	*Philenoptera violacea* (Klotzsch) Schrire	Mpanda	Bark	Cough and chest pains	Sun-dried and pounded to powder separately and mixed with other medicinal plants. Add warm water as and when needed. Let it soak for 60mins.	Drink the decoction; ½ a cup, 3 times a day	N/A
Fabaceae	*Rhynchosia minima* (L.) DC.	Nzunguza	Root	Erectile dysfunction	Fresh roots are boiled alongside other plants	Drink the decoction; ½ a cup, 3 times a day	N/A
Fabaceae	*Rhynchosia totta* (Thunb.) DC.	Nkonondoga	Root	Erectile dysfunction	Fresh roots are boiled alongside other plants	Drink the decoction; ½ a cup, 3 times a day	N/A
Fabaceae	*Senegalia nigrescens* (Oliv.) P.J.H. Hurter	Nkosho	Bark	Snake bite	Cut the bark into three parts and pierce with wooden nail. Boil the pierced bark in water	Massage the beaten area using the hot medicated water; Drink the remaining decoction	N/A

Fabaceae	*Vachellia nilotica* (L.) P.J.H. Hurter & Mabb.	Nsu-ntema	Seed	Thrush	Pounded into paste	Paste and apply on the affected area	HIV-1 Reverse Transcriptase inhibition [56]
Fabaceae	*Xanthocercis zambesiaca* (Baker) Dumaz-le-Grand	Mbengawonye	Root	Blood Cleansing	Sun-dry the roots and them pound to powder. Add half a teaspoon to 2L of cold water.	Drink the decoction for 2-3 times a day.	Antimycobacterial activity against *Mycobacterium smegmatis*; with a minimum inhibitory concentration (MIC) value of 0.106 mg/ml [57]
		Mbengawonye	Root & Leaf	Weight loss	Grind the leaves and roots, sun-dry and then pound to powder. Add the powder of this plant (alongside a mixture of other plants) to water as and when is needed.	Add 2 full teaspoons of the powder to 1L of water and drink the decoction, 3 times a day.	
		Mbengawonye	Root & Bark	Cough and chest pains	Pounded fresh and sun-dried; sift into powder and mixed with other prescribed and measured medicinal plant in water; ½ a teaspoon each.	Drink the decoction; ½ a cup, twice a day.	

		Mbengawonye	Root	Cough and chest pains	Sun-dried and pounded into powder and mixed with other medicinal plants. Add ½ teaspoon to 1L of “Mageu” (Fermented maize meal) or in a small bowl of porridge	Drink the decoction if it is with “Mageu”; or eat the meal if it is soft porridge	
Hyacinthaceae	*Drimia sanguine*a (Schinz) Jessop	Tjinyami	Root	STIs	Mix the plant’s fruit with other prescribed medicines (a butterfly) in warm water.	Drink the decoction; ½ a cup, once a day.	Ethnomedicine for Chlamydia in Blouberg South Africa [50]
		Sekaname se se hibidu	Root	STIs	Boiled while fresh or sun-dried then pounded into powder and add water alongside other prescribed medicinal plants.	Drink the decoction; one full cup; 3 times a day until the rash, or discharge come out and is cleared.	
Hyacinthaceae	*Ledebouria cooperi* (Hook.f.) Jessop	Phalalume	Root	STIs and Herpes	Pound while fresh alongside other prescribed medicinal plants. Add burnt grass powder and mix in a 2L bottle of warm water.	Drink the decoction; ½ a cup; 3 times a day	N/A

		Phalalume	Fruit	STIs	Cut and pounded fresh, sun-dried; sift into powder and mix with other medicinal plant in warm water; ½ a teaspoon.	Drink the decoction; ¼ of a cup	
		Phalalume	Root	Weight loss	Sun-dried and pounded to powder separately and then mixed with other medicinal plants in cold water	Drink the decoction; half a cup	
Hyacinthaceae	*Ornithogalum seineri* (Engl. & K.Krause) Oberm.	Tjinyami	Root	Blood cleansing; High blood pressure	Pounded into paste; boil in water	Drink the decoction; ½ a cup, twice a day	N/A
Lamiaceae	*Clerodendrum serratum* (L.) Moon	Sedupapula	Root	Diarrhoea; STIs	For Diarrhoea: Boiled in water while fresh. Secondly, boil roots and use the medicated water to make soft porridge For STIs: Boiled while fresh or sun-dried then pounded into powder and add water.	For Diarrhoea: Drink the decoction; 3 tablespoons, 3 times a day Also, eat the medicated soft porridge, once a day. For STIs: Drink the decoction; one full cup; 3 times a day until the	N/A

						rash, or discharge come out and is cleared.	
Lamiaceae	*Leonotis nepetifolia* (L.) R.Br.	Shababagulu	Stem & Fruit	Colic (Uterus disorders)	Grind into powder; mix with other medicinal plants and add water. Also add a ¼ teaspoon of the powder to soft porridge and give to the patient to eat.	Drink the decoction; also taken with the prepared soft-porridge meal	N/A
Lamiaceae	*Ocimum americanum* L.	Tothodzani	Whole plant	Frequent fevers	Pound and /or grind fresh plant and added to bathing water.	Bathing so as to apply the medicine all over the body.	N/A
		Ntatabadzimu	Root & Leaf	Paralysis & Arthritis	Pounded fresh and mixed with other prescribed medicinal plants. Boil in water.	Bath and massage the patient using the medicated water	
Lamiaceae	*Vitex zeyheri*	Ntalabani	Root	Erectile dysfunctio	Sun-dry the roots and then pound into powder; mix this	Drink the soup or have it as a normal meal	N/A

	Sond. Ex Schauer			n & impotence	powder with the other 2 prescribed plants powders. Cook meat, scoop a cup full of broth/soup and add one teaspoon (1) of the medicinal powder.	alongside porridge; 3 times a day.	
		Ntalabani	Root	Skin rashes	Sun-dry the roots and then pound to powder	The powder (¼ of the teaspoon) is added to soft-porridge and taken as a meal.	
Loranthaceae	*Agelanthus lugardii* (N.E.Br.) Polhill & Wiens	Bulimbo gwe nkosho (palamela)	Branch	STIs	Pound some of it fresh and roast some. Crash roasted medicine into powder.	Smear the powder on the genital rash/sore	N/A
Loranthaceae	*Erianthemum ngamicum* (Spragueue) Danser	Bolimbo-gwe-mpani	Leaf	Diarrhoea	Sun-dried or used as fresh. When fresh; the roots are boiled and given to the patient. When sun-dried; pound into powder, take a quarter of	Drink the decoction; a quarter, 3 times a day	N/A

					teaspoon and add to warm water.		
		Bolimbo-gwe-mpani	Leaf	Diarrhoea	Pound the leaves, add cold water and give to the patient to drink.	Drink the decoction	
Lyrthraceae	*Punica gra natum* L.	Garenati	Root	Diarrhoea	Boil the roots and then cool down.	Drink the decoction; ½ a cup, 3 times a day	HIV-1 entry inhibition; ED50 (endpoints for 50% inhibition of infection) in the range of 1/16 and 1/64 serial dilutions. Also blocked the interaction of the virus with the CD4 receptor together with co-receptors [58]
Malvaceae	*Adansonia digitata* L.	Mbuyu/ mowana	Bark	Frequent fevers	Pounded to powder together with the mixture of another plant; ¼ of the teaspoon is dissolved in 2L of water	Drink the decoction; ½ a cup; twice a day	*In vitro* inhibition of HIV-1 reverse transcriptase; 26.5% inhibition by 50 µg/ml root-bark methanolic extract.

							Anti-HIV-1 protease (PR) activity; 75% and 74% inhibition by 50 µg/ml leaf and fruit extracts, respectively [59]
Malvaceae	*Sida spinosa* L.	Leheto la basadibagolo	Root	Diarrhoea	Boil roots in water to drink alongside other prescribed medicinal plants. Secondly, boil the mixed roots and use the water to make porridge.	Drink the medicated water; 3 tablespoons, 3 times a day. Also, eat porridge once a day	Ethanolic extracts displayed concentration-dependent antibacterial activity against a wide array of bacteria including; S*taphylococcus aureus, Bacillus subsitlis, Pseudomonas aeroginosa, Escherichia coli, Candida albicans & Aspergillus nige* [60-61]
Myrothamnaceae	*Myrothamnus flabellifolia* Welw.	Nfadzimuke	Branch	Stroke	Pounded into powder and mixed with other medicinal plants. Add water. The powder is also mixed with Vaseline	Drink the decoction; any preferred quantity, once a day The powder-vaseline paste is applied to the skin to relax the nerves, veins and arteries.	*In vitro* inhibition of HIV-1 reverse transcriptase activity; 34 µM (IC50) [62]

		Nfadzimuke	Leaf	Weight loss	Soak leaves in hot water. Scoop a tablespoon of mixture to a bowel of porridge feed to the patient.	Taken with the prepared soft-porridge meal.	
		Mafavuke	Whole plant	HIV	Boiled and mixed with FG-branded tea leaves (which are bought over the counter) and condensed milk. Allow the medicine to cool and give to patient to drink.	Drink the decoction; full cup; 3 times a day	
Olacaceae	*Ximenia americana* L.	Moretlologa	Root	STIs	Boiled while fresh or sun-dried then pounded into powder and add water alongside other prescribed medicinal plants.	Drink the decoction; one full cup; 3 times a day until the rash, or discharge come out and is cleared.	*In vitro* inhibition of both HIV-1 (IIIB) strain (EC50: 8.3.1. µg/ml) and HIV-2 (ROD) strain (EC50:27.1µg/ml) replication on MT-4 cells. [63]
		Nswanja bakhwa	Root	Diarrhoea; Menstrual cycle disorders including	For Menstrual cycle disorders: Cut the root into 3 pieces and then tie using the bark of another medicinal plant.	For Menstrual cycle disorders: Drink the decoction 3 days; 1 full cup, 2 times a day. Then dump the remnants into a	

				excessive bleeding	Add water. For Diarrhoea: Scrap-off the root-bark and add cold water to it.	designated hole. For Diarrhoea: Drink the decoction; ½ a cup, 4 times a day	
Passifloroideae	*Adenia gla uca* Schinz	Monna-ga-apara	Tuber/b ud	Weight loss	Pounded into powder. Boil the powder with milk. Alternatively, add the powder to soft porridge.	Drink the decoction or eat the medicated soft-porridge meal; twice a day	N/A
Pedaliaceae	Dicerocary um eriocar pum (Decn e.) Abels	Igogo	Root	Corona & respiratory disorder (difficulty in breathing)	Sun-dried and pounded into powder. Take ¼ teaspoon of the powder and add water and give to the patient	Drink the decoction; twice a day	N/A
Pedaliaceae	*Harpagoph ytum procu mbens* var. *sublobatu m* (Engl.) Stapf	Sengaparile	Fruit	HIV	Cooked to prepare thin meat soup or boiled to prepare medicated water — alongside another medicinal plant	Drink the decoction; 3 times a day	Ethnomedicine for HIV/AIDS and headache in DRC, South- Africa, Tanzania and Nigeria [64-65]

Phyllanthaceae	*Bridelia mollis* Hutch.	Nfufu	Bark	Headaches	Sun-dried and lit on some fire once needed	Smoked & inhaled through nostrils	N/A
Plumbaginaceae	*Plumbago zeylanica* L.	Matsogomabe	Root	Skin rashes & STIs	Pounded fresh into paste and mixed with other measured medicinal plants in bathing water. For STIs: Sun-dried and pounded to powder. Mix ¼ teaspoon of the powder with other measured medicinal plants in 2L of water.	For skin rashes: Bathing; sized with a single table spoon in 5L of water. For STIs: Drink the decoction.	*ex vivo* blockage of HIV-1c (MJ4) p24 antigen using human PBMCs; 150 µg/ml) exhibited 90.0 ± 2.9% viral replication inhibition [49]
		Matsogomabe	Root and Fruit	Persistent Cough	Cooked and prepared using goat milk.	Drink the decoction; ½ a cup, once a day	
		Matjisa	Root	Cough and chest pains	Sun-dried and pounded into powder and mixed with other medicinal plants. Add ½ teaspoon to 1L of “Mageu” (Fermented maize meal) or in a small bowl of porridge.	Drink the decoction if it is with “Mageu”; or eat the meal if it is soft porridge.	

		Matsogomabe	Root	STIs	Pounded and mixed with other prescribed medicines. Add ½ teaspoon of the powder to 2L of warm water.	Drink the decoction; 3 times a day	
Rhamnaceae	*Ziziphus mucronata* Willd.	Mokgalo	Root	STIs	Boiled while fresh or sun-dried then pounded into powder and add water alongside other prescribed medicinal plants.	Drink the decoction; one full cup; 3 times a day until the rash, or discharge come out and is cleared.	Ethnomedicine for Gonorrhoea, syphilis boils, pneumonia, cough in Livingstone, Southern Province, Zambia. [38]
		Ntjetjeni	Root	Back and waist pain	Used while fresh. Mixed with the sun-dried powder of another medicinal plant; Boil and use the resultant medicated water to prepare soft-porridge.	Taken with the prepared soft-porridge meal; once daily for 2 consecutive days.	
		Ntjetjeni	Leaf	Sores/herpes (Wounds on sex organs)	Pound while fresh to make a paste of the medicine which would be stuck on the wound.	Paste and smear over the sore	

Rubiaceae	*Gardenia volkensii* K.Schum.	Ntala/ Nkwakwasi	Fruit	Persistent cough	Roasted and then pounded into powder alongside another prescribed herbal plant. Add coarse salt to some powder; alternatively, add honey.	Lick the powder mixture on a base of a teaspoon.	N/A
		Ntala	Fruit	Persistent cough	Cut and crash. Sun-dry, pound into powder and sift. Mix with other prescribed and measured medicinal plants powder.	Orally administer the powder to patient. For children: ¼ teaspoon of the powder, 2 times a day; add glycerin to improve taste. For adults: ½ teaspoon of the powder; twice a day.	
		Ntala	Root and Fruit	Persistent cough	Pound the roots into powder and roast fruits, then pound into a powder too; then mix the two powders	Lick the powder (¼ of the teaspoon); 3 times a day	

Rubiaceae	*Vangueria infausta* Burch.	Nzwigwa	Root	A combination of HIV-related symptoms and/or conditions	Sun-dried and pounded to powder. Add 2 table spoons of the powder to 2L of water alongside other prescribed medicinal plants powder.	Drink the decoction; a full cup	Ethnomedicine for Coughs in Rundu, Kavango East Region, Namibia [30]
Rutaceae	*Vepris za mbesiaca* S. Moore	Ndamoyo	Root	Loss of Appetite; Fatigue	Boil fresh roots and give the patient the medicate water to drinks. Sun-dry and pound into powder. Then add to porridge.	Drink the decoction; ¼ a cup; 3 times a day. Add ¼ a teaspoon and eat porridge; twice a day.	N/A
		Ndamoyo	Root	Weight loss	Sun dried and pounded to powder.	Lick the powder (¼ of the teaspoon)	
		Ndamoyo	Root	Frequent fevers	Pound the roots alongside other medicinal plants. Then cook the plant's mixture with animal's spleen while it is still dripping blood. Give the patient to drink.	Drink the medicine; twice a day	

		Ndamoyo	Root	Fontanelle disorder	Sun-dried and pounded into a powder. Add the powder to goat fat and inhale; also add some of the powder to soft-porridge to be eaten by the patient	Inhalation; also taken with the prepared soft-porridge meal	
Scrophulariaceae	*Aptosimum lineare* Marloth & Engl.	Makgonatsotlhe	Whole plant	A combination of HIV-related symptoms and/or conditions	Uproot the whole plant, chop and boil in water	Drink the decoction; 1 full cup; 3 times a day	N/A
Simaroubaceae	*Kirkia acuminata* Oliv.	Modumela	Leaf & Root	Persistent cough; Cough and chest pain	Boil fresh roots and leaves together. Also, sun dry both roots and leaves, pound into powder and add into 2L of boiled water	Drink the decoction; ½ a cup, 3 times a day for 5 consecutive days	Ethnomedicine for Diarrhoea in Lusaka, Zambia [48]

Solanaceae	*Datura stramonium* L.	Nfutenbwa	Leaf	STIs, heart disease and mental disorder	Boil the leaves in water and the patient drinks the medication	For STIs: Drink the decoction; ½ a cup; twice a day For heart disease & mental disorder: Drink the decoction; ½ a teaspoon	Ethnomedicine for Chlamydia in Blouberg, South Africa. [50]
Solanaceae	*Solanum incanum* L.	Duthugwagulu	Root	Frequent fevers	Pound and/or grind fresh roots and add cold water	Drink the decoction; ½ a cup, one time a day for 3 consecutive days.	Ethnomedicine for skin infections, skin cancer in Rundu, Kavango East Region, Namibia. [30]
		Duthugwagulu	Root	STIs	Pounded and mixed with other prescribed medicines. Add ½ teaspoon of the powder to 2L of warm water	Drink the decoction; 3 times a day	
		Morolwana omonye	Root	STIs & Kidney disorder	Boiled while fresh or sun-dried then pounded into powder and add water alongside other prescribed medicinal plants.	For STIs: Drink the decoction; one full cup; 3 times a day until the rash, or discharge come out and is cleared. For Kidney disorder: Drink the decoction; one full cup; 3 times a day	

		Duthugwagulu	Root	Persistent cough	Roast the roots over fire, pound to powder and mix with other medicinal plants and sometimes with honey.	Take the powder and swallow; small amount once a day.	
		Duthugwagulu	Root	Cancer	Sun-dried and pounded into powder. Take ¼ teaspoon of the powder and add water and give to the patient.	Drink the decoction; twice a day.	
		Duthugwagulu	Leaf & Root	Frequent fevers	Pound and /or grind the fresh leaves and roots and add the medicine to bathing water.	Bathing so as to apply the medicine all over the body.	
		Duthugwagulu	Root	STIs	Cut the fresh roots into small pieces and pound into paste. Soak in warm water.	Drink the decoction; ½ a cup; 3 times a day.	
		Thuthugwana/tholwana	Root & Leaf	Migraine (Headache); Colic	Sun-dried and pounded into powder. Add cold water and give the patient to drink.	Drink the decoction; twice a day.	

		Thululi	Root	Skin rashes & STIs	For Skin rashes: Pounded fresh and mixed with other measured medicinal plants in bathing water For STIs: Sun-dried and pounded to powder. Take ¼ teaspoon of the powder and mix with other plants in water	For Skin rashes: Bathing; sized with a single table spoon in 5L of water. For STIs: Drink the decoction	
Solanaceae	*Solanum panduriforme* E. Mey	Nthuthugwana	Root	Diarrhoea	Sun-dried or used as fresh. When fresh; the roots are boiled and given to the patient. When sun-dried; pound into powder, take ¼ teaspoon of the powder and add to warm water.	Drink the decoction; ½ a cup, 3 times a day.	Ethnomedicine for diarrhoea infections in Sesheke District, Western Province, Zambia [66] Ethnomedicine for gonorrhoea in Limpopo Province, South Africa [67]
		Nthuthugwana	Root	Stomach pains	Roots are tied and boiled in water and poured into 2L bottle.	Drink the 2L decoction; one full cup, once a day.	

		Nthuthugwa	Fruit & Root	Persistent cough	Cut and crash. Sun-dry, pound into powder and sift. Mix with other prescribed and measured medicinal plants powder	Orally administer the powder to patient. For children: ¼ teaspoon of the powder, 2 times a day; add glycerin to improve taste. For adults: ½ teaspoon of the powder; twice a day.	
		Nthuthugwana	Root	Fontanelle disorder	Sun-dried and pounded into a powder. Add the powder to goat fat and inhale; also add some of the powder to soft-porridge to be eaten by the patient.	Inhalation; also taken with the prepared soft-porridge meal.	
Strychnaceae	*Strychnos cocculoides* Baker	Ntamba/ mogorogorwana	Root	STIs	Pounded into powder and mixed with other medicinal plants and added to water	Drink the decoction; ¼ of a cup; twice a day	Ethnomedicine for HIV/AIDS related infections in Zambia [48]
Tiliaceae	*Grewia damine* Gaertn.	Ntewadani	Root	STIs	Cut the fresh fruit into small pieces and add cold water	Drink the decoction alongside other plants; ½ a cup, 3 times a day	Ethnomedicine for treating bacterial infections including syphilis [68-69]

Velloziaceae	*Xerophyta retinervis* Baker	Nthuthe	Whole plant	Persistent cough and Tuberculosis	Pounded to powder. Dissolve the powder (½ of the teaspoon) in 2L of water, together with the mixture of another plant	Orally administer the medicated water; ½ a teaspoon; twice a day	N/A
Verbenaceae	*Lippia javanica* (Burm.f.) Spreng.	Nvavani	Leaf	Malaria	Boil fresh or dry leaves in water and give the medicated hot water to the patient to drink as tea. The patient may or may not add sugar.	Drink the medicated water when warm. ½ a cup, 2 times a day.	Ethnomedicine for Malaria, Fever, Influenza, lung infections in South Africa. [70]
		Nvavani	Root & Leaf	Cough and chest pains	Pounded fresh and sun-dried; sift into powder. Mix ½ teaspoon of the powder of this plant with other measured medicinal plants in water.	Drink the decoction; ½ a cup, twice a day	

		Nvavani	Leaf	Persistent Cough	Pounded into powder and mixed with other medicinal plant	Take the medicine orally; 1 table spoon; 3 times a day	
Vitaceae	*Cyphostemma cirrhosum* (Thunb.) Desc. Ex Wild & R.B.Drumm.	Nlima	Root	STIs	Pounded into powder and mixed with other medicinal plants and added to water.	Drink the decoction; ¼ of a cup; twice a day	N/A
		Besu-le-pkhwizi	Root	Skin Rashes	Sun-dry and then pound to powder	The powder (a quarter of the teaspoon) is added to soft-porridge and taken as a meal.	
		Besu-le-pkhwizi	Root	Blood cleansing	Sun-dry and then pound to powder	Add half a teaspoon to 2L of cold water; Drink the decoction for 2-3 times a day.	

From these interviews with 13 THPs, we documented 83 plants from 39 taxonomical families (**Table 3.2; Fig 3.2**). Plants of the family Fabaceae were most frequently cited (21 plants, or 25.0%), followed by plants of the family Euphorbiaceae (5 plants, or 6.0%) (**Fig 3.2)**. Less cited plant families included Boranginaceae, Amaranthaceae, Passifloroideae, Simaroubaceae, Velloziaceae, Capparaceae, which represented 1 or 1.2% of plants each. THPs most frequently reported use of plant roots in their ethnomedicine (60, 53.1%), followed by leaves, bark and fruits, at 17 (15.0%), 13 (11.5%) and 10 (8.8%) respectively, with tubers and stems used the least (1, 0.9%) **(Fig. 3.3**).

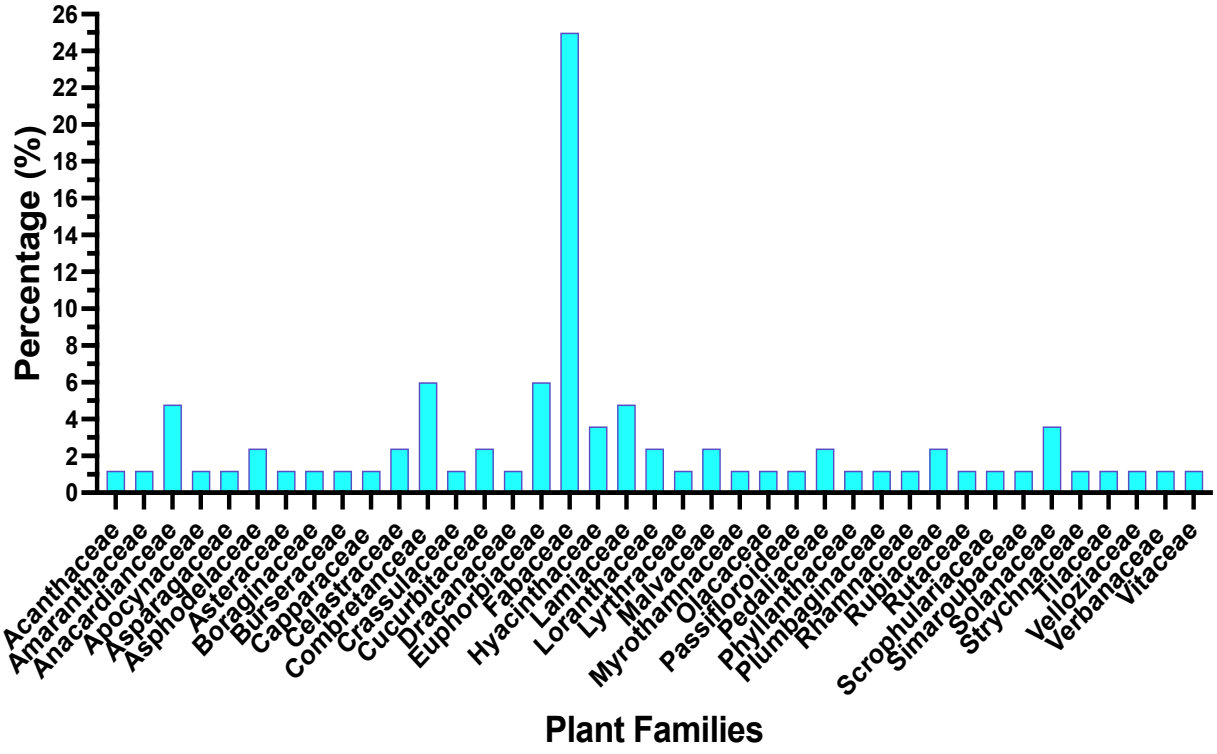

Figure 3.2: Percentage of the plant families used for management of HIV/AIDS-related diseases and other health conditions in Tutume subdistrict (Central Botswana, Africa)

The thematic analysis of our data **(Table 3.3**), indicated that in choosing which medicinal plant is fresh enough to be picked and used for medicine, 12 out of 13 (92.3%) THPs in Tutume subdistrict consider maturity of the plant as one of the indicators; 3 (23.1%) of the THPs mention that they can also taste the plant while 5 (38.5%) of them claim they may also use leaf colour as a determinant factor for freshness of use. Whereas storage of

medicines may differ from one plant to another, most of the THPs (92.3 in%) in this area say they store their medicines as powder; 4 (30.8%) said they store their medicines in liquid form depending on the type of the medicinal plant. Thirty-eight percent (38.5%, or 5 out of 13) sun-dry the plant parts and keep them for use while 15.4% or 2 of the 13 THPs use their medicine fresh from collection and this could differ from one plant to another and the disease being treated. In terms of sources of medicinal plants (theme 3), 100% of THPs say they collect some of their medicines from the bush; however, 12 (92.3%) THPs grow at least a portion of their medicinal plants themselves, while 30.8% (4 out of 13) also buy from markets. Another theme that arose from data analysis was seasonality of harvesting for TCMs, where 12 or 92.3% of the THPs harvest their medicines in summer, 1 out of 13 (8.3%) THPs said harvesting of medicinal may also occur in winter depending on the type of the plants needed, and 1 (8.3%) THP mentioned that harvesting is conducted throughout the year. The fifth (5^{th}) theme was availability and scarcity of medicinal plants, where 12 (92.3%) of THPs said availability of medicinal plants is seasonal (during rainy season), while the THP (8.3%) who harvests TCM throughout the year, mentioned that medicinal plants are available throughout the year. Additionally, 5 out 13 (38.5%) THPs indicated that the plants they use in their TCM are scarce, while 46.2% (6) said the plants are easily available. THPs defined scarcity arose from several factors including environmentally unfriendly harvesting habits, including excessive harvesting by healers themselves (1, 8.3%), lack of underground ground water (1, 8.3%), and overgrazing by animals (1, 8.3%). All THPs (100%) were interested in working and cooperating with biomedical researchers and clinicians to advance their ethnomedicines through scientific research and collaborations, with a primary goal to improve the primary health care and the lives of their patients. They want to be helped, where possible, with the planting and growing of medicinal plants which are either increasingly becoming scarce or are officially endangered species so that they are available for harvest and use throughout the year.

Table 3.3: Thematic analysis of the TCM qualitative data from THPs in Tutume subdistrict

Sub-themes	**(No.) of THPs**	**Percentage (%) of THPs**
Theme 1: Identification criteria for freshness of the medicinal plants		
Maturity	12	92.3%
Leaf colour	5	38.5%
Taste	3	23.1%
Theme 2: Storage of Medicines		
Dry	5	38.5%
Liquid	4	30.8%
Powder	12	92.3%
Used as fresh straightway	2	15.4%
Theme 3: Sources for the medicinal plants		
Buy from markets	4	30.8%
Collect from the field	13	100%
Grow for themselves	12	92.3%
Theme 4: Seasonality of harvesting medicinal plants		
Summer (rainy season)	12	92.3%

All year round	1	8.3%
Winter	1	8.3%
Theme 5: Availability and scarcity of medicinal plants		
Seasonal (summer and winter)	12	92.3%
All year round	1	8.3%
Easily available	6	46.2%
Not easily available	9	69.2
Scarce	5	38.5%
Environmentally unfriendly harvesting methods, including excessive harvesting by healers	1	8.3%
Insufficient underground ground water	1	8.3%
Overgrazing by animals	1	8.3%
Theme 6: Interest in working with biomedical researchers and clinicians		
Interested:	13	100%
Not interested	0	0%

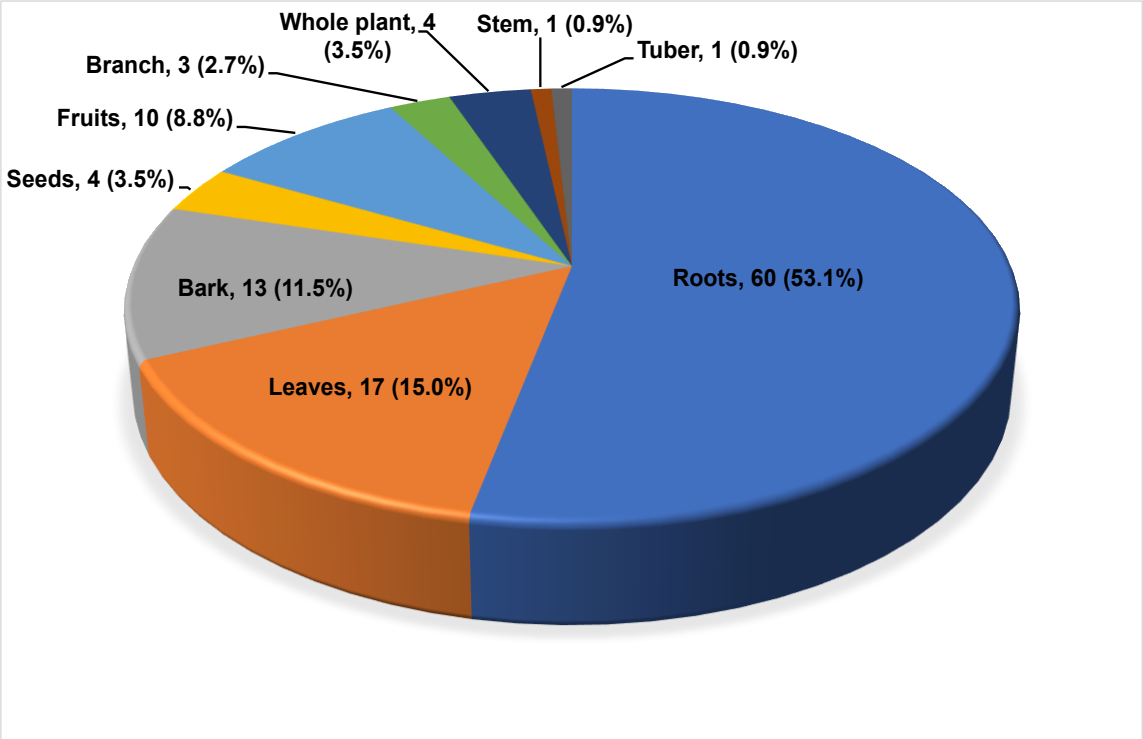

Figure 3.3: Frequency distribution of medicinal plant parts used to manage HIV/AIDS-related diseases and other health conditions in Tutume subdistrict (Central Botswana, Africa): N (%)

Of the 83 plants documented in this study, 32 (38.6%) were used to treat STIs. 4 (4.8%) were used as specific regimens for HIV-1 directly, while 16 (19.3%) were used specifically for diarrhoea which is part of the HIV-1 diagnostic symptoms. Nine (10.8%) and 7 (8.4%) were used to treat weight loss and frequent fever, respectively, while 17 (20.5%) were used to treat persistent cough and/or cough that is accompanied by chest pains. Finally, 30 (36.1%) were used to treat various other health conditions unrelated to HIV/AIDS, which THPs sometimes attend to **(Fig. 3.4).** Additionally, we mention that some of the medicinal plants identified in this study are used in mixtures, whereas others are administered as single-plant medicine depending on the healer's medicinal knowledge, the health condition being treated and their individual approach to primary health care.

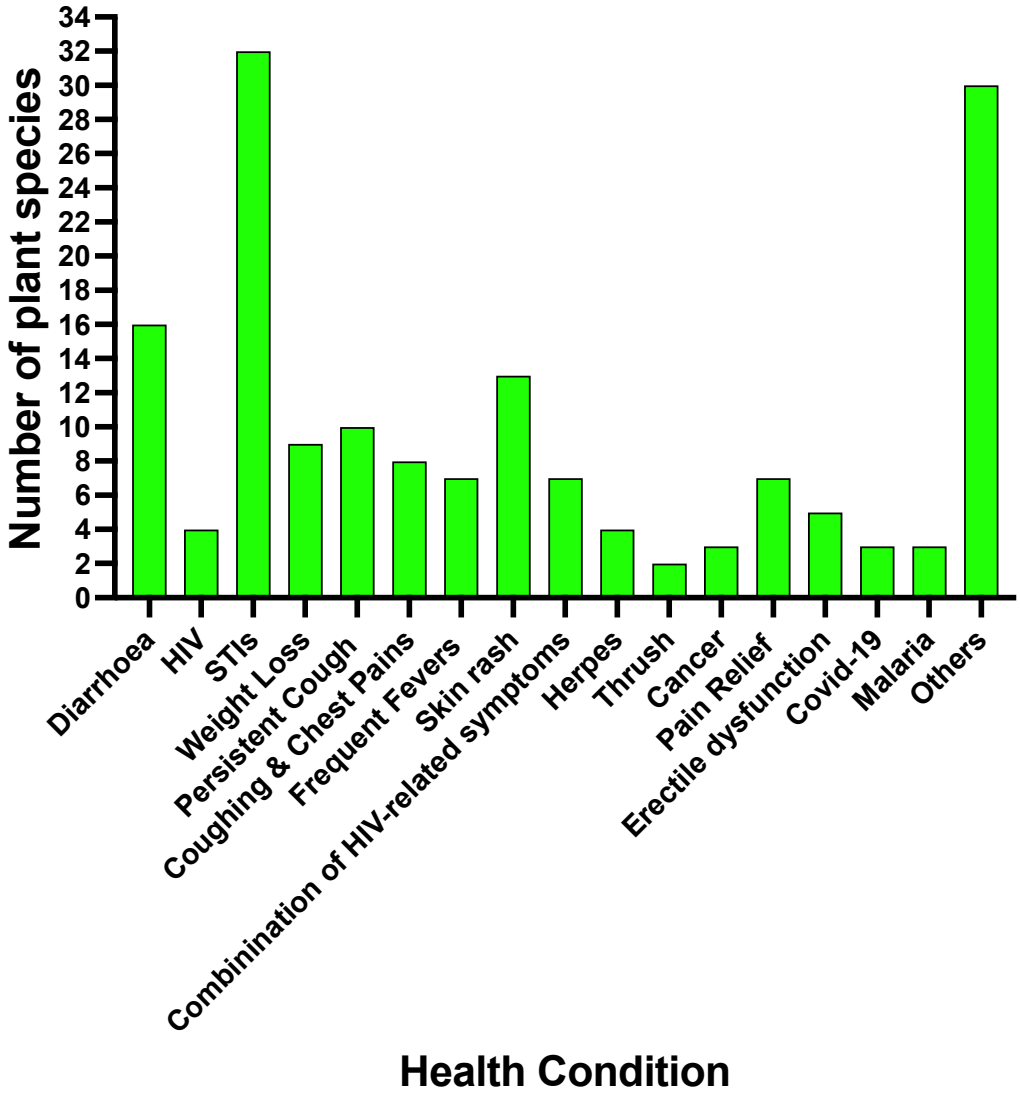

Figure 3.4: Number of plants species used to treat various health conditions in Tutume subdistrict (Central Botswana, Africa)

A detailed literature search identified that 45 (54.2%) of plants documented here have been previously reported for comparable antiviral, antimicrobial and/or pharmacologic activities that are linked to the ethnomedical uses this survey unearthed (**Table 3.2**), while 38 (45.8%) have not been reported, to the best of our knowledge.

3.5. Discussion

Here we report an ethnomedical survey of TCMs of botanical origin used for the management of HIV/AIDS and its associated comorbidities by the BaKalanga peoples of the Tutume subdistrict in Central Botswana. Through the snowballing technique we recruited and worked with 13 THPs whose combined wealth of experience in ethnomedicine practice totaled 221 years (**Table 3.1**). To the best of our knowledge, this survey is the first of its kind in Tutume subdistrict, documenting medicinal plants used for the management of HIV/AIDS-related infections and other general health conditions by the BaKalanga peoples in this region.

From this survey, we found that plants from the 39 families were represented, Fabaceae (25%) being the most predominant. To a first approximation, these results are consistent with results from parallel surveys from other regions of Southern Africa. For example, Chinsembu (2015), found out that Fabaceae (24.4%) was the most used plant family for the management of HIV/AIDS opportunistic diseases by traditional healers in Rundu, Kavango East Region, Namibia, with plants from a total of 27 families used for HIV-1 management (30). Similarly, Kazhila (2016) found that Fabaceae (22%), dominated the list of the 39 plant families used to manage HIV/AIDS opportunistic infections in Livingstone, Southern Province, Zambia (71). In contrast, in another ethnobotanical survey of plants traditionally used to manage HIV/AIDS-related conditions in Makete District, Southern Highlands of Tanzania, a total of 27 plant families predominantly represented by plants from Compositae and Leguminosae were reported (13.5% for both) (72). In an ethnopharmacological survey of plant-based HIV/AIDS medicines in Caprivi region (Namibia), plants of the Combretaceae (14.0 %) were most represented out of 28 identified plant families (73). This trend of the predominance of a single or few plant families in all these regions, including the region for the present study (Tutume subdistrict, Central Botswana), could possibly be a reflection of the shared indigenous medicinal knowledge within the region by the knowledge holders, in addition to the type of floral biota available in the respective regions. Our study also shows that traditional healers in Central Botswana region mostly use roots (53.1%) in their ethnomedicine (**Fig. 3.3**), although leaves, bark, fruits, tubers, seeds and, in some instances, the whole plant were also used in some cases, consistent with other regional ethnomedicinal surveys, where various

plants parts are used to treat health disorders depending on the type of the disease and the type of the medicinal plant (71-76).

The thematic analysis of our qualitative data identified five themes which were overarchingly used by THPs of the IKalanga origin in Tutume subdistrict namely: sources for the medicinal plants; storage of medicines; identification criteria for freshness of the medicinal plants; harvesting time for the medicinal plants and lastly, the availability and scarcity of medicinal plants (**Table 3.3**). The majority of THPs (12 or 92.3%) in this area reported that they store their medicines as powder, though others store their medicine in liquid form, dried, or fresh. The availability and harvesting of the medicinal plants in Tutume subdistrict, according to THPs in this area (92.3%), was largely seasonal and largely specific to summer / rainy season. All THPs (100%) reported that their sources of medicinal plants are largely collections from the bush, though some of the healers grow some of the medicinal plants or bought them from markets. Determinants of the freshness of the medicinal plant to be used primarily included maturity as the leading indicator, used by 12 (92.3%) of healers, although leaf colour and tasting the plant were used by 23.1 and 38.5% of healers, respectively. These patterns of results are also consistent with other regional studies (71-76).

In our ethnomedical study we specifically inquired about medicinal plants used for the management of HIV/AIDS and associated comorbidities and opportunistic infections including persistent cough, frequent fevers, diarrhoea, cough and chest pains, weight loss, skin rashes, tuberculosis, cancer, thrush, and herpes and other STIs (26-29). To this end, a considerable number of plants (32 or 38.6%) as shown in **Fig. 3.4** were used to treat sexually transmitted infections (STIs); 4 out of 83 (4.8%) were used specifically as HIV treatment; 17 (20.5%) and 16 (19.2%) were used for cough related condition and diarrhoea respectively. We also observed and noted that the preparations of the medicinal decoctions, their use and dosages may differ depending on the healer, consistent with observations made in other studies. [55-59] The difference in preparations of the plant-based ethnomedicines may depend on the type of the health condition being treated. Notably, 45 (54.2%) of the 83 plants documented in this pharmacologic survey, have ethnomedical uses and antiviral properties that have been reported in other regional ethnomedical surveys. However, 38 (45.8%) of plants documented here have not been investigated *in vitro*, *ex vivo* and/or *in vivo* and should therefore be prioritized for investigation in future

bioassay-based studies. Furthermore, there is need to elucidate the biological mechanisms for those plants that have been reported in various ethnomedicinal surveys.

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Chapter 4.
Identification of novel HIV-1 latency reversing agents from a library of marine natural products

4.1. Abstract

Natural products originating from marine and plant materials are a rich source of chemical diversity and unique antimicrobials. Using an established *in vitro* model of HIV-1 latency, we screened 257 pure compounds from a marine natural product library and identified 4 (psammaplin A, aplysiatoxin, debromoaplysiatoxin, and previously-described alotaketal C) that induced expression of latent HIV-1 provirus in both cell line and primary cell models. Notably, aplysiatoxin induced similar levels of HIV-1 expression as prostratin but at up to 900-fold lower concentrations and without substantial effects on cell viability. Psammaplin A enhanced HIV-1 expression synergistically when treated in combination with the protein kinase C (PKC) activator prostratin, but not the HDAC inhibitor (HDACi) panobinostat, suggesting that psammaplin A functions as a latency-reversing agent (LRA) of the HDACi class. Conversely, aplysiatoxin and debromoaplysiatoxin synergized with panobinostat but not prostratin, suggesting that they function as PKC activators. Our study identifies new compounds from previously untested marine natural products and adds to the repertoire of LRAs that can inform therapeutic “shock-and-kill”-based strategies to eliminate latent HIV-infected reservoirs.

Keywords: HIV-1; latency reversal; natural products; psammaplin A; aplysiatoxin; debromoaplysiatoxin

4.2. Introduction

While current licensed HIV-1 therapies inhibit virus replication, they do not act on latently-infected CD4+ T cells which have HIV-1 provirus incorporated within their genomes. As these proviruses can reactivate at any time to produce infectious virus, novel approaches are needed to eliminate these HIV-1 reservoirs (1-3). One therapeutic strategy, frequently termed “shock-and-kill” [4], proposes the treatment of latent HIV-infected cells with latency-reversing agents (LRAs) to induce proviral expression (“shock”), after which these cells may be eliminated by viral cytopathic effects or host immune responses (“kill”). Numerous LRAs have been identified that belong to distinct functional classes, primarily histone deacetylase inhibitors (HDACi) and protein kinase C (PKC) activators (5, 6). However, while several of these LRAs reproducibly induce HIV-1 proviral expression *in vitro* and/or *in vivo*, to date no LRA has appreciably reduced the size of the inducible viral reservoir in clinical trials, underscoring a need for new LRAs with improved efficacy (5-7). Toward this goal, several studies show that treatment of cells with combinations of LRAs from distinct functional classes yields synergistic responses that are significantly greater than the expected additive effects of these agents (8-15). However, as outcomes from clinical trials with LRA combinations are not yet reported, continued discovery of additional LRAs and LRA combinations remains a priority.

Natural products obtained from plant and marine sources are a rich source of diverse chemical compounds, including HIV-1 inhibitors and novel LRAs (16, 17). Moreover, screens for novel LRAs from natural product libraries often result in “hit” rates of 1.0% or more (18, 19), indicating that even small natural chemical libraries may be sufficient to identify new agents of interest. Here, we describe the results from a screen for novel LRAs from a library of 257 pure and structurally diverse natural compounds derived from marine invertebrates and microorganisms. These compounds were assembled over many years by the laboratory of RJA (20-24); however, many of these compounds have no known molecular targets or have only been reported to possess basic cytotoxic or antimicrobial activity. We previously screened this library for compounds that inhibit HIV replication and identified 6 with 50% effective concentrations (EC_{50}s) of 3.8 μM or less, including at least one (bengamide A) that acts by mechanisms which are distinct from licensed antiretrovirals (25). Here we describe 4 new LRAs identified from this library, including 1 HDACi and 3 PKC activators.

4.3. Testing and reconfirming latency reversal activities of established LRAs to be used as positive controls in the investigation of the new test agents

4.3.1. Materials, reagents and cells

The control LRA TNFα (Sigma-Aldrich, Oakville, ON, Canada) was dissolved in PBS plus 0.1% bovine serum albumin to a stock concentration of 5 µg/mL. The control LRA panobinostat (HDACi; Sigma-Aldrich) was dissolved in DMSO to a stock concentration of 30 mM. The control LRA prostratin (PKC activator; Selleck Chemicals, Houston, TX, USA) was dissolved in PBS to a stock concentration of 1.5 mM.

J-Lat Full Length T cell lines (clones 9.2, including 8.4. and 10.6, which were also used for novel LRAs experiments) were obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (contributed by Dr. Eric Verdin) (26). These cells are derived from Jurkat cells and contain a transcriptionally-silent, HIV-1 proviral genome encoding a frameshift mutation within Env, and where Nef is replaced with a GFP reporter (26)]. As a result, GFP expression in these cells indicates reactivation of HIV-1 from latency. Cells were maintained in R10+ medium [RPMI 1640 with HEPES and L-Glutamine (Lonza, Mississauga, ON, Canada), 10% fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma-Aldrich)] at 37 °C and 5% CO_2.

For latency-reversal assays, J-Lat cells were re-suspended in R10+ medium to a concentration of 10^6 cells/mL. $2*10^5$ cells were then seeded into 96-well plate wells with control LRAs (and also tests agents or compounds) at desired final concentrations. Control cells were incubated at a final concentration of up to 0.3% DMSO vehicle control. In no case did final test concentrations of DMSO exceed 0.3%, which had no observable effects on cell viability or GFP expression (data not shown). Cells were incubated at 37 °C and 5% CO_2 for 24 hours, after which a minimum of 5000 cells from each culture was analyzed by flow cytometry (Guava EasyCyte 8HT, EMD Millipore). Flow cytometry data were analyzed using FlowJo v. 8.8.7 software (FlowJo LLC, Ashland, OR, USA), and results were reported as the mean ± s.e.m. from at least 3 independent experiments.

4.3.2. Control LRAs reactivate HIV-1 virus in J-Lat 9.2 cells

Compounds were initially assessed in J-Lat 9.2 cells based on observations by us and others (12, 14-15) that this cell line responds to LRAs from multiple functional classes but rarely achieves GFP expression in all cells, thereby enabling comparative dose-response studies across large concentration ranges in addition to accurate studies of synergistic effects of LRA combinations. Representative flow cytometry data using this cell line and control LRAs are shown in **Figure 4.1A**. For each experiment, control J-Lat cells treated with 0.1% DMSO were first gated for typical forward and side-scatter profiles consistent with viable, healthy cells (data not shown) (14, 15). This subset was then assessed for GFP expression across all cell cultures, with the GFP-positive gate set such that the average of GFP-positive cells in DMSO-treated control cells was 0.05% (**Figure 4.1A,** top). For each culture treated with compound, the extent of latency reversal was then measured as the percentage of GFP-positive cells. For example, in one representative experiment, treatment of J-Lat cells with 0.1 µg/mL panobinostat (~0.15 µM) resulted in 13.9% GFP-positive cells, while treatment with 10 µg/mL prostratin (~12 µM) resulted in 6.2% GFP-positive cells (**Figure 4.1A,** center and bottom).

Consistent with published data (8, 15, 27), both control LRAs exhibited dose-dependent expression of GFP across multiple concentrations (**Figure 4.1B**). For example, maximal GFP expression in J-Lat cells was observed after treatment with 1.4 µM panobinostat (17.1 ± 2.7%) or 12 µM prostratin (6.4 ± 0.9%) (**Figure 4.1B**). Using the approach of Hashemi et al. (28) and normalizing results relative to the average GFP response for 12 µM prostratin (the concentration at which maximal prostratin activity was observed), the EC_{50}s for panobinostat and prostratin were calculated to be 0.10 ± 0.02 and 7.1 ± 2.8 µM, respectively (**Table 4.1**). These results confirm that panobinostat is approximately 70-fold more potent than prostratin, which is also consistent with previous reports (8, 15, 27).

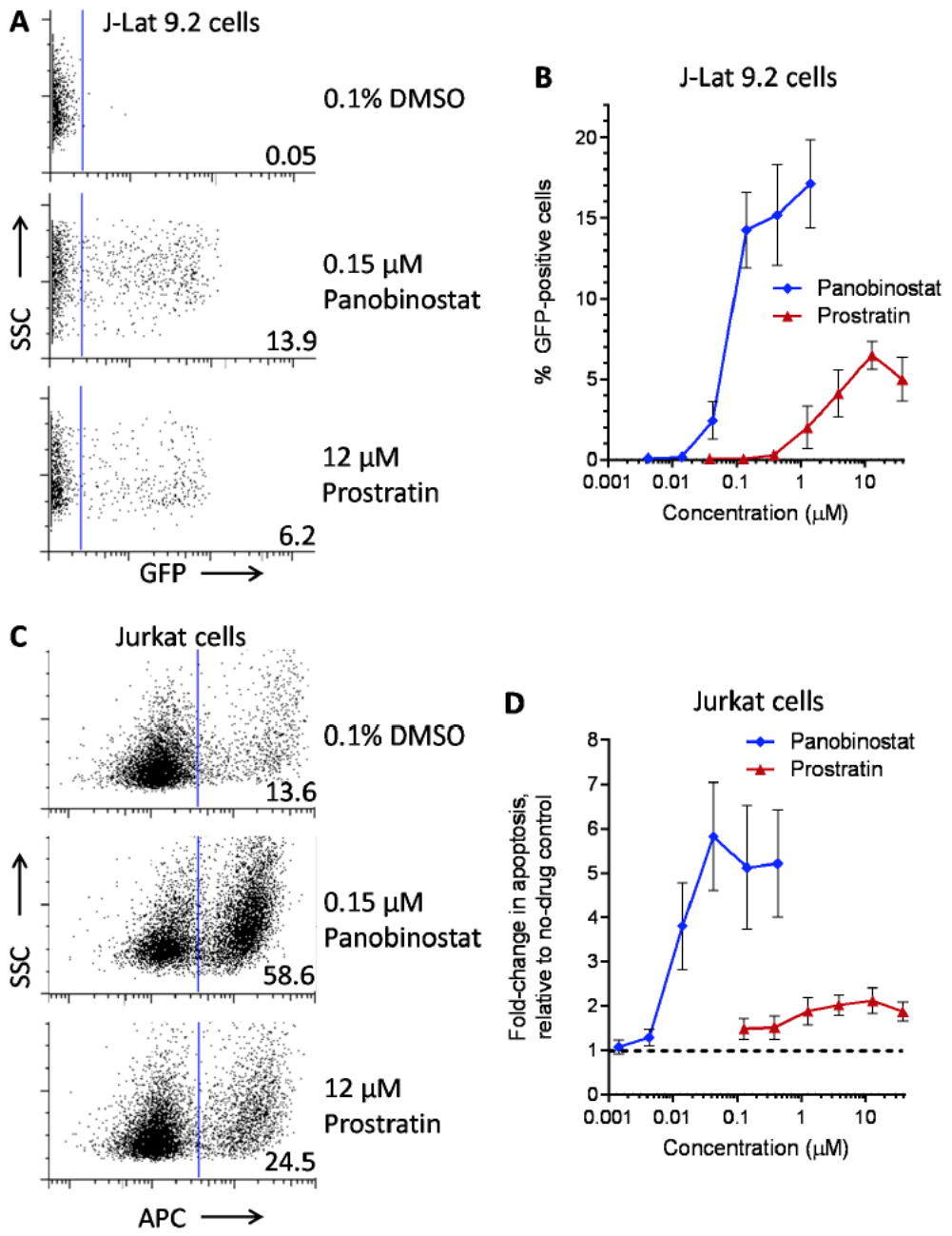

Figure 4.1: HIV-1 latency reversal and cellular toxicity assessments of control LRAs.

A, Representative GFP expression in J-Lat cells treated with 0.1% DMSO (top), 0.15 µM panobinostat (center), or 12 µM prostratin (bottom). Numbers to the right of the blue bar indicate percent GFP-positive (i.e., HIV-expressing) cells. **B**, Effects of control LRAs on HIV-1 provirus expression in J-lat cells. **C**, Representative effects of compounds on apoptosis in Jurkat cells, as measured by annexin V staining. Numbers to the right of the blue bar indicate percent APC-positive (i.e., apoptotic) cells. **D**, Effects of control LRAs on apoptosis in Jurkat cells, expressed as fold-increase in apoptosis relative to control cultures treated with 0.1% DMSO (dotted line).

4.3.3. Cellular viability assessment of established control LRAs

To directly assess the impact of control LRAs on cell viability (*i.e.,* in a manner independent of provirus expression), parental Jurkat cells (Clone E6-1, American Type Culture Collection; Manassas, VA, USA), which do not harbor integrated HIV-1 provirus, were prepared and treated with LRAs as described above. Following 24 hours' incubation, compound toxicity was assessed by measuring surface expression of the early apoptotic marker annexin V by flow cytometry (by staining with annexin V-APC; BioLegend, San Diego, CA, USA). Results were reported as the fold-increase in annexin V-positive cells relative to 0.1% DMSO-treated control cells (mean ± s.e.m) from at least 3 independent experiments. Representative data are shown in **Figure 4.1C**. Here, treatment with 0.1% DMSO resulted in 13.6% of cells with APC-fluorescence above the bulk of the cell population which is presumed to lack surface expression of annexin V (top). In contrast, treatment with 0.15 μM panobinostat resulted in 58.6% APC-positive cells (*i.e.,* a 4.3-fold increase in apoptosis from DMSO control) (center), while 12 μM prostratin resulted in 24.5% APC-positive cells (*i.e.,* 1.8-fold increase from DMSO control) (bottom). As expected, control LRAs increased cellular apoptosis in a dose-dependent manner (**Figure 4.1D**). For example, treatment with 0.045 μM panobinostat induced a 5.8 ± 1.2-fold increase in apoptosis, indicating poor cellular tolerance at concentrations that induced latency reversal, while at least 10 μM prostratin induced no more than a 2.0 ± 0.3-fold increase in apoptosis (**Figure 4.1D**). Taken together, our control experiments confirm that panobinostat is a more potent, yet also more toxic, LRA than prostratin (8, 15, 27).

Table 4.1: 50% effective concentrations (EC50s) of LRAs.
EC50s were calculated in J-lat 9.2, 8.4, and 10.6 cells based on the percent of GFP expression relative to controls treated with 12, 38, or 3.8 μM prostratin, respectively, using the approach of Hashemi et al. (28) n.d., not determined.

LRA	EC50 (μM; mean ± s.e.m.)			Mechanism of Action	Ref.
	J-Lat 9.2	J-Lat 8.4	J-Lat 10.6		
Panobinostat	0.10 ± 0.02	0.073 ± 0.010	0.041 ± 0.003	HDACi	[5,6]
Prostratin	7.1 ± 2.8	10 ± 1	1.8 ± 0.4	PKC activator	[5,6]
Psammaplin A	1.9 ± 0.3	1.5 ± 0.1	1.5 ± 0.1	HDACi	[29–31]
Aplysiatoxin	0.045 ± 0.021	0.011 ± 0.003	0.0033 ± 0.0012	PKC activator	[32–34]
Debromoaplysiatoxin	0.92 ± 0.14	0.52 ± 0.02	0.081 ± 0.029	PKC activator	[32,34]
Alotaketal C	1.3 ± 0.2	n.d.	n.d.	PKC activator	[14]

4.4. Investigating and evaluating latency reversal activities of novel LRAs

4.4.1. Discovery of new LRAs from a library of marine natural products

We next screened 257 structurally-diverse pure compounds derived from marine natural products at 2.5 μg/mL for latency reversal activity in J-Lat 9.2 cells. Of these, nine (3.5%) compounds resulted in cytolysis and disruption of cell morphology as observed by light microscopy, consistent with widespread cell death (data not shown), and were not considered further. Of the remaining 248 compounds, four induced GFP expression in at least 4% of cells. This “hit” rate of 1.6% is in line with previously reported screens of natural product libraries (~0.5-1.1%) (18, 19) and supports the notion that pure natural product libraries are enriched for bioactive LRAs compared to synthetic small molecule libraries, where reported hit rates of ~0.1% are more frequent (28, 35-36).

Structures of the four identified compounds are shown in **Figure 4.2A**. Notably, psammaplin A, originally isolated from the two-sponge associate *Poecillastra* sp. and *Jaspis* sp. (29), was previously identified as an HDACi with anti-tumor activity (30, 31). In contrast, aplysiatoxin and debromoaplysiatoxin, which differs from aplysiatoxin by the loss of a bromine atom in the phenol ring, are toxins produced by blue-green algae and potent PKC activators (32-34). However, not all HDACis and PKC activators possess potent HIV latency modulatory functions (14, 37-38), and none of these compounds have been

investigated as HIV-1 LRAs. Finally, alotaketal C, originally isolated from *Phorbas* sp., is a potent activator of cyclic AMP and PKC signaling that we recently characterized for its HIV-1 latency reversal activity (14, 20) and is thus not assessed further here. All natural compounds were confirmed ≥ 95% pure by NMR and LC/MS (data not shown) and dissolved in DMSO to stock concentrations of 5 mg/mL.

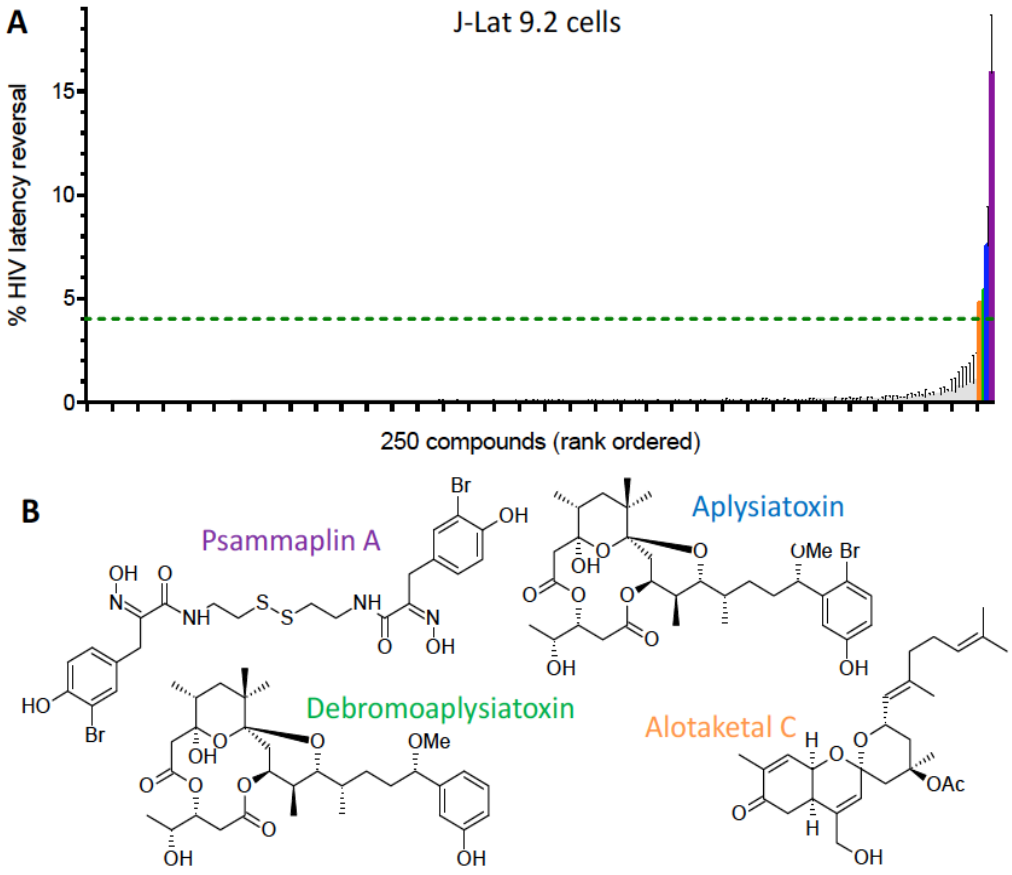

Figure 4.2 : Discovery of new LRAs from marine natural products.
A, HIV latency reversal in J-Lat 9.2 cells, as assessed by measuring GFP reporter expression in the presence of 250 compounds from marine natural products at 2.5 µg/mL. Colored bars denote 4 of 250 compounds (1.6%) that induced GFP expression in at least 4% of cells (dotted line). **B,** Structures of 4 LRAs identified from screening of 257 pure natural products at 2.5 µg/mL.

4.4.2. New LRAs reactivate HIV-1 virus in J-Lat 9.2 cells

Each new LRA induced dose-dependent reversal of HIV-1 latency in J-Lat cells across multiple concentrations (**Figures 4.3B-C**). The average maximum responses observed for each compound ranged from 1.4 to 2.7-fold more than controls treated with 12 μM prostratin. For example, psammaplin A induced GFP expression in up to 17.6 ± 4.0% of cells at 5 μg/mL (3.8 μM). When results were normalized to the average GFP response for 12 μM prostratin (28), psammaplin A's EC_{50} was calculated as 1.9 ± 0.3 μM, approximately 19-fold higher than the EC_{50} of panobinostat (**Table 4.1**). In contrast, aplysiatoxin induced GFP expression in 9.4 ± 0.1% cells with as little as 0.15 μg/mL (0.1 μM) and yielded a calculated EC_{50} of 0.045 ± 0.021 μM. Aplysiatoxin is therefore 160-fold more potent than prostratin and 2.2-fold more than panobinostat, identifying it as a particularly potent LRA in J-lat 9.2 cells. Debromoaplysiatoxin induced GFP expression in 7.2 ± 1.5% of cells at 1.5 μg/mL (1.3 μM) and yielded a calculated EC_{50} of 0.92 ± 0.14 μM, or 7.7-fold more potent than prostratin.

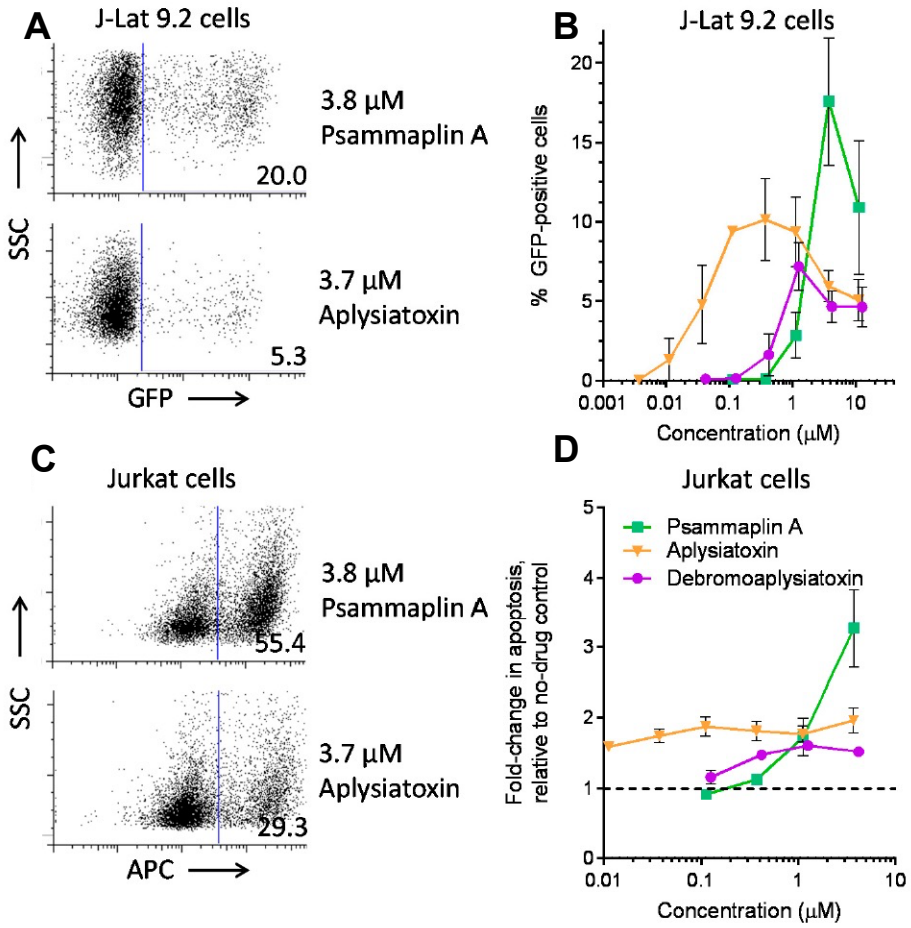

Figure 4.3: Assessment of latency reversal profiles of new LRAs on J-Lat 9.2, including cell toxicities

A, Representative GFP expression in J-Lat 9.2 cells treated with 3.8 µM psammaplin A (top) or 3.7 µM aplysiatoxin (bottom). Numbers to the right of the blue bar indicate percent GFP-positive (i.e., HIV-expressing) cells. B., Numbers to the right of the blue bar indicate percent GFP-positive (i.e., HIV-expressing) cells. (**B)** Effects of LRAs on HIV-1 provirus expression in J-Lat cells. **(C**) Representative effects of compounds on apoptosis in Jurkat cells, as measured by annexin V staining. Numbers to the right of the blue bar indicate percent APC-positive (i.e., apoptotic) cells. **(D**) Effects on apoptosis in Jurkat cells, expressed as fold-increase in apoptosis relative to control cultures treated with 0.1% DMSO (dotted line). Data presented in panels **A** and **C** originate from the same experiments shown in **Figure 1A**, **C**, respectively.

4.4.3. Toxicity assessment of novel LRAs in J-Lat 9.2 cells

With the same approach used to assess cellular viability of established control LRAs (Annexin V-FITC dead cell apoptosis detection by flow cytometry) in Jurkat cells, it was observed that 3.8 µM psammaplin A induced a 3.3 ± 0.6-fold increase in annexin V staining, with extensive cell death observed at higher concentrations (data not shown). In

contrast, no more than 2.0 ± 0.2 and 1.6 ± 0.1-fold increases in annexin V staining were observed for aplysiatoxin and debromoaplysiatoxin, respectively (**Figure 4.3C-D**). Thus, psammaplin A appeared to be toxic at concentrations that induced latency reversal, while both aplysiatoxin and debromoaplysiatoxin were largely well-tolerated across all concentrations.

4.4.4. New LRAs reactivate HIV-1 virus in multiple cell lines

Similarly, using J-Lat assay used for control LRAs, it was shown that these agents displayed similar dose-response profiles in J-Lat 8.2 cells, indicating that they act on HIV provirus independent of its integration site (**Figure 4A**). For example, when results were normalized to the average GFP response for 38 μM prostratin (*i.e.*, the concentration at which maximal activity was observed in J-Lat 8.2 cells), the EC_{50} of psammaplin A was calculated as 1.5 ± 0.1 μM, or approximately 20.5-fold higher than the EC_{50} of panobinostat (**Table 4.1**). Aplysiatoxin induced detectable GFP expression at concentrations as low as 0.0001 μM, with a calculated EC_{50} of 0.011 ± 0.003 μM, or 900-fold more potent than prostratin and 6.6-fold more than panobinostat. Similarly, an EC_{50} of 0.52 ± 0.02 μM was calculated for debromoaplysiatoxin, or 19.2-fold more potent than prostratin.

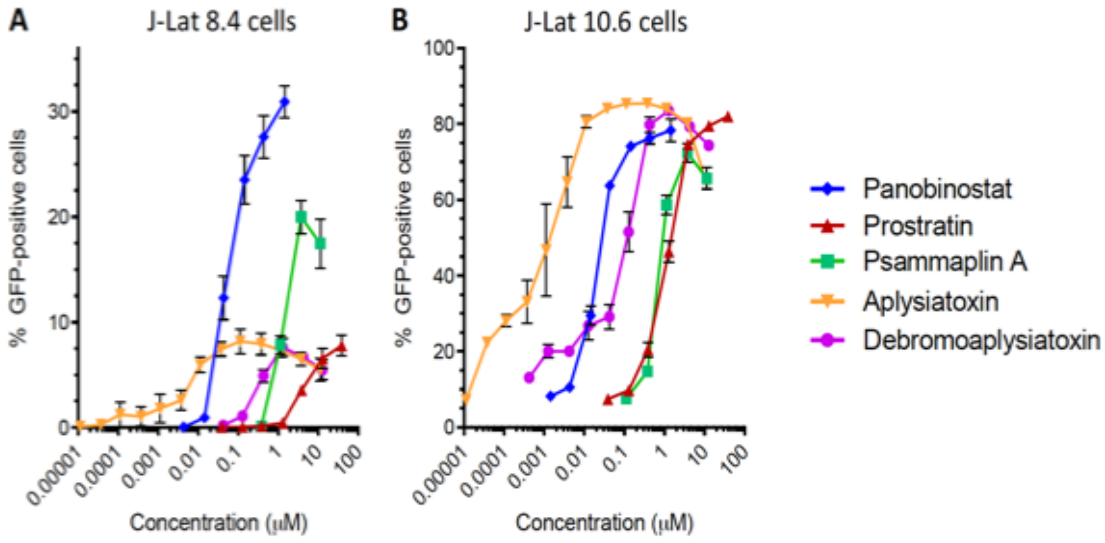

Figure 4.4 Natural products reverse latency in multiple cell line models.
(**A, B**) Effects of LRAs on GFP expression in J-Lat 8.4 (**A**) and 10.6 cells (**B**). (**C**) Effects of LRAs on intracellular viral p24Gag expression in J-Lat 10.6 cells. Data are presented as fold-change in supernatant p24Gag relative to cells treated with0.1% DMSO vehicle control (dotted line)

In J-Lat 10.6 cells, we observed that all LRAs were capable of inducing GFP expression in at least two-thirds of cells (**Figure 4.4B**), indicating robust efficacy. However, we also observed an average of 7.5% of J-Lat 10.6 cells spontaneously expressing GFP in the absence of LRAs, consistent with previous reports (39), indicating a lower barrier to HIV latency reversal compared to J-Lat 9.2 and 8.4. Nevertheless, when results were normalized to the average GFP response for 3.8 μM prostratin (one of three concentrations where maximum activity was observed), the EC_{50} of psammaplin A was again calculated as 1.5 ± 0.1 μM, or approximately 36.6-fold higher than the EC_{50} of panobinostat (**Table 4.1**). Moreover, GFP expression was observed with aplysiatoxin concentrations as low as $3.7*10^{-5}$ μM (37 pM) and a calculated EC_{50} of 0.0033 ± 0.0012 μM, or 540-fold more potent than prostratin and 12.4-fold more than panobinostat. Finally, the calculated EC_{50} of debromoaplysiatoxin (0.081 ± 0.029 μM) was 22.2-fold more potent than prostratin. Thus, the rank-order of potency for all LRAs was consistent across all cell lines.

4.4.5. New LRAs induce HIV-1 protein expression

To confirm that LRAs induce HIV protein expression in addition to the GFP reporter, J-Lat 10.6 cells were also stained with the HIV-1 $p24^{Gag}$ antibody KC57-RD1 (Beckman Coulter, Indianapolis, IN, USA) and processed using the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences, Mississauga, ON, Canada) prior to flow cytometric analysis. Results were then reported as the fold-increase in $p24^{Gag}$-positive cells relative to 0.1% DMSO-treated control cells (mean ± s.e.m) from at least 3 independent experiments. All LRAs were observed to induce at least 9.7-fold increased $p24^{Gag}$-positive cells, with the same rank order as observed for GFP expression (**Figure 4.5**). This confirms that LRAs also induce viral protein expression.

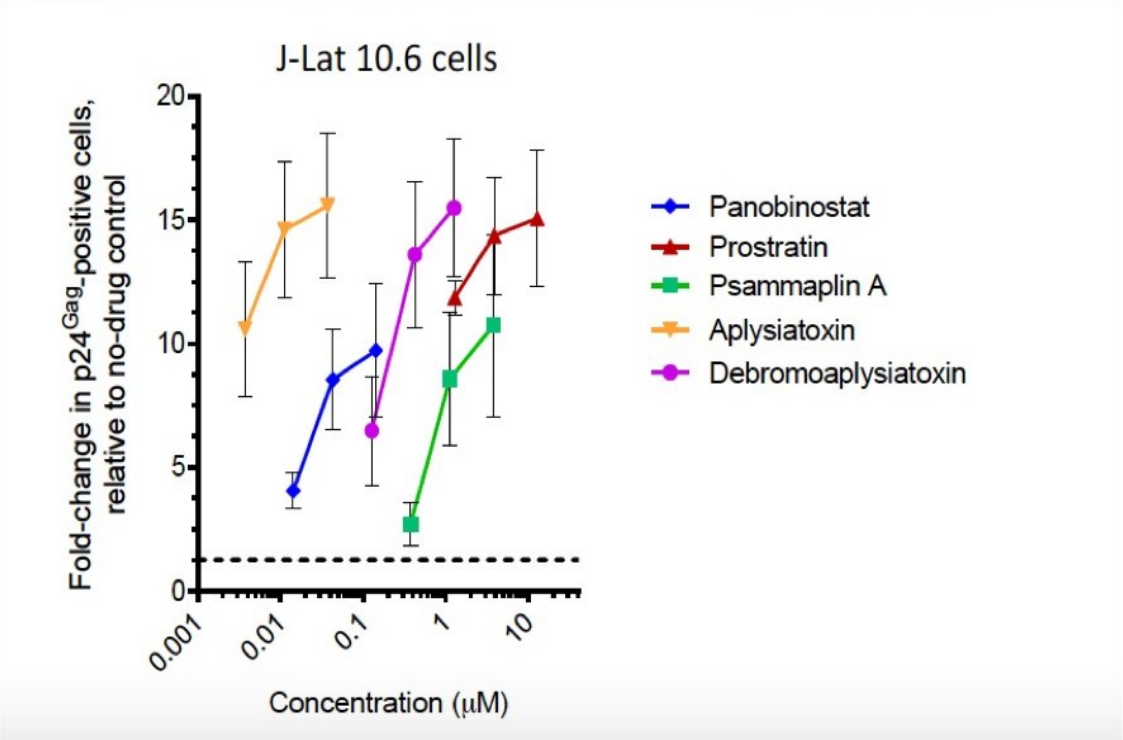

Figure 4.5: Natural products induce HIV-1 protein expression *in vitro*.
Effects of LRAs on intracellular viral p24Gag expression in J-Lat 10.6 cells. Data are presented as fold-change in supernatant p24Gag relative to cells treated with0.1% DMSO vehicle control (dotted line)

4.4.6. LRAs induce proviral expression in primary human cells

To investigate whether LRAs induce proviral expression in primary human cells, we obtained peripheral blood mononuclear cells (PBMCs) from three HIV-infected donors on stably-suppressive antiretroviral therapy for at least three years (**Figure 4.6**). Study protocols were approved by the Institutional Reviews Boards of Simon Fraser University and the University of British Columbia – Providence Health Care Research Institute. Frozen PBMC aliquots were thawed and allowed to recover in R10+ medium at 37 °C, 5% CO_2 for 24 hours at $2.5*10^6$ cells/mL. PBMCs were then incubated at 10^6 cells/mL with positive control cell activators PMA (100 ng/mL) plus ionomycin (1 μg/mL), 3.8 μM psammaplin A, 1.1 μM aplysiatoxin, 1.3 μM debromoaplysiatoxin, or 0.1% DMSO vehicle control. All conditions were performed in duplicate. Following 24 hours' incubation at 37 °C and 5% CO_2, supernatant $p24^{Gag}$ protein was quantified by ELISA (Xpress Bio, Frederick, MD), and cell viability was measured by flow cytometry using Guava Viacount, a DNA intercalating dye (Millipore). In most cases, each LRA caused an increase in

supernatant $p24^{Gag}$ above background; however, consistent with other studies (10-12), substantial donor-to-donor variation was observed (**Figure 4.6A**). For example, while treatment with PMA + ionomycin induced an average 62.3 ± 53.9% increase in supernatant $p24^{Gag}$ relative to untreated cells, psammaplin A resulted in a 85.0 ± 72.0% increase. Similarly, aplysiatoxin and debromoaplysiatoxin induced increases of 46.9 ± 38.6 and 48.1 ± 52.5%, respectively. No major changes in cell viability were observed, with a maximum 15.4 ± 3.8% reduction in viability in the presence of 3.8 μM psammaplin A (**Figure 4.6B**). These results indicate that LRAs have the capacity to activate latent HIV-1 provirus in primary human cells isolated from persons with long-term viremia suppression on antiretroviral therapy. However, the evident induction of viral latency reversal in primary cells by our newly discovered latency modulating agents did not yield statistically significant changes. It is plausibly suggested that increasing the study population may provide a better environment for improved statistical significance tests and analysis.

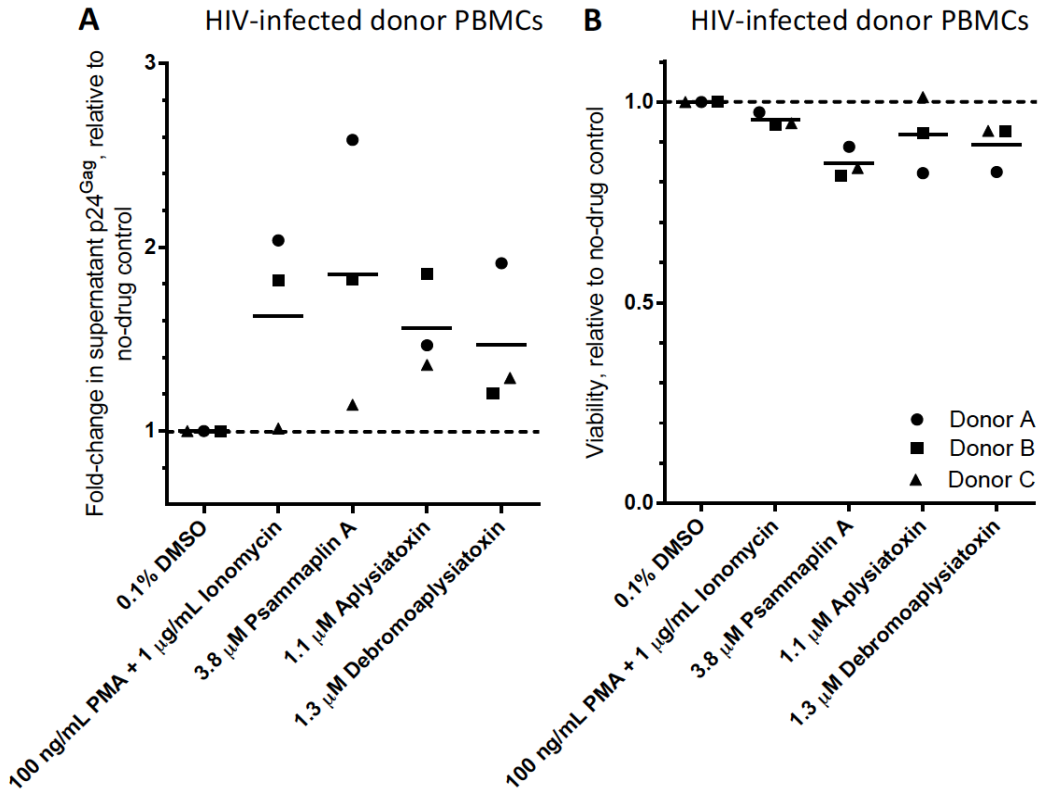

Figure 4.6 : Effects of LRAs on PBMCs from three HIV-infected donors.
A, $p24^{Gag}$ viral protein levels in PBMC culture supernatants following 24 hours' treatment with control compounds or candidate LRAs for 24 hours. **B,** Viability of PBMCs after 24 hours' treatment with LRAs, as measured by Viacount cell-permeable dye. In both panels, shapes represent PBMCs from individual donors.

4.4.7. Synergism tests for the new LRAs to predict their mechanisms

As described previously (8-15), treatment of cells with combinations of LRAs acting through different mechanisms tends to result in synergistic effects on HIV-1 latency reversal, while treatment with compounds acting through similar mechanisms tends to yield at most additive responses. These observations can therefore be used to identify potential functional classes of novel LRAs (14, 15). To demonstrate this, we assessed GFP expression in J-Lat 9.2 cells treated with novel LRAs in combination with control LRAs, including the pro-inflammatory cytokine TNFα, the HDACi panobinostat, and the PKC activator prostratin (**Figure 4.7**). In these studies, synergism was observed in all cases where control LRAs were applied in combination: for example, treatment of J-Lat 9.2 cells separately with 0.01 μg/mL TNFα or 0.15 μM panobinostat induced 22.3 ± 2.5% and 8.9 ± 1.8% GFP-positive cells, respectively, whereas treatment of cells with both compounds simultaneously led to 49.0 ± 1.2% GFP-positive cells, which is ~1.6-fold higher than would be expected if the effects of these two compounds were strictly additive (*i.e.*, 31.2%; **Figure 7**). Similarly, treatment with TNFα plus 12 μM prostratin, or panobinostat plus prostratin, induced responses that were 1.6- and 2.6-fold higher than expected for additive effects, respectively. These levels of synergism were statistically significant ($p < 0.05$; Student's unpaired t-test with a two-sided, Bonferroni correction) as measured by the Bliss independence model, which was calculated as described previously (10, 11).

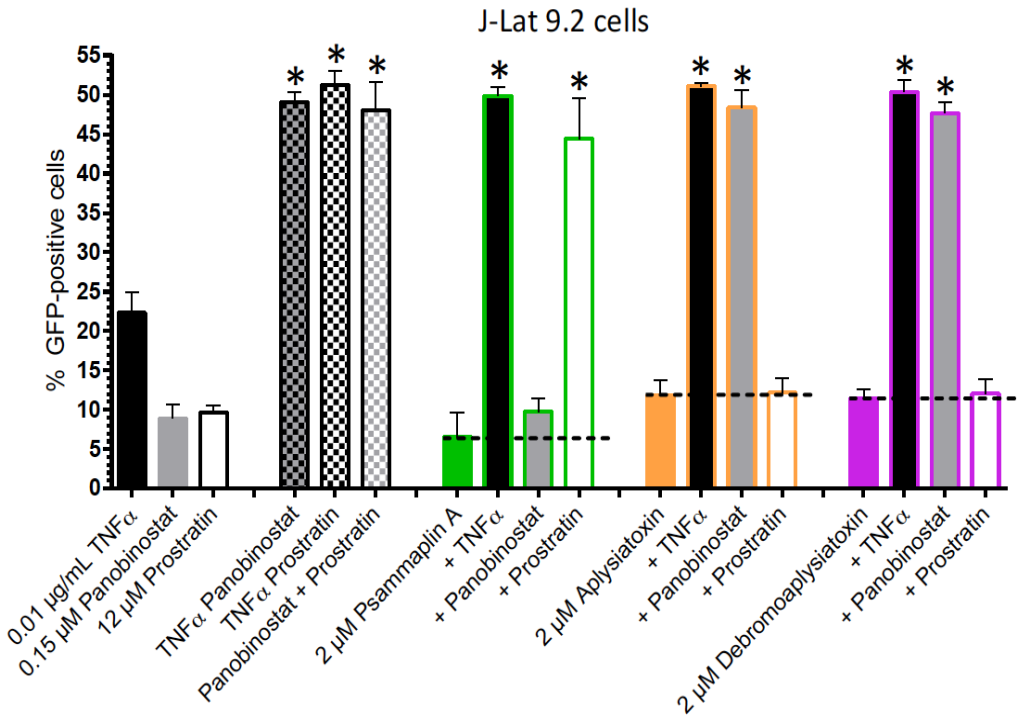

Figure 4.7: Effects of LRAs and LRA combinations on HIV-1 provirus expression in J-Lat cells.
*, $p < 0.05$ using the Bliss independence model [10, 11].

Treatment of J-Lat 9.2 cells with 2 µM psammaplin A in addition to TNFα or prostratin induced 49.8 ± 1.1 and 44.4 ± 5.1% GFP-positive cells, representing 1.7- and 2.7-fold increases in GFP expression over what would be expected from strictly additive effects, respectively. However, treatment of J-Lat cells with psammaplin A plus panobinostat induced only 9.8 ± 1.7% GFP-positive cells, or 0.6-fold of what would be expected by strictly additive effects (**Figure 4.7**). These results are consistent with the known function of psammaplin A as an HDACi (30-31). Conversely, treatment of cells with 2 µM aplysiatoxin plus TNFα or panobinostat induced 51.1 ± 0.5 and 48.4 ± 2.2% GFP-positive cells, respectively, or 1.5- and 2.3-fold increases in GFP- positive cells over expected additive effects, while co-treatment with prostratin induced only 12.2 ± 1.8% GFP-positive cells, or 0.6-fold of expected additive effects. These observations are consistent with the known function of aplysiatoxin as a PKC activator (33-34). Similar results were found using debromoaplysiatoxin: co-treatment with TNFα or panobinostat induced 50.4 ± 1.5 and 47.6 ± 1.5% GFP-positive cells, respectively, or 1.5 or 2.3-fold over expected additive effects, while co-treatment with prostratin induced 12.0 ± 1.8% GFP-positive cells, or 0.6-

fold of expected additive effects, indicating that its latency reversal activity is also likely due to activation of PKC. All synergistic effects were statistically significant as measured with the Bliss independence model. Taken together, these results indicate that the latency reversal properties of these pure natural products in J-Lat cells are consistent with their previously reported functional properties.

4.5. Conclusion

In summary, we identify four new LRAs derived from marine natural products that can be added to the repertoire of known HIV-1 shock-and-kill agents. The likely mechanisms of action for all four compounds, supported by prior studies, are consistent with established functional classes, including one HDACi (psammaplin A) and three PKC activators (aplysiatoxin, debromoaplysiatoxin, and previously-described alotaketal C) (14). Dose response profiles suggest that psammaplin A is a less potent LRA than panobinostat, while aplysiatoxin and debromoaplysiatoxin are more potent than prostratin. These trends were confirmed in two additional J-Lat cell lines and in PBMCs from three HIV-infected donors, indicating that latency reversal occurs independent of proviral integration site and that these compounds can reverse latency in primary cells. The contributions of these mechanisms to HIV-1 latency reversal were further supported by synergism studies, described both here and elsewhere [14].

Our previous discovery of multiple novel HIV inhibitors from this chemically-diverse library (*e.g.*, (25)) led us to hypothesize that we might also identify new modulators of HIV latency with distinct molecular mechanisms. However, while this screen instead identified only compounds of the HDACi and PKC activation classes, the results described here should not preclude future screening of natural product-derived compound libraries for additional LRAs which may act by novel mechanisms of action. Conversely, as the kinetic properties of both HDACis and PKC activators do not necessarily correspond with latency reversal efficacy (14, 37-38), testing of library compounds with both known and unknown molecular functions remains warranted. In support of this, we notably identify one PKC activator, aplysiatoxin, that is particularly potent across multiple cell models, with activity observed in J-Lat 8.4 and 10.6 cells at picomolar concentrations. Finally, we note that additional latency reversal mechanisms by these agents may remain undetected. For example, we previously described alotaketal C as an LRA of the PKC activator class (14), but it is

additionally reported to function as an activator of cyclic AMP signalling (20), which may also modulate HIV-1 latency reversal (40-41).

Taken together, this study highlights the benefits of natural product-based screens for LRA discovery. The identification and evaluation of new LRAs will support the development of novel therapeutic combinations and clinical approaches that can reduce or eliminate latent HIV-1 reservoirs.

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Chapter 5.

The African natural product knipholone anthrone and its analogue anthralin (dithranol) enhance HIV-1 latency reversal

5.1. Abstract

A sterilizing or functional cure for HIV is currently precluded by resting CD4+ T cells that harbor latent but replication-competent provirus. The “shock-and-kill” pharmacological approach aims to reactivate provirus expression in the presence of antiretroviral therapy and target virus-expressing cells for elimination. However, no latency-reversal agent (LRA) to date effectively clears viral reservoirs in humans, suggesting a need for new LRAs and LRA combinations. Here we screened 216 compounds from the pan-African Natural Products Library and identified knipholone anthrone (KA) and its basic building block anthralin (dithranol) as novel LRAs that reverse viral latency at low micromolar concentrations in multiple cell lines. Neither agent’s activity is dependent on protein kinase C (PKC), nor do they inhibit class I/II histone deacetylases. However, they are differentially modulated by oxidative stress and metal ions and induce distinct patterns of global gene expression from established LRAs. When applied in combination, both KA and anthralin synergize with LRAs representing multiple functional classes. Finally, KA induces both HIV RNA and protein in primary cells from HIV-infected donors. Taken together, we describe two novel LRAs that enhance the activities of multiple “shock-and-kill” agents, which in turn may inform ongoing LRA combination therapy efforts.

Key words: HIV-1; Latency reversal agents; “shock-and-kill”; natural products; Knipholone anthrone; Anthralin

5.2. Introduction

As extensively reviewed in chapter 1, the use of combination antiretroviral therapy (cART) has been a resounding success in terms of reducing HIV/AIDS related morbidity and mortality (by 47% since 2010) as well as HIV transmission (by 52% since the summit in 1997) (1). As of 2020, 73% of people living with HIV globally were reliably accessing cART (UNAIDS (2020) (1). cART significantly improves the quality of life of patients, albeit not curative as it does not target resting CD4+ T cells that persistently bear integrated and immunologically invisible provirus, capable of reactivating to produce infectious virus, at any time upon cessation of treatment — as such cART must be taken for life (2-5).

One approach toward developing a sterilizing or functional HIV cure, as reviewed in chapter 1, is termed “Shock-and-Kill” or latency reversal. “Shock-and-Kill,” could theoretically eliminate an individual’s viral reservoir and/or reduce the viral reservoir to a point that cART-free remission is achievable, provided that sufficiently effective LRAs and immune enhancers can be identified (6-7). Numerous LRAs have been described representing different functional classes. The majority represents protein kinase C (PKC) activators and histone deacetylase (HDAC) inhibitors, although agents that act by other mechanisms such as BET bromodomain and DNA methyltransferase inhibition are also intensively studied (8-9). However, as discussed earlier on, LRAs tested to date in humans have shown limited clinical success due to extensive toxicity, poor efficacy, inconsistent viral reactivation, and/or insufficient engagement of cellular “kill” mechanisms (8-10). New LRA-based strategies, including LRA combinatorial approaches (of different functional classes than synergistically enhance latency reversal) are likely to be needed to circumvent these issues. Thus, discovery of LRAs that enhance the activities of existing LRA clinical candidates would support efforts to identify optimized LRA combinations.

Evidence continues to grow showing that pure compounds isolated from natural products are a rich source of unique chemical diversity and new LRAs. For example, we previously screened a library of 257 compounds originating from marine natural products and identified 4 (1.6%) that reversed HIV latency in both cell line and primary cell models (11), as discussed in chapter 4. LRA “hit” rates of 1.0% or more have also been reported by others (12-13). Based on these observations, we hypothesized that new LRAs that can enhance the activities of existing agents could be isolated from additional natural product-

based compound libraries. Here we describe the results of a screen of the pan-African Natural Products Library (pANAPL), which contains compounds originating from African medicinal plants (14-15). From this screen, we identified and characterized knipholone anthrone (KA), in addition to its synthetic analogue anthralin (dithranol), as novel LRAs that synergize with established HIV latency reversal agents.

5.3. Materials and methods

5.3.1. Cells and reagents

Jurkat T cells (Clone E6-1) were obtained from the American Tissue Culture Collection (ATCC). J-Lat T cells (clones 8.4, 9.2, and 10.6) and OM-10.1 cells were obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (contributed by Drs. Eric Verdin and Salvatore Butera, respectively) (16, 17). Cells were cultured in R10+ media [RPMI 1640 with HEPES and L-Glutamine, 10% Fetal Bovine Serum (FBS), 100 U/mL of penicillin, and 100 µg/mL of streptomycin (Sigma)].

PBMCs were collected from three uninfected donors in addition to three HIV-infected donors on stably-suppressive combination antiretroviral therapy for at least three years. Study protocols were approved by the Institutional Review Boards of Simon Fraser University and the University of British Columbia – Providence Health Care Research Institute (REB: H15-03077, approved 8 March 2016 and H16-02474, approved 15 November 2016) and abide by the Declaration of Helsinki principles. CD4+ T cells were also obtained from an additional three HIV-infected donors on stably-suppressive combination antiretroviral therapy (< 50 copies/mL plasma viral load) for three years. These study participants were recruited in accordance with the human subject research guidelines of the US Department of Health and Human Services under the supervision of the Wistar and Philadelphia FIGHT institutional review boards. Written informed consent was obtained from all study participants.

KA was obtained from pANAPL chemical stocks and as described previously (18-20). Anthralin, prostratin, panobinostat, 5-aza-2'-deoxycytidine (Aza-CdR), GÖ-6983, deferoxamine, glutathione, and bathocuproine were commercially obtained from Sigma. (+)-JQ1 was obtained from Cayman Chemical Co (Ann Arbor, MI, USA). Annexin V-APC

and HIV $p24^{Gag}$ antibody KC57-RD1 were purchased from BioLegend (San Diego, CA, USA). CD25-FITC and CD69-Phycoerithrin (PE) were purchased from BD Biosciences (Mississauga, ON, Canada).

5.3.2. *In vitro* latency reversal assays

J-Lat and OM-10.1 cells were re-suspended in fresh R10+ to a concentration of 10^6 cells/mL, and $2x10^5$ cells were aliquoted into 96-well plates alongside test agents at defined concentrations or 0.1% DMSO vehicle control and incubated for 24 hrs. For each experiment, all conditions were performed in duplicate. Following incubation, for experiments using OM-10.1 cells and select experiments with J-Lat cells, $p24^{Gag}$ viral antigen was detected by staining cells with anti-$p24^{Gag}$ antibody and using the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences, Mississauga, ON, Canada) according to manufacturers' instructions prior to flow cytometric analysis. 5,000 live cells (as estimated from cells displaying the characteristic forward- and side-scatter parameters of cells treated with 0.1% DMSO) (15) from each cell culture were collected for detection of green fluorescent protein (GFP) and/or $p24^{Gag}$ expression by flow cytometry (Guava EasyCyte 8HT, EMD Millipore).

5.3.3. Detection of cell viability and T cell activation markers

To detect *in vitro* cell viability directly, Jurkat cells were treated with test agents at defined concentrations or 0.1% DMSO vehicle control in duplicate for 24 hours and stained with annexin V-APC according to manufacturer's instructions (BioLegend). To detect markers of cell activation, Jurkat T cells were stained with CD25-FITC or CD69-PE antibodies using the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences, Mississauga, ON, Canada) according to manufacturers' instructions. Flow cytometry was then performed as described above.

Viability in uninfected PBMCs was measured in the presence of test agents for 24 hours using Guava ViaCount Reagent (Millipore) and flow cytometry as described previously (21).

5.3.4. *In vitro* HDAC activity

Cellular HDAC activity was measured in the presence of test agents using the HDAC-Glo I/II Assay kit (Promega) according to manufacturer's instructions. Briefly, Jurkat cells were re-suspended in phenol red and FBS-free RPMI 1640 at $3.0x10^5$ cells/mL, and 10 µL cell cultures were aliquoted into white 384-well plates in the presence of test agents or 0.1% DMSO diluted in 10 µL of HDAC-Glo Buffer. Following incubation at 37 °C for 90 minutes, 20 µL of HDAC-Glo I/II Reagent plus 1% Triton X-100 was added to each well, mixed, and incubated at room temperature for 30 minutes. Luminescence was detected using an Infinity M200 multimode plate reader (Tecan Life Sciences). Wells containing only media were processed in parallel to control for signal background. For each experiment, 4 replicates of each condition were performed.

5.3.5. RNA sequencing and data analysis

RNA sequencing and data analysis were performed as described previously (22). RNA was extracted from cells using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen) with on-column DNAse treatment (Qiagen RNase-Free DNase Set). 100 ng of DNase treated total RNA was then used for library preparation using the Quant-Seq 3' mRNA-Seq Library Preparation Kit (Lexogen, Vienna, Austria). Library quantity was determined by qPCR (KAPA Biosystem, Wilmington, MA), and library size was determined using the Agilent TapeStation and DNA High Sensitivity D5000 ScreenTape (Agilent, Santa Clara, CA). Libraries were pooled in equimolar amounts and denatured, and high-output, single-read, 75 base pair sequencing was performed using a NextSeq 500 (Illumina, San Diego, CA).

RNAseq data were aligned against the human genome (version hg19) using STAR (23). Raw read counts were estimated using RSEM v1.2.12 software (24) with Ensemble transcriptome information version GRCh37.13. Raw counts were normalized and tested by DESeq2 (25) to estimate significance of differential expression, where genes that passed the FDR < 5% threshold were considered significant. Gene set enrichment analysis of significant genes was performed using Ingenuity Pathway Analysis software (Qiagen) using the "canonical pathways" category. Nominal p values were adjusted for multiple testing using the Benjamini-Hochberg procedure to estimate the FDR (26). Pathways enriched at FDR < 5% and with a predicted activation |Z-score| > 2 in at least one treatment were reported. Predicted activation Z-score was calculated by Ingenuity

Pathway Analysis software based on the direction of gene expression changes and known effect on pathway activity.

5.3.6. Measures of HIV-1 latency reversal and viability in primary CD4+ T cells

CD4+ T cells were isolated from the PBMCs of three HIV-infected ART-suppressed individuals using the EasySepTM Human CD4+ T Cell Enrichment Kit (STEMCELL) and allowed to recover in RPMI plus 20% FBS at 37 °C for 24 hours. For each donor, 106 CD4+ T cells were then cultured in 1 mL of RPMI plus 20% FBS plus test agent and 100 U/mL IL-2 (Sigma-Aldrich) at defined concentrations for an additional 24 hours. Total RNA was then extracted using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen) with on-column DNase treatment. Cell-associated total elongated HIV-1 RNA was then quantified with a qPCR TaqMan assay using LTR-specific and control PCR primers and conditions described previously (27). Nucleic acid input from 5 µL of isolated total RNA was normalized for cell number by comparing the 18S housekeeping gene copy number to co-amplified copy number standards. Viral RNA per million cells was then determined by comparing results to co-amplified HIV copy number standards.

Viability of CD4+ T cells from 3 donors without HIV was measured using the culture conditions described above and ViaCount dye (Millipore) according to manufacturer's instructions.

5.3.7. Measures of HIV-1 latency reversal and viability in PBMCs

3x105 PBMCs were incubated in 200 µL R10+ plus for 24 hours at 37 °C before addition of test agents at defined concentrations or 0.1% DMSO in duplicate and further incubation for an additional 24 hours. Total cell lysates and supernatants were then measured for detection of p24Gag protein by ELISA (Xpress Bio, Frederick, MD) according to manufacturer's instructions. Results were normalized to kit p24Gag protein standards to calculate total p24Gag per sample (pg/mL). Viability of PBMCs from 6 donors without HIV was measured using the culture conditions described above and ViaCount dye (Millipore) according to manufacturer's instructions.

5.3.8. Cellular data analysis

Flow cytometry data were analyzed using FlowJo v. 10.5.3 software (FlowJo LLC, Ashland, OR). For studies using flow cytometry, background GFP signals in J-Lat cells treated with 0.1% DMSO were set to an average of 0.05 – 0.15%-positive cells, while background GFP signals in CEM-GXR cells treated with 0.1% DMSO were set to an average of 1.0%. For flow cytometry experiments measuring CD25/CD69 and $p24^{Gag}$, background fluorescence signals were set to an average of 1.0%.

Synergism from LRA combinations was calculated using the Bliss independence model as described previously (11, 28). Here, synergy was defined by the equation:

$$f_{axy,P} = fa_x + fa_y - (fa_x)(fa_y)$$

where $f_{axy,P}$ represents the predicted fractional response due to drugs x + y assuming strictly additive effects, given the observed fractional responses of drug x (fa_x) and drug y (fa_y). The experimentally observed fractional response due to drugs x + y ($f_{axy,O}$) was then compared with the predicted fractional response:

$$\Delta f_{axy} = f_{axy,O} - f_{axy,P}$$

For a given drug combination, a $\Delta f_{axy} > 0$ indicates a fractional response greater than what is expected for additive effects. The statistical significance of this difference (*i.e.* $f_{axy,O}$ vs. $f_{axy,P}$) was determined using Student's paired (for cell lines) or unpaired (for PBMCs) t-test, where a two-sided p value of 0.05 was considered significant.

All data are reported as mean ± SD from at least 3 independent experiments. For *in vitro* drug combination/synergy studies, all data are reported as mean ± SD from at least 4 independent experiments.

5.3.9. HIV-1c replication inhibition assay

In a separate study (18), KA's anti-HIV profile *ex vivo* was determined as previously described (29) using PBMCs isolated from HIV-1 negative individuals (generously donated to the researchers by Botswana National Blood Bank). The PBMCs were infected with HIV-1c molecular clone MJ_4 (30), and then treated with several concentrations of KA alongside azidothymidine (AZT, Zidovudine, Retovir®, GlaxoSmithKline) as a positive control. After 7days incubation, HIV-1c (MJ_4) p24 antigen production was measured by ELISA (XpressBio, Frederick, MD, USA) in accordance with the manufacturer's instructions. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Sigma) was used to assess viability of PBMCs treated with different concentrations of the compounds as previously described (29). GraphPad Prism 4.0 software (GraphPad Software, La Jolla, CA, USA) was used to calculate dose–response data performed in triplicate determination (reported as mean ± S.D.); from which the best-fit dose response curves were generated and EC_{50} and CC_{50} determined.

5.4. Results

5.4.1. Discovery of novel LRAs from pure natural products

To identify new LRAs from natural product sources, we used the Jurkat-derived J-Lat 9.2 cell line, which contains a non-infectious HIV provirus where premature stop codons are engineered into *env* and where *nef* is replaced with a GFP reporter (16). Detection of GFP in these cells, as measured by flow cytometry, thus indicates HIV provirus expression. Using this assay, we screened 216 pure compounds from the pANAPL at 5 µg/mL for 24 hours and identified one compound, KA (**Fig. 5.**), which at 5 µg/mL (~12 µM) induced GFP expression in 6.1 ± 5.2% of cells (mean ± SD). Based on this observation, we then screened 16 additional anthrones from pANAPL and commercially available sources at 10 µM (**Appendix B)** and observed that anthralin (dithranol; **Fig. 5.1**) also induced 6.9 ± 2.4% GFP-positive cells. A third anthrone, prinoidin (Fig. 1), was also observed to induce 2.8 ± 0.7% GFP-positive cells; however, it was not explored further due to its limited availability. No other assessed anthrones induced latency reversal. KA and anthralin were therefore selected for further study.

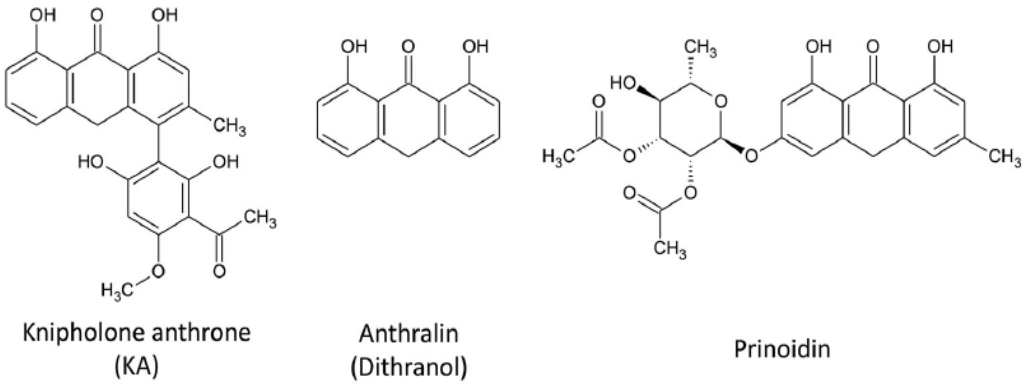


Figure 5.1: Structures of identified LRAs.

5.4.2. KA and anthralin reverse HIV latency in multiple in vitro cell models

To investigate the *in vitro* latency reversal properties of these anthrones in detail, we next measured the dose-response profiles of KA and anthralin in live J-Lat 9.2 cells. In parallel, we also assessed the activities of control LRAs including the PKC activator prostratin and the HDAC inhibitor panobinostat. Examples of latency reversal in J-Lat 9.2 cells, as measured by GFP expression, are shown in Fig. 5.2A. In these studies, treatment of J-Lat 9.2 cells with 10 μM of prostratin induced 15.8 ± 2.7% GFP-positive cells, while stimulation with 0.3 μM panobinostat resulted in 40.5 ± 3.8% GFP-positive cells (**Fig. 5.2B**). In contrast, 10 μM KA resulted in 7.1 ± 1.6% GFP-positive cells, while 10 μM anthralin induced 7.2 ± 0.5% positive-cells. Using the approach of Hashemi *et al.* (2017) (28) and normalizing to the average GFP response for 10 μM prostratin as described previously (11), the relative 50% effective concentrations (EC_{50}s) for prostratin, panobinostat, KA, and anthralin were calculated to be 5.4 ± 1.4, 0.14 ± 0.02, 10.4 ± 1.0, and 12.1 ± 1.7 μM, respectively (**Table 5.1**).

Table 5.1: Relative activities of LRAs in vitro.
Data are presented as relative EC50s (in μM) normalized to the average GFP response for 10 μM prostratin (i.e., the concentration required to induce 50% of the signal observed by 10 μM prostratin) (28).

Cell line	Prostratin	Panobinostat	KA	Anthralin
	μM	μM	μM	μM
J-Lat 9.2	5.4 ± 1.4	0.14 ± 0.02	10.4 ± 1.0	12.1 ± 1.7
J-Lat 8.4	5.0 ± 0.9	0.46 ± 0.12	7.4 ± 2.6	12.6 ± 1.9
J-Lat 10.6	1.9 ± 0.9	0.64 ± 0.09	16.0 ± 5.5	16.2 ± 1.2
OM10.1	2.7 ± 1.0	1.6 ± 0.5	>30	>30

To investigate whether latency reversal due to KA and anthralin were independent of proviral integration site in J-Lat cells, we next assessed their dose-response profiles in the related cell lines J-Lat 8.4 and J-Lat 10.6 (**Fig. 5.2C-D**). While results were broadly consistent with those from J-Lat 9.2 cells, a few differences were observed. For example, while 10 μM prostratin induced 5.0 ± 1.1% GFP-positive, live J-Lat 8.4 cells, 0.3 μM panobinostat induced GFP in only 1.4 ± 0.9% of J-Lat 8.4 cells, indicating that this cell line is less responsive to this HDAC inhibitor. In contrast, KA and anthralin induced 5.0 ± 2.5% and 1.8 ± 0.8% GFP-positive live cells, respectively (**Fig. 5.2C**). When normalized to 10 μM prostratin, the relative EC_{50}s of prostratin, panobinostat, KA, and anthralin were 5.0 ± 0.9, 0.46 ± 0.12, 7.4 ± 2.6, and 12.6 ± 1.9 μM, respectively (**Table 5.1**). Similarly, in live J-Lat 10.6 cells, which in our hands (and as described previously (31-32)), induced spontaneous GFP expression in 7.6 ± 0.2% of cells and as described previously (31, 33), 10 μM prostratin induced 76.0 ± 0.8% GFP-positive cells, while 0.3 μM panobinostat induced GFP in 23.7 ± 1.8% of cells. Comparatively, 36.2 ± 13.7% and 30.8 ± 2.4% of cells were induced by 10 μM of KA and anthralin, respectively (**Fig. 5.2D**). When normalized to 10 μM prostratin, we recorded relative EC_{50}s of 1.9 ± 0.9, 0.64 ± 0.09, 16.0 ± 5.5, and 16.2 ± 1.2 μM for prostratin, panobinostat, KA, and anthralin (**Table 5.1**). The observation that the latency reversal properties of KA and anthralin are broadly consistent across multiple cell lines suggests that their activities are not dependent on specific proviral integration sites, at least in Jurkat-derived T cell lines.

To determine whether KA and anthralin's latency reversal was independent of cell type, we next treated OM-10.1 cells, which are derived from HL-60 promyelocyte cells and

contain an integrated, replication-competent provirus. Similar to J-Lat 10.6 cells, we observed that OM10.1 cells spontaneously expressed $p24^{Gag}$ protein in 2.1 ± 0.2% of cells. Treatment of OM-10.1 cells with 10 μM prostratin for 24 hours stimulated virus expression in 62.4 ± 4.4% of live cells, while 0.3 μM panobinostat induced provirus expression in only 12.0 ± 4.5% of cells. Notably, 10 μM KA elicited only 3.3 ± 0.7% $p24^{Gag}$-positive live cells, while 10 μM anthralin induced 5.2 ± 1.2% $p24^{Gag}$-positive live cells (**Fig. 5.2E**). While improved responses were observed with 30 μM KA (27.4 ± 6.5% $p24^{Gag}$-positive live cells), extensive toxicity precluded measurements of 30 μM anthralin. When normalized to 10 μM prostratin, the relative EC_{50}s for prostratin, panobinostat, KA, and anthralin were 2.7 ± 1.0, 1.6 ± 0.5, > 30, and > 30 μM, respectively (**Table 5.1**). Thus, KA and anthralin may more effectively reverse latency in T cell-derived lines.

Taken together, these results suggest that both KA and anthralin induce provirus expression across multiple cell lines and proviral integration sites, although KA and anthralin's activities varied depending on the cell line.

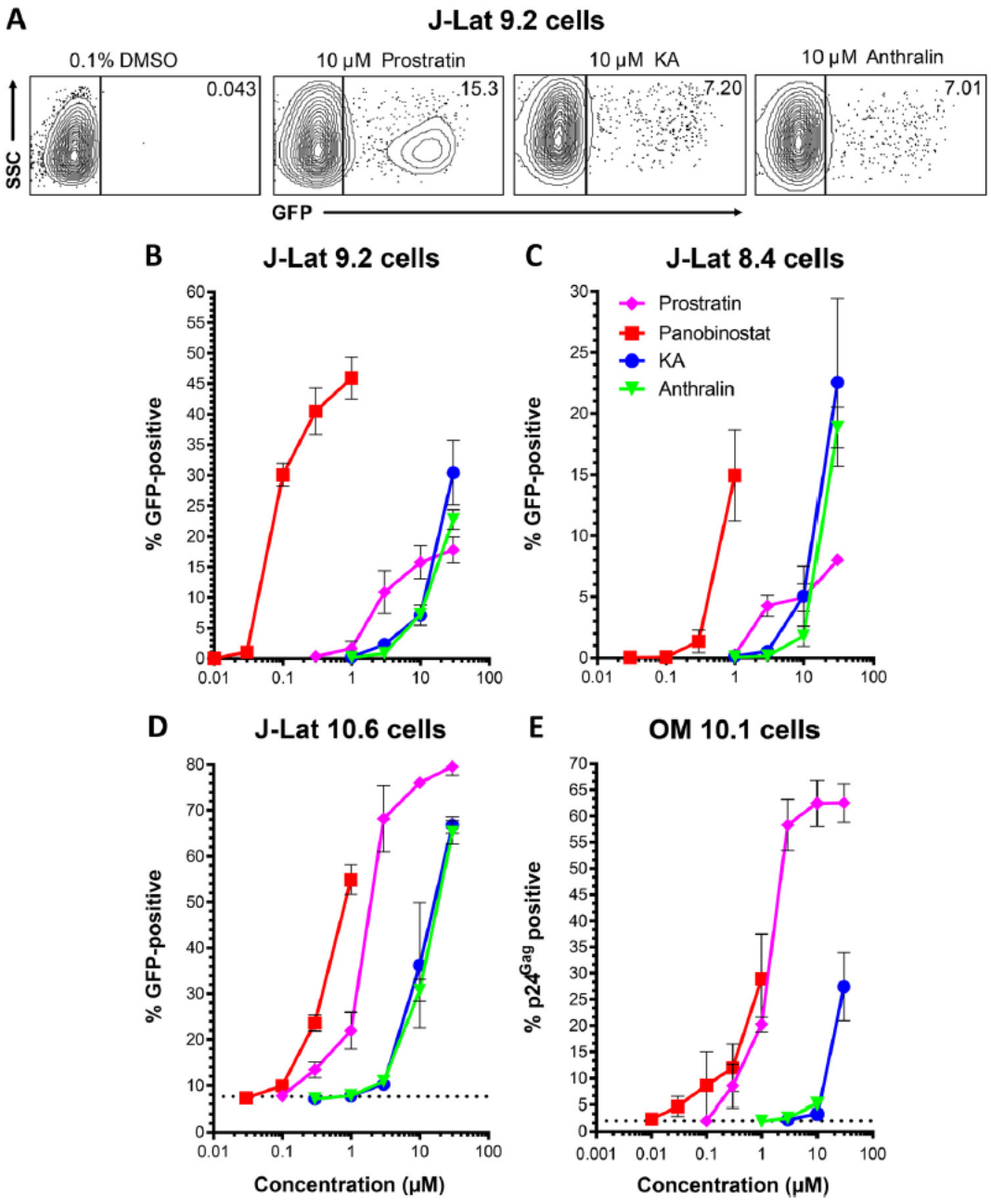


Figure 5.2: KA and anthralin reverse HIV latency in vitro.
(**A**) Representative flow cytometry data showing latency reversal, as measured by GFP expression, in J-Lat 9.2 cells. Values indicate percent GFP-positive cells for each condition. (**B-D**) Dose response profiles of control LRAs panobinostat and prostratin, in addition to KA and anthralin, are shown in J-Lat 9.2 (B), J-Lat 8.4 (**C**), and J-Lat 10.6 (**D**) T cells. (**E**) Dose response profiles of LRAs in OM10.1 promyeloid cells, as measured by cellular expression of viral $p24^{Gag}$ protein. Dotted lines in (**C**) and (**D**) indicate baseline levels of spontaneous latency reversal. Error bars, S.D.

5.4.3. Effects of KA and anthralin on apoptosis and cell activation markers

To directly assess the impact of KA and anthralin on cell viability, we next treated Jurkat cells (the parental cell line of J-Lat cells) with LRAs as described above. Following treatment, cells were then assessed for surface expression of the early apoptotic marker annexin V by flow cytometry. **Fig. 5.3A** shows representative results of apoptotic cells in the presence of LRAs, where control data are consistent with previous results (11), while **Fig. 5.3B** shows dose-response profiles where data were normalized to the percent apoptotic cells in control cell cultures treated with 0.1% DMSO. In this assay, 10 μM prostratin induced only a 1.8 ± 0.4-fold increase in apoptosis, while 0.3 μM panobinostat induced a 4.6 ± 1.4-fold increase, consistent with our previous results (11). Like panobinostat, both KA and anthralin also induced apoptosis: for example, 10 μM KA resulted in a 3.4 ± 0.6-fold increase in apoptotic cells, while 10 μM anthralin caused a 5.6 ± 1.1-fold increase (**Fig. 3B**). These results suggest that, like the HDAC inhibitor panobinostat, both KA and anthralin induce apoptosis *in vitro* at concentrations that also induce latency reversal.

To assess whether KA and anthralin may also affect T cell activation, Jurkat cells were treated with LRAs for 24 hours and stained for the T cell activation markers CD69 and CD25. **Fig. 5.3C-D** show representative results of CD69 and CD25 expression in live-gated Jurkat cells in the presence of LRAs, respectively, while **Fig. 5.3E-F** show dose-response profiles normalized to marker expression in control cells treated with 0.1% DMSO. As expected, based on previous observations (32, 34), 10 μM prostratin induced a 7.2 ± 4.0-fold increase in CD69 expression, relative to cells treated with 0.1% DMSO, while no detectable increase in CD69 expression was observed with any concentration of panobinostat (**Fig. 5.3E**). Notably, 10 μM KA induced a 9.3 ± 6.4-fold increase in CD69 expression, while 30 μM induced up to 22.5 ± 7.1-fold increase. In contrast, 10 μM anthralin induced only a 2.0 ± 0.6-fold increase in CD69-positive cells, while 30 μM induced a 6.4 ± 4.4-fold increase. Also consistent with previous observations (32, 34), 10 μM prostratin induced an 11.8 ± 1.3-fold increase in CD25 expression, while no more than a 2.0 ± 0.8-fold increase in CD25 expression was observed with 0.3 μM panobinostat (**Fig. 3F**). However, neither KA nor anthralin induced CD25 expression to levels approximating

those of prostratin: no increase was observed for KA at any concentration, while 10 μM anthralin induced only a maximal 3.6 ± 2.2-fold increase. These observations indicate that KA is a particularly potent inducer of at least a subset of T cell activation markers *in vitro*, while anthralin can also induce T cell activation markers at high concentrations.

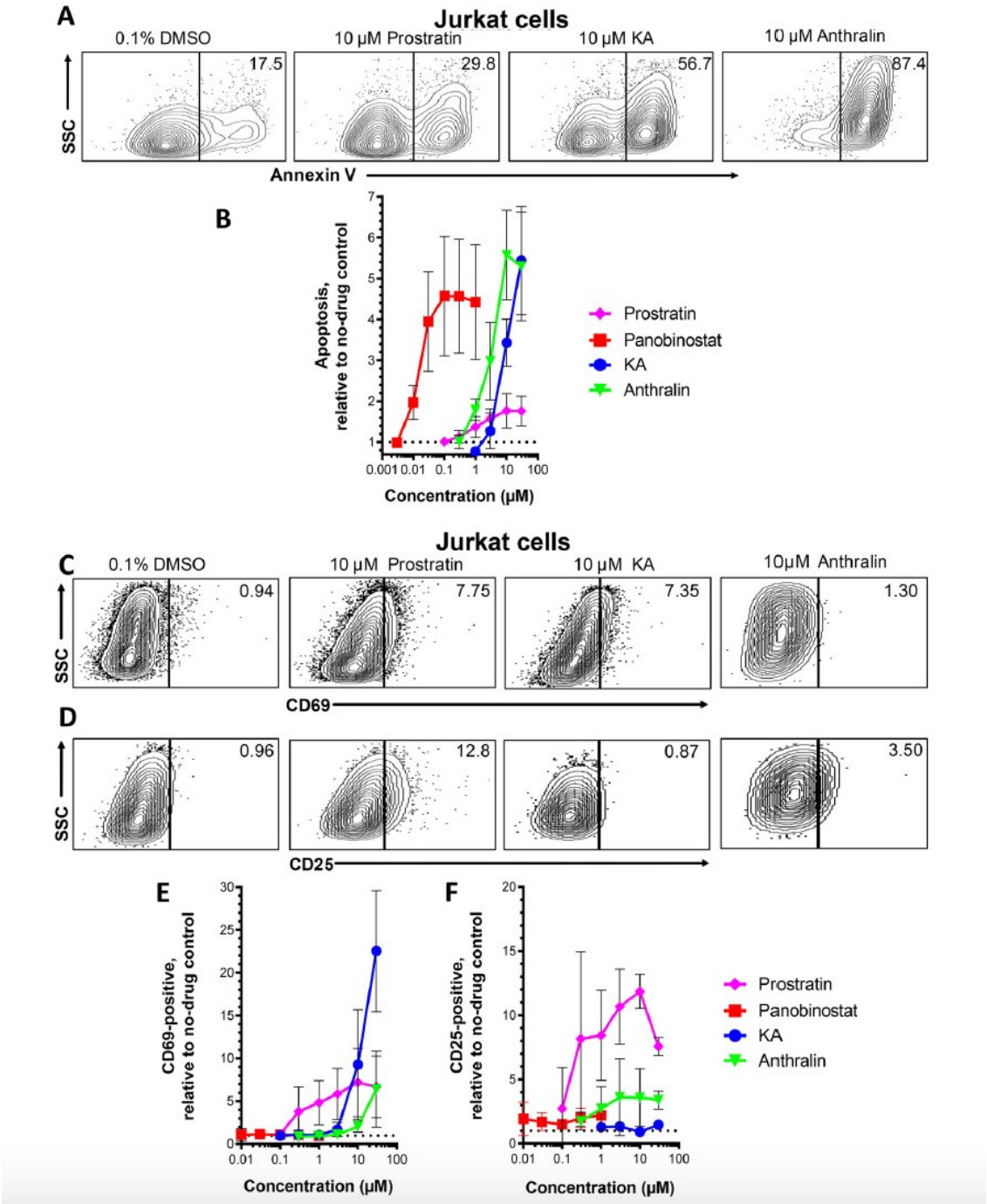


Figure 5.3*:* Effects of LRAs on in vitro cell viability and expression of cell activation markers. **A**, representative flow cytometry data showing Jurkat cell apoptosis, as measured by annexin V detection. Values indicate percentage of annexin-positive cells. **B**, dose-response profiles of panobinostat, prostratin, KA, and anthralin on apoptosis in Jurkat cells. Data are presented as -fold

increase in annexin V–positive cells relative to cells treated with 0.1% DMSO (dotted line). **C** and **D**, representative flow cytometry data showing Jurkat cells stained for CD69 **(C**) or CD25 expression (**D**). Values indicate percentage of CD69- or CD25-positive cells, respectively. **E** and **F**, dose-response profiles of LRAs on CD69 **(E)** and CD25 **(F**) expression in Jurkat cells. Data are presented as -fold increases in each marker relative to cells treated with 0.1%DMSO (dotted lines). Error bars, S.D.

5.4.4. KA and anthralin do not function as PKC activators or HDAC inhibitors

To date, numerous LRAs have been reported to act through two major cellular pathways: PKC activation and HDAC inhibition (8-9). To determine whether KA and anthralin function as PKC activators, we asked if their activities in live J-Lat 9.2 cells were antagonized by the pan-PKC inhibitor GÖ-6983 (35). Following 24 hours' treatment with LRAs, additional co-treatment with 1 μM GÖ-6983 resulted in complete suppression of GFP expression induced by 10 μM of the PKC activator prostratin (0.4% GFP-positive cells relative to cells treated with only prostratin), but not by 0.3 μM of the HDAC inhibitor panobinostat (**Fig. 5.4A**). Furthermore, no loss of GFP expression was observed when 1 μM GÖ-6983 was added to J-Lat cultures treated with either 10 μM KA or anthralin (in both cases > 95% of cells treated without GÖ-6983 maintained GFP). This indicates that KA and anthralin do not function as PKC activators *in vitro*.

To assess whether KA and anthralin function as HDAC inhibitors, we used a commercially available HDAC-Glo I/II Assay kit which quantifies class I and II HDAC activity in live Jurkat cells via a cell permeable, acetylated, luminogenic peptide substrate. With this approach, HDAC inhibitors should antagonize cellular deacetylation of the fluorogenic substrate and reduce luminescence readout. As expected, panobinostat was a potent cellular HDAC inhibitor in this assay: 0.1 μM inhibited 96.4 ± 1.2% of luminescence, while 0.3 nM inhibited 52.5 ± 1.2% (**Fig. 5.4B**). In contrast, up to 30 μM prostratin did not affect cellular HDAC activity. Similarly, neither KA nor anthralin had any activity at up to 30 μM, indicating that these LRAs also do not function as HDAC inhibitors *in vitro*.

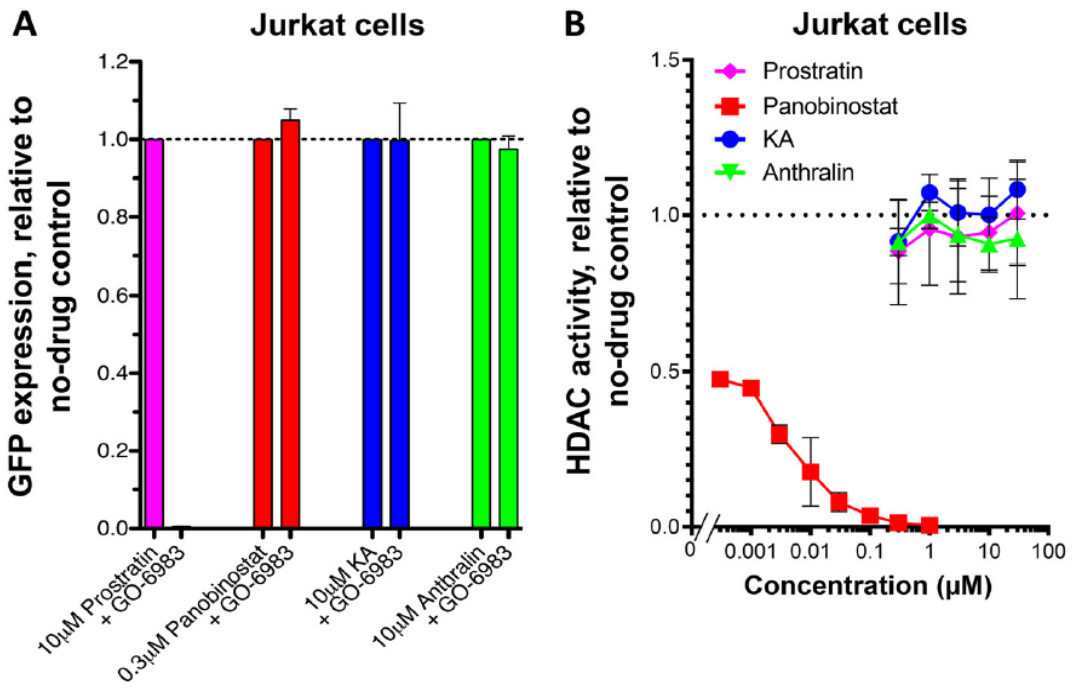


Figure 5.4*:* KA and anthralin are not PKC activators or HDAC inhibitors.
A, effects of panobinostat, prostratin, KA, and anthralin on latency reversal in J-Lat 9.2 cells in the presence of pan-PKC inhibitor GÖ-6983. **B**, effects of LRAs on cellular HDAC activity, as measured by HDAC-Glo assay. Error bars, S.D.

5.4.5. Latency reversal by KA and anthralin are regulated by reactive oxygen species and/or metal ions

Both KA and anthralin are reported to promote oxidative stress in cells (36-39). KA is also reported to chelate metal ions (36). If one or more of these properties are required by KA or anthralin for latency reversal, then blocking these pathways should antagonize these LRAs. To test this hypothesis, we first treated J-Lat 9.2 cells with 10 μM prostratin, 0.1 μM panobinostat, 10 μM KA, or 10 μM anthralin for 24 hours in the presence or absence of modulators of oxidative stress or free metal ions (**Fig. 5.5**). Modulators included glutathione (a scavenger of reactive oxygen species), deferoxamine (an iron chelator), and bathocuproine (a copper (I) chelator).

In the presence of increasing concentrations of glutathione, we observed that anthralin-induced GFP, but not GFP induced by KA, prostratin or panobinostat, was inhibited (**Fig. 5.5A**). While complete inhibition of anthralin's activity was not observed, treatment of J-Lat 9.2 cells with 100 μM glutathione reduced anthralin-dependent latency reversal to 45.0

± 6.5% of cells treated with anthralin in the absence of glutathione. In contrast, no inhibition of KA was observed with glutathione concentrations as high at 3 mM. Glutathione in the absence of LRAs had no effect on GFP expression (data not shown). These observations suggest that the latency reversal properties of anthralin, but not KA or control LRAs, are dependent on cellular oxidative stress.

In contrast, treatment of J-Lat 9.2 cells with increasing concentrations of deferoxamine resulted in enhanced latency reversal when co-incubated with KA, where 300 μM boosted the activity of 10 μM KA to 130.7 ± 10.6% of cells expressing GFP in the absence of deferoxamine (**Fig. 5.5B**). In the presence of 10 μM anthralin, 300 μM deferoxamine caused a 2.6 ± 0.4-fold increase in GFP expression relative to cells treated with anthralin alone. In contrast, both prostratin and panobinostat were slightly inhibited by 300 μM deferoxamine (69.5 ± 3.9% and 77.1 ± 17.1% GFP-positive cells, respectively). These results suggest that the activities of both KA and anthralin are either inhibited by iron ions or otherwise stimulated by deferoxamine *in vitro*.

Finally, treatment of cells with 100 μM bathocuproine inhibited GFP expression induced by 10 μM KA, where only 39.6 ± 11.8% of GFP-positive cells were observed relative to KA-treated cells without bathocuproine. This suggests that KA's activity is dependent on the presence of copper ions. GFP expression induced by anthralin and panobinostat were also affected by 100 μM bathocuproine (50.2 ± 24.6% and 21.9 ± 17.1% of GFP expression, respectively; **Fig. 5.5C**), suggesting that their activity is also dependent on copper. In contrast, prostratin’s activity was enhanced in the presence of bathocuproine where, for example, 100 μM boosted GFP expression to 159.1 ± 44.4% of cells treated with prostratin alone. Taken together, these results suggest that the latency reversal properties of anthralin are dependent on both oxidative stress and metal ions, while KA’s properties are dependent only on metal ions.

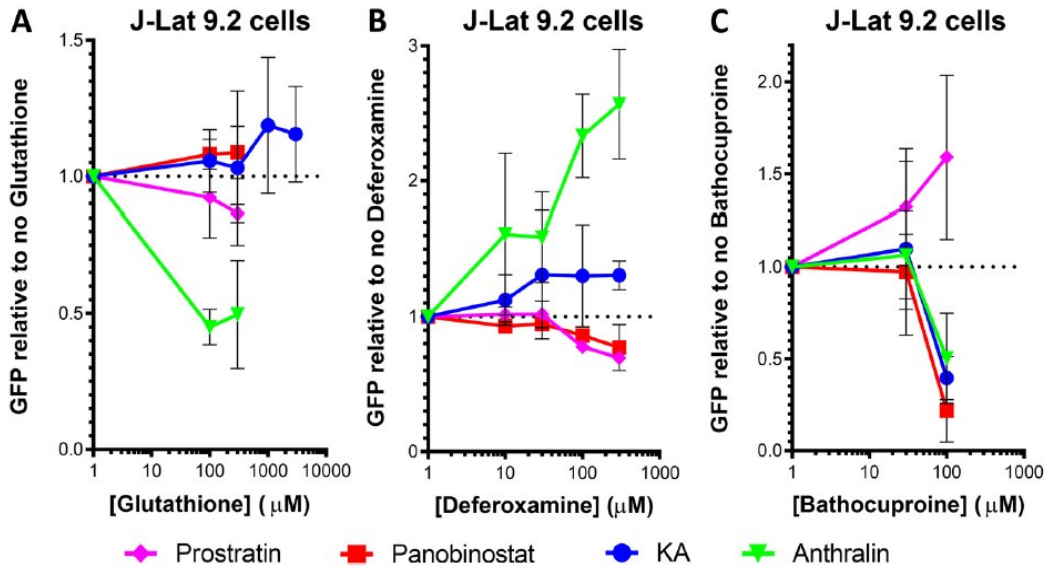


Figure 5.5: Effects of LRAs on HIV latency reversal in J-Lat 9.2 cells in the presence of the anti-oxidant GSH (A), iron chelator deferoxamine (B), and copper chelator bathocuproine (C).
Error bars, S.D.

5.4.6. KA and anthralin induce distinct gene expression profiles in vitro

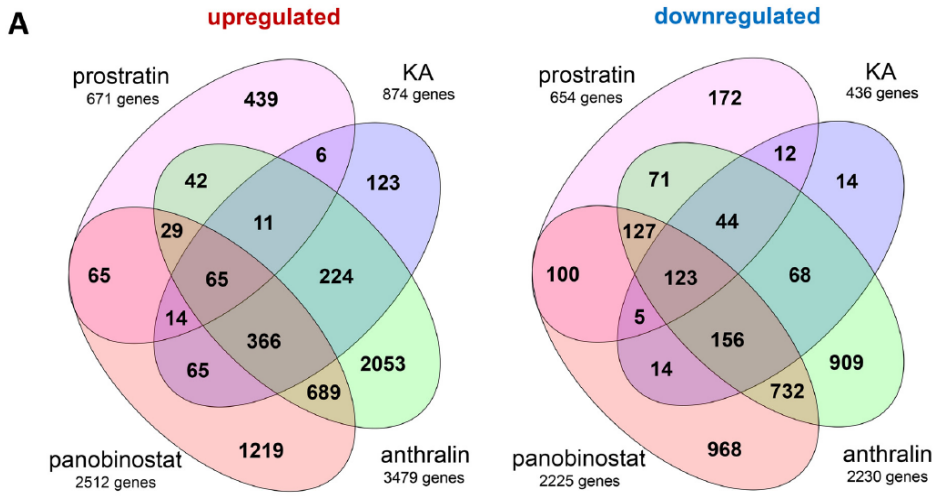
To identify potential mechanisms by which KA and anthralin may induce HIV transcription *in vitro*, we next treated 3 independent preparations of J-Lat 10.6 cells for 24 hours with 0.1% DMSO vehicle control, prostratin (10 μM), panobinostat (0.1 μM), or KA or anthralin (10 μM). Cells were then harvested for RNA extraction, and global transcriptional profiles were assessed by RNAseq. High-quality sequence data were obtained for all samples with the potential exception of cells treated with anthralin, which contained approximately 21.5% of total transcriptome reads observed in other samples and which was suggestive of cellular toxicity. From these data, we identified a total of 8707 unique differentially-expressed genes (false discovery rate (FDR) < 5%) affected by at least one treatment when compared to DMSO-treated samples. Anthralin and panobinostat had the largest effects on overall transcription with overlap of over 2000 genes (**Fig. 5.6A**).

The four sets of genes affected by each individual treatment were then analyzed for enrichment of canonical pathways using Ingenuity Pathway Analysis (IPA) (**Fig. 5.6B**). Consistent with the established role of prostratin as a PKC agonist, we identified distinct upregulation of genes involved in PKCθ signaling (Z-score = 4.1; see Experimental Procedures) and NF-κB activation by viruses (Z-score = 2.7; **Fig. 5.6B**). Additionally, we

found activation of CD28 signaling and cytokine signaling and production, while PD-1/PD-L1 pathways were inhibited, consistent with T cell activation (**Fig. 5.6B**). In contrast, cells treated with panobinostat activated cellular senescence, unfolded protein response, and the antiproliferative role of TOB (Transducer of ErbB2) in T cell signaling, suggestive of reduced cellular activation and mild-to-moderate cytotoxicity. Also consistent with induction of T cell quiescence in cells treated with panobinostat, genes affected by prostratin were frequently induced in the opposite direction of T cell activation and cytokine signaling (**Fig. 5.6B**).

Cells treated with KA also had several affected gene pathways that were also observed in panobinostat-treated cells. However, KA-treated cells also had particularly strong and unique activation of the sirtuin signaling pathway (Z-score = 2.2). This result suggests that KA might disproportionately support latency reversal via sirtuin-mediated enhancement of Tat deacetylation and priming to initiate new cycles of viral transcription (39). Additional pathways unique to KA treatment included inhibition of gluconeogenesis and glycolysis (Z-scores = -2.2 and -2.6, respectively; **Fig. 5.6B**).

Finally, although cells treated with anthralin resulted in the largest number of significantly affected genes (N = 5709; **Fig. 5.6A**), no signaling pathways were identified at FDR < 5% significance. However, among the most strongly affected pathways unique to anthralin treatment was cAMP-mediated signaling, which exhibited borderline significance ($p = 0.003$; FDR = 0.16; Z-score = 4.0), which is suggestive of anthralin uniquely acting in part by driving cAMP or protein kinase A-mediated viral transcription (40, 41). Taken together, these results suggest that KA and anthralin affect gene expression and induction of pathways distinct from those of control LRAs and further support that they induce latency reversal by distinct mechanisms.



B

Canonical Pathways	Prostratin N	Prostratin pval	Prostratin FDR	Prostratin Z	Panobinostat N	Panobinostat pval	Panobinostat FDR	Panobinostat Z	KA N	KA pval	KA FDR	KA Z	Anthralin N	Anthralin p	Anthralin pval	Anthralin Z
NF-κB Activation by Viruses	17	$1x10^{-6}$	0%	2.7	15	0.003	1%	-0.3	4	0.49	62%	na	4	1	100%	na
PKCθ Signaling in T Lymphocytes	25	$7x10^{-7}$	0%	4.1	29	$4x10^{-5}$	0%	0.4	16	0.001	1%	0.3	13	0.071	43%	2.8
CD28 Signaling in T Helper Cells	24	$2x10^{-8}$	0%	3.9	31	$1x10^{-8}$	0%	-0.6	14	$8x10^{-4}$	1%	0	6	1	100%	2.2
IL-2 Signaling	14	$3x10^{-6}$	0%	3.2	13	0.002	1%	-0.6	4	0.28	41%	na	3	1	100%	na
IL-9 Signaling	14	$5x10^{-10}$	0%	3.1	8	0.005	2%	0.7	3	0.18	30%	na	2	0.53	77%	na
IL-15 Signaling	18	$6x10^{-8}$	0%	2.4	14	0.004	2%	-0.5	3	1	100%	na	2	1	100%	na
PD-1, PD-L1 cancer immunotherapy	26	$4x10^{-11}$	0%	-2.6	21	$2x10^{-4}$	0%	1.3	6	0.32	45%	0.8	3	1	100%	na
Senescence Pathway	34	$5x10^{-6}$	0%	3.7	43	$6x10^{-5}$	0%	2.3	26	$2x10^{-4}$	0%	2.4	27	0.002	16%	2.5
Unfolded protein response	3	0.58	65%	na	13	$6x10^{-4}$	0%	2.3	9	$7x10^{-4}$	1%	2.8	6	0.078	43%	2.2
Antiproliferative Role of TOB in T Cells	5	0.055	10%	1.3	10	0.001	1%	2.5	6	0.007	4%	2.4	2	1	100%	na
Sirtuin Signaling Pathway	33	$4x10^{-5}$	0%	-0.7	43	$2x10^{-4}$	0%	1.4	40	$2x10^{-10}$	0%	2.2	19	0.21	56%	1.7
Gluconeogenesis I	1	1	100%	na	0	1	100%	na	5	0.005	3%	-2.2	0	1	100%	na
Glycolysis I	0	1	100%	na	0	1	100%	na	7	1E-04	0%	-2.6	0	1	100%	na
cAMP-mediated signaling	15	0.24	32%	0	23	0.22	33%	2.4	15	0.079	19%	1.4	23	0.003	16%	4.0

Figure 5.6: Effects of LRAs on in vitro global gene expression as measured by RNAseq. (**A**) Venn diagrams showing number of significantly upregulated or downregulated genes (FDR < 5%) in J-lat 10.6 cells treated with prostratin, panobinostat, KA, or anthralin, when compared to cells treated with 0.1% DMSO. (**B**) Ingenuity Pathway Analysis results of genes identified in (A) that passed FDR < 5% and |Z-score| > 2 thresholds. For each pathway, data listed include Z-scores (Z) for predicted pathway state (where positive and negative values indicate activation or inhibition by treatment, respectively), number of affected genes (N), p value (pval), and FDR. P-values < 0.05, FDR < 5% (or < 20% for anthralin) thresholds are highlighted. Number of genes and Z-scores are highlighted as scales.

5.4.7. KA and anthralin synergize with multiple LRAs

When applied in combination, LRAs with similar mechanisms of action tend to exhibit additive effects, while LRAs representing different functional classes frequently result in synergistic (*i.e.,* greater than additive) effects (11, 27, 42-43). To examine whether KA and anthralin can enhance the activities of established LRAs, we treated J-Lat 9.2 cells with KA or anthralin in combination with control LRAs at concentrations that induce sub-maximal GFP expression. We first assessed whether 3 μM KA and 10 μM anthralin could

synergize with control LRAs tested at a single concentration. Control LRAs included prostratin (10 μM), panobinostat (0.1 μM), TNFα (10 ng/mL), the BET bromodomain inhibitor JQ1 (0.7 μM) (27, 42-43), and the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (Aza-CdR; 1 μM) (44). For each combination, synergism was assessed using the Bliss independence model (27, 42-43), where a calculated Δf_{axy} value > 0 indicates evidence of synergistic effects (see Experimental Procedures). In this study, we conservatively defined evidence of synergism as a Δf_{axy} value > 1.

In all cases, KA enhanced the activities of control LRAs (**Fig. 5.7A-B**). For example, when administered alone, 3 μM KA induced GFP in 5.5 ± 1.7% of live cells, while 10 μM prostratin induced GFP in 12.4 ± 3.1% of live J-Lat 9.2 cells. However, when cells were co-incubated with 10 μM prostratin and 3 μM KA, we observed 38.2 ± 9.4% GFP-positive cells (**Fig. 5.7A**). This represented a 2.1-fold increase over what would be expected by additive effects (~17.9%) and significant evidence of synergism as measured by the Bliss independence model (Δf_{axy} = 20.3 ± 5.0; p = 7.8×10^{-4}; **Fig. 5.7B**). KA also synergized with 0.1 μM panobinostat, which induced 21.9 ± 6.2% GFP expression on its own but 45.0 ± 4.1% GFP expression in combination with KA (Δf_{axy} of 17.6 ± 7.2; p = 0.0055; **Fig. 5.7A-B**). Similarly, while 10 ng/mL TNFα induced 16.8 ± 3.3% GFP-positive cells on its own, addition of KA resulted in 43.2 ± 5.1% GFP positive cells (Δf_{axy} of 20.8 ± 1.7; p = 9.7×10^{-6}). When 3 μM KA was combined with 0.7 μM of the BET bromodomain inhibitor JQ1 (which exhibited no LRA activity on its own in J-Lat cells), we observed 9.8 ± 5.1% GFP-positive cells and a borderline significant Δf_{axy} of 4.2 ± 3.7 (p = 0.064). Finally, while 1 μM Aza-CdR also exhibited no activity on its own in live J-Lat cells, addition of KA increased GFP-positive cells to 16.0 ± 4.5% GFP-positive cells and a Δf_{axy} of 10.4 ± 3.0 (p = 0.0015). These results indicate that 3 μM KA significantly synergizes with 4 of 5 control LRAs at these concentrations *in vitro*.

Similar results were observed when control LRAs were co-incubated with anthralin (**Fig. 5.7C-D**). For example, when administered alone, 10 μM anthralin induced 4.5 ± 2.3% GFP-expression in live J-Lat 9.2 cells, while 10 μM prostratin induced 11.0 ± 3.1% GFP-positive cells in paired experiments. However, when cells were co-incubated with 10 μM prostratin plus anthralin, we observed 32.5 ± 8.4% live GFP-positive cells (**Fig. 5.7C**). This represented a 1.8-fold increase over the expected additive effects (~16.5%) and a

significant Δf_{axy} of 17.0 ± 9.0 (p = 0.0056; **Fig. 5.7D**). In addition, 10 μM anthralin also enhanced the activity of 0.1 μM panobinostat and 10 ng/mL TNFα, (Δf_{axy} = 4.2 ± 3.2 and 13.2 ± 3.9; p = 0.023 and 4.0×10^{-4}, respectively; **Fig. 5.7C-D**). Co-administration of 10 μM anthralin with 0.7 μM JQ1 also resulted in a Δf_{axy} value of 11.1 ± 3.0 (p = 0.0053), while anthralin plus 1 μM Aza-CdR resulted in a Δf_{axy} value of 5.1 ± 0.8; p = 0.0013). Thus 10 μM anthralin significantly synergized with all control LRAs at these concentrations *in vitro*. In contrast, no obvious enhancement of GFP expression was observed when KA was combined with anthralin (data not shown), although the poor viability of cells treated with both agents made interpretation of these data difficult.

We next asked what concentrations of KA and anthralin were required to achieve synergy with a control LRA. In this experiment, J-Lat 9.2 cells were treated with 10 μM prostratin in the presence of multiple concentrations of KA or anthralin (**Fig. 5.7E-F**). We observed additionally synergistic provirus expression when 10 μM prostratin was co-incubated with 1 and 10 μM KA (**Fig. 5.7E**), with calculated Δf_{axy} values of 10.9 ± 5.1 and 19.1 ± 6.2, respectively (p = 0.0088 and 0.0023, respectively; **Fig. 5.7F**), in addition to the previously-described synergy with 3 μM KA (*i.e.,* **Fig. 5.7A**). Prostratin also synergized with 1, 3, and 30 μM anthralin (**Fig. 5.7E**), where Δf_{axy} values were calculated as 4.7 ± 3.6, 5.7 ± 4.0, and 16.1 ± 11.6, respectively (p = 0.024, 0.018, and 0.019, respectively, **Fig. 5.7F**), in addition to the previously-described synergy with 10 μM anthralin (**Fig. 5.7C**).

In summary, these results indicate that both KA and anthralin synergize with control LRAs representing multiple functional classes and at concentrations as low as 1 μM.

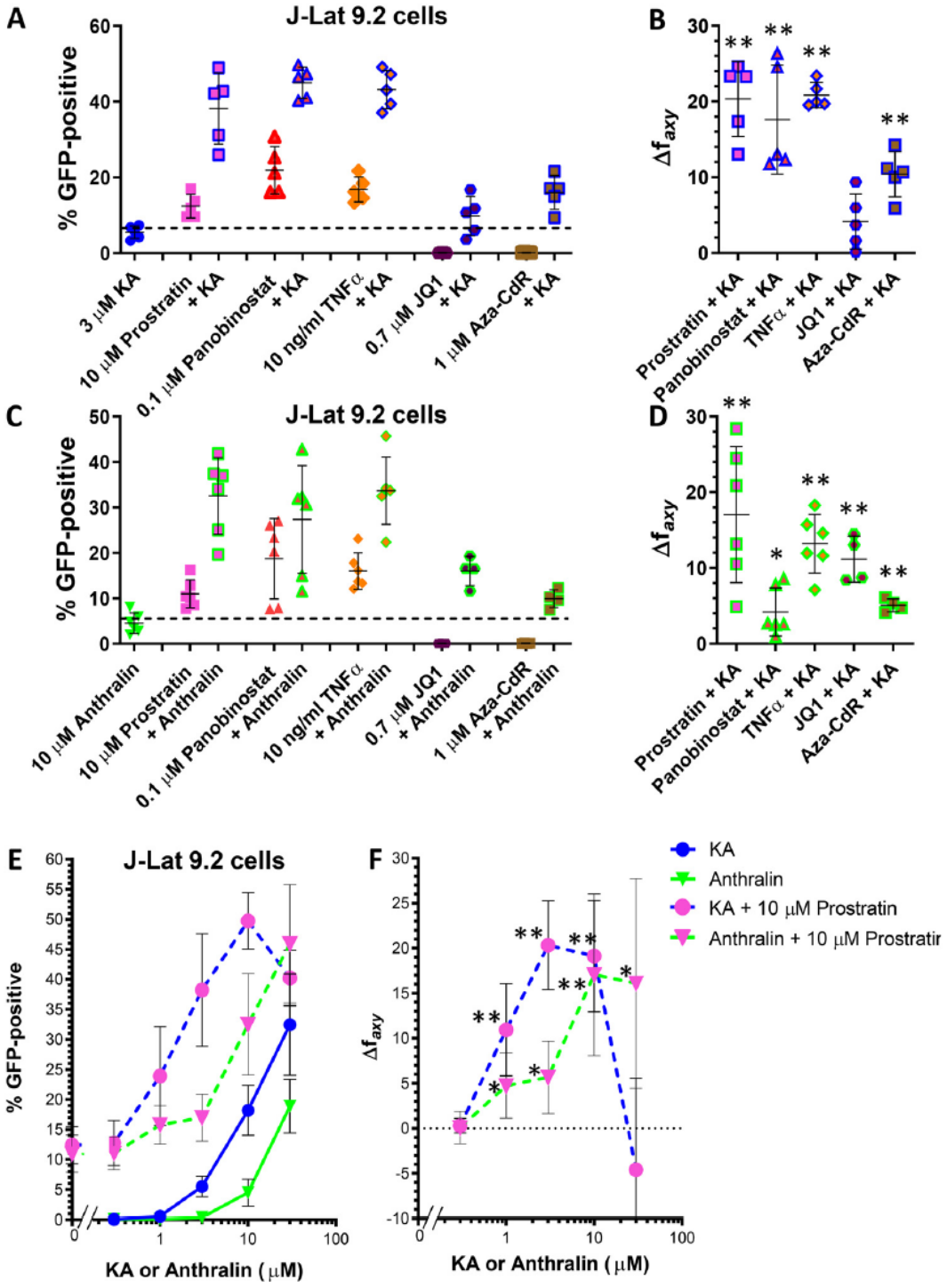


Figure 5.7: KA and anthralin synergize with LRAs from multiple functional classes in vitro. (**A**) Effects of 3 μM KA on latency reversal in J-Lat 9.2 cells in the presence of 10 μM prostratin, 0.1 μM panobinostat, 10 ng/mL TNFα, 0.7 μM JQ1, and 1 μM Aza-Cdr. (**B**) Extent of synergism in J-Lat 9.2 cells treated with KA plus control LRAs in (**A**), as measured by the Bliss Independence model. (**C-D**) Effects of 10 μM anthralin on latency reversal (**C**) and synergism (**D**) in J-Lat 9.2 cells in the presence of control LRAs. Data are arrayed as described in panels (**A-B**). **(E-F**) Effects of KA and anthralin on latency reversal **(E**) and synergism (**F**) in the presence of 10 μM prostratin. In panel (**E**), "0" on the x-axis indicates the activity of 10 μM prostratin in the absence of KA or

anthralin. Data shown for 10 μM prostratin plus 3 μM KA or 10 μM anthralin are the same data shown in panels (**A-D**). *, $p < 0.05$ and **, $p < 0.01$ between the observed and predicted responses assuming strictly additive effects (i.e., Bliss independence model-based synergism) from at least 4 independent experiments. Error bars, S.D.

5.4.8. KA but not anthralin induces HIV-1 expression *ex vivo*

We next sought to investigate whether KA and/or anthralin can reactivate HIV proviruses in primary cells directly isolated from HIV-infected, cART-suppressed individuals. However, we first assessed the extent to which LRAs affected viability of isolated CD4+ cells obtained from 3 uninfected donors, as determined using ViaCount cell viability dye (21). LRAs assessed in this assay included 10 μM prostratin, KA, or anthralin, while 100 ng/mL phorbol myristate acetate (PMA) + 0.1 μg/mL ionomycin was applied as a positive control. For each condition, 10^6 cells were cultured in 1 mL of media in the presence of test agents and 100 U/mL IL-2 for 24 h before assessment of cell viability. Consistent with *in vitro* observations (**Fig. 5.3A-B**), the effects of LRAs on cell viability after 24 hours' incubation extended to primary CD4+ cells (**Fig. 5.8A**). For example, CD4+ cells treated with PMA + ionomycin resulted in 93.3 ± 6.8% viability relative to cells treated with 0.1% DMSO, while 10 μM prostratin resulted in 86.2 ± 11.4% viability. Cells treated with 10 μM KA resulted in 54.2 ± 27.9% viability, indicating detectable but moderate cytotoxicity. In contrast, almost complete loss of cell viability was observed with CD4+ cells treated with 10 (2.0 ± 1.6% viability; **Fig. 5.8A**) or 3 μM anthralin (data not shown).

We next assessed whether PMA + ionomycin, prostratin, KA, or anthralin at the same concentrations and treatment durations described above could induce viral RNA expression, as measured by quantitative PCR (qPCR), from 10^6 isolated CD4+ T cells obtained from 3 HIV-infected donors (cultured in 1 mL of media; **Fig. 5.8B**). qPCR results were normalized to co-amplified 18S housekeeping gene copy number and respective copy number standards to determine absolute copies of HIV per million CD4+ cells (**Appendix C**). Following treatment, PMA + ionomycin increased viral RNA expression in two of three donors compared to baseline expression in cells treated with 0.1% DMSO control, (average across 3 donors of 576 ± 486 viral RNA copies per million CD4+ cells treated with PMA + ionomycin vs. 435 ± 311 copies / million cells in DMSO-treated cells; **Appendix C**). Similar results were also observed in CD4+ cells treated with prostratin (average across 3 donors of 916 ± 765 copies / million cells). Notably, increased viral RNA expression was also observed in 2 of 3 donors treated with KA (average 3365 ± 4563

copies / million cells; **Fig. 5.8B; Appendix C**). These results suggest that KA induces total viral RNA expression in primary CD4+ T cells on par with established LRAs. In contrast, CD4+ cells treated with anthralin resulted in extensive cell death, as observed by microscopy, and viral RNA was not detected (data not shown). To validate whether KA enhances HIV production *ex vivo*, we also assessed its effects on peripheral blood mononuclear cells (PBMCs). When $3x10^5$ PBMCs from 6 uninfected donors were cultured at $1.5x10^6$ cells/mL and assessed for viability following 24 hours' incubation with PMA + ionomycin, we observed 51.0 ± 24.7% viability relative to PBMCs treated with 0.1% DMSO (**Figure 5.8C**). However, cells treated with 10 μM KA resulted in 38.3 ± 20.8% viability, indicating elevated but comparable toxicity when compared to control PMA + ionomycin in whole PBMC cultures. In contrast, near-complete loss of cell viability was again observed with PBMCs treated with 10 (2.8 ± 2.6% viability; **Fig. 5.8C**) or 3 μM anthralin (data not shown).

We next treated $3x10^5$ PBMCs from three additional HIV-infected donors (separate from those in **Fig. 5.8B**) with PMA + ionomycin or KA at $1.5x10^6$ cell/mL for 24 hours, as described above, and quantified combined lysate and supernatant $p24^{Gag}$ protein using a commercially-available ELISA kit (**Fig. 5.8D**) (11). Data were then normalized to internal kit standards to calculate total $p24^{Gag}$ protein. Per donor, PMA + ionomycin induced an average 1.6 ± 0.5-fold increase in $p24^{Gag}$ relative to PBMCs treated with 0.1% DMSO, with 2 of 3 donors exhibiting at least 1.8-fold increases. Across all donors, this resulted in a combined average of 6.0 ± 1.1 pg/mL $p24^{Gag}$ protein in PMA + ionomycin treated PBMCs vs. 5.0 ± 2.5 pg/mL in DMSO-treated PBMCs (**Fig. 5.8D**). In contrast, KA induced an average 1.5 ± 0.5-fold increase per donor, with 1 donor inducing a 2.1-fold increase in supernatant $p24^{Gag}$, and an average of 5.9 ± 2.7 pg/mL detected in PBMCs across all donors. These results further support KA as having *ex vivo* efficacy. Conversely, extensive cell death, as visualized microscopically, and no $p24^{Gag}$ protein were detected from PBMCs treated with 10 or 3 μM anthralin (data not shown), further supporting poor *ex vivo* activity of this LRA.

Taken together, these results suggest that KA, but not anthralin, enhances the production of both viral RNA and protein from HIV-positive CD4+ T cells and PBMCs, respectively. Whereas there is clear evidence of *ex vivo* HIV-1 latency reversal activity by our novel LRAs, their activities were not statistically significant. However, it is our assumption that

recruitment of more HIV-1 infected donors in our future supplementary studies could be helpful in comprehensively and adequately addressing the statistical significance question.

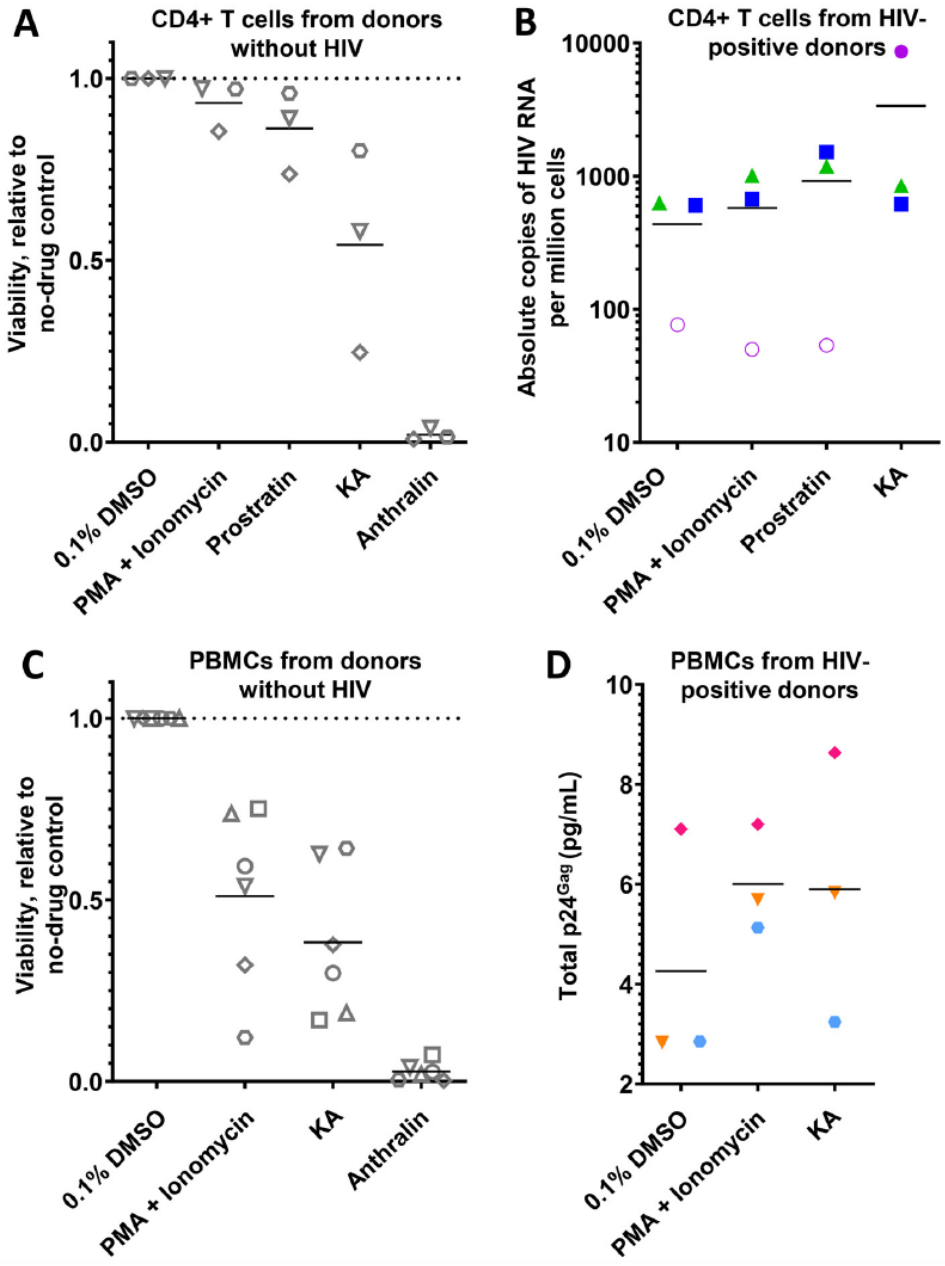


Figure 5.8: Effects of LRAs on primary cells.
(**A**) Effects of LRA treatments (100 ng/mL PMA + 1 µM ionomycin, or 10 µM prostratin, KA, or anthralin) on viability of CD4+ T cells from 3 uninfected donors after 24 h, as measured by ViaCount viability stain. **(B**) Effects of LRAs on total HIV RNA copies per million CD4+ T-cells isolated from 3 cART-suppressed, HIV-infected donors. Empty circles indicate the limit of detection, where no viral RNA was observed. (**C**) Effects of LRA treatments on viability of PBMCs from 6 uninfected donors after 24 h, as measured by ViaCount viability stain. **(D**) Effects of LRAs on $p24^{Gag}$ production

in total cell lysate and cell culture supernatants of PBMCs from 3 additional cART-suppressed, HIV-infected donors. For each panel, shapes and/or colors denote individual donors.

5.4.9. Knipholone Anthrone (KA) inhibits of HIV-1c replication

In a different study we conducted (18), KA displayed anti-HIV-1_c activity and cytotoxic effects in PBMCs infected with HIV-1c molecular clone MJ_4 at multiple concentrations (in the range of 0.01 to 50 µg/ml) as shown in **Fig. 5.9**. Quantification of HIV-1 p24 antigen production by ELISA show that KA (between the range of 0.5 to 50 µg/ml) inhibits HIV-1_c (MJ_4) replication by more than 60%. For example, at a concentration of 15 µg/ml KA exhibited 79.0 ± 1.0% inhibition of virus replication which was the highest, while 50µg/ml of KA yielded 70.6 ± 4.2% inhibition (**Fig. 5.9A**). The antiviral activity of KA as measured by the inhibition of p24 antigen production was concentration-dependent (**Fig. 5.9C**) with an EC_{50} value of 1.8 µg/ml (4.3 µM), a value which was considerably greater than that of the positive control AZT (EC_{50}: 0.49 µM) (data not shown). However, KA displayed dose-dependent cytotoxicity (**Fig. 5.9B & D**). For instance, 15 µg/ml KA resulted in 51.3 ± 3.1% PBMCs viability while only 28.0 ± 2.0% remained viable at 50 µg/ml (**Fig 5.9B**).

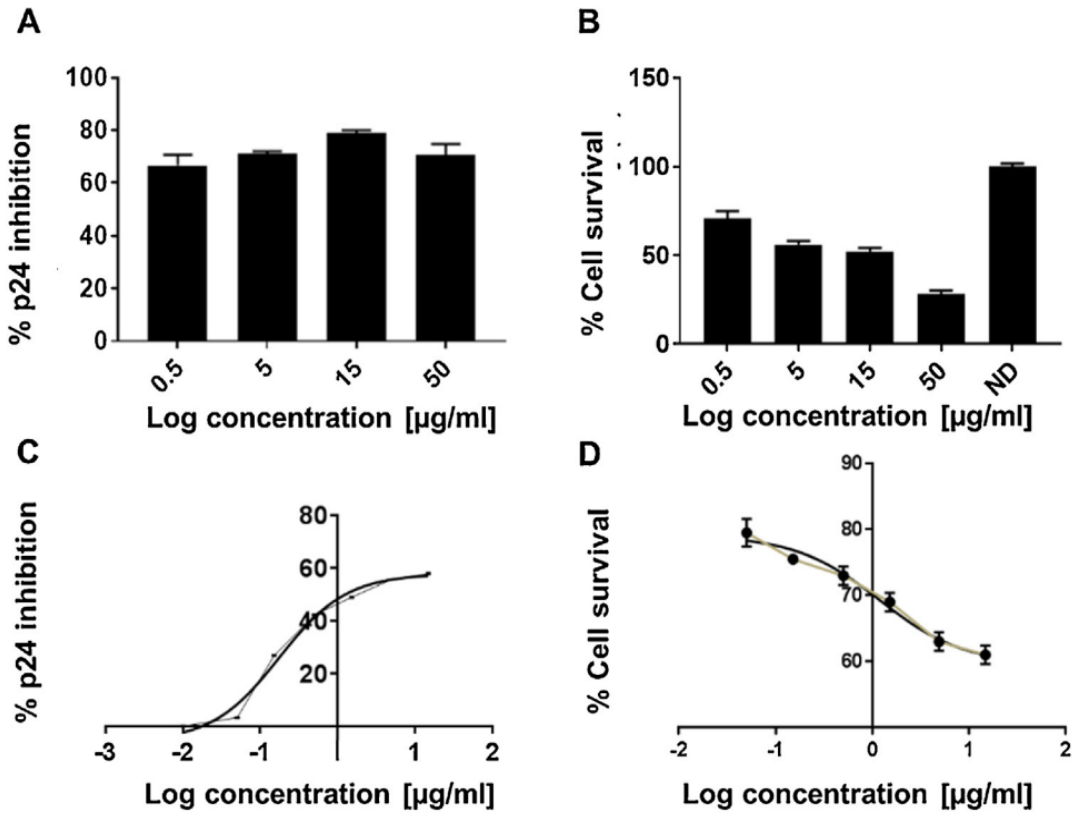


Figure 5.9: Antiviral activity of KA in HIV-1$_c$ (MJ$_4$) infected-PBMCs.
(**A**) PBMCs were infected for 2h at 37˚C with HIV-1$_c$ (MJ$_4$) clone and exposed to multiple concentrations of KA. The effects of compounds on p24^{Gag} production in cell culture supernatants relative to no drug were reported after 7 days of incubation. (**B**) KA's effects on mock-transfected PBMCs viability as measured by the MTT cell Proliferation Assay (ND; no drug). (**C**) Dose response curve showing viral p24^{Gag} antigen inhibition relative to no drug control. (**D**) dose-dependent cytotoxicity of KA-treated, HIV-1$_c$ (MJ$_4$)-infected PBMCs. For C **& D** grey represents the actual measurements and black represents the best fit curve. Data are reported as means and S.D. from an experiment performed in triplicate.

5.5. Discussion

New LRAs and LRA combinations are needed to improve existing "shock and kill" HIV therapeutic approaches (8, 45). Here, we screened 216 compounds from pANAPL and 18 chemical analogues to identify KA, anthralin, and prinoidin as novel LRAs, which continues to support that chemical libraries of pure compounds from natural products, can be rich sources of LRAs (11-14). Although both KA and anthralin, due to their toxicities, are unlikely to be used as LRAs in humans, their distinct activities *in vitro* make them useful probes to further understand the cellular mechanisms of HIV latency and latency reversal. This in turn can aid in the development of future LRAs with reduced toxicities, in addition to LRA combinations with improved efficacies and fewer side effects in clinical studies.

KA is a phenylanthraquinone which was originally isolated from Ethiopian *Kniphofia foliosa* and initially reported to display antiprotozoal activity *in vitro* (46-47). KA is also reported to possess both pro- and anti-oxidant activities, depending on the reaction partners and culture or solvent conditions (36, 47). Since its initial discovery, several phenylanthraquinones have been isolated from plants of South Africa, Botswana, Lesotho, Germany, Australia, and Japan (48). In contrast, anthralin (dithranol) is a KA-like molecule and a licensed topical therapy for psoriasis, dermatitis and eczema, where mechanisms of action include the induction of excessive oxidative stress in targeted cells (37-38) in addition to induction of anti-proliferative and pro-apoptotic signaling pathways (49). To our knowledge this is the first report of anthrones affecting HIV latency.

We observed that both KA and anthralin reversed HIV latency across multiple cell-line models with dose-dependence, indicating that their activities were independent of viral integration site, at least in lymphoid-derived cell lines. When assessed *in vitro*, both KA and anthralin also induced cellular apoptosis at levels approximating those of the control HDAC inhibitor panobinostat. Both KA and anthralin, like prostratin, also induced expression of T cell activation markers CD69 and CD25, although KA induced exceptionally strong (*i.e.*, up to 22-fold) upregulation of CD69 but not CD25, while anthralin induced weak CD69 and CD25 expression. Thus, despite the observed toxicity and pro-activation properties of KA and anthralin, their distinct profiles, when compared to prostratin and panobinostat, suggested that their effects on T cells may differ from these control PKC activator and HDAC inhibitor functional classes of LRAs. In support of this, our results using both a pharmacological pan-inhibitor of PKC signaling and a cell-based HDAC activity assay indicate that KA and anthralin are neither PKC activators nor HDAC inhibitors.

Based on the known cellular mechanisms of KA and anthralin (36-38), we initially hypothesized that both would reverse HIV latency by promoting “oxidative stress” or enhanced redox traffic, namely the formation of redox-reactive radicals derived from oxygen and from the anthrones themselves. The effects of reactive oxygen species on HIV latency reversal have been described extensively (50), and agents that intensify and uncouple cellular redox processes have been reported as LRAs or inducers of latency reversal *in vitro* and *ex vivo* (12, 51). Redox-reactive species like superoxide induce T cell

activation but also promote cell death, while factors that mitigate redox-reactive species production tend to favor the maintenance of HIV latency (50). In support of this model, a scavenger of oxidizing species, glutathione, inhibited *in vitro* latency reversal induced by anthralin, consistent with the known reactivity of anthralin in promoting oxidative stress. Contrary to our initial hypothesis, however, latency reversal by KA was not inhibited by glutathione, indicating that the latency-reversal properties of KA cannot solely rest on the anthrone moiety it has in common with anthralin, but that one or more distinct mechanisms of action appear to operate.

The latency reversal effects of both KA and anthralin, in addition to panobinostat, were further inhibited by the copper-sequestering agent bathocuproine. Anthrones are reported to show selectivity toward copper ions and are more easily oxidized in the presence of copper (52). This corroborates that the oxidation reaction could be responsible for driving latency reversal (18, 36). In line with this, latency reversal by KA and anthralin were enhanced by the iron chelator deferoxamine, indicating that KA and anthralin maintain latency reversal following iron sequestration, and that iron inhibits them. Superoxide also leads to oxidative dimerization of anthrone derivatives, and physiologically, superoxide is reduced by copper or iron-containing enzymes (53). Taken together, latency reversal by KA and anthralin appears to require a specific oxidation pathway that is connected to superoxide metabolism and promotes cellular activation, and this pathway is tightly modulated by particular metal ion species.

Despite years of research on both KA and anthralin (36-38, 48), direct molecular targets of these compounds are not yet elucidated. While the exact molecular targets of KA and anthralin in the context of HIV latency reversal also require further investigation, analysis of *in vitro* global gene expression following KA or anthralin treatment indicate distinct expression profiles when compared to control LRAs prostratin or panobinostat. Most notably, KA treatment robustly induced expression of genes related to sirtuin signaling pathways, while responses to anthralin treatment were suggestive of upregulation of cAMP-mediated signaling. To our knowledge, this is the first report linking these agents to these signaling pathways. These leads warrant further validation in cellular models, although studies would likely require derivatives of anthralin and perhaps KA with reduced cytotoxicities.

We further showed that both KA and anthralin synergize with the activities of LRAs representing PKC activators, HDAC inhibitors, cytokines, BET bromodomain inhibitors, and DNA methyltransferase inhibitors. In most cases, this synergism was statistically significant as measured using the Bliss Independence model. These results suggest that future compounds with improved preclinical profiles but similar mechanisms of action to KA or anthralin may be capable of enhancing the activities of existing LRAs which currently exhibit sub-optimal efficacies in clinical studies (8, 10).

The ability of KA to promote latency reversal was further confirmed *ex vivo*, as it enhanced production of both viral RNA from CD4+ T-cells and $p24^{Gag}$ protein from PBMCs from HIV-infected donors. The moderate toxicity observed with KA also raises the possibility of its use at lower concentrations, where toxicity is less likely, as part of future LRA combination therapies with synergistic effects on latency reversal. The extent to which combination LRA therapies that include KA can be optimized in primary cells also require further study. In contrast to KA, we were unable to demonstrate *ex vivo* latency reversal by anthralin, where severe oxidative stress is likely driving cell toxicity even at relatively low concentrations. In contrast to our results, a previous study identified 5-hydroxynaphthalene-1,4-dione (5HN) as a novel LRA in latently-infected, Bcl-2-transduced primary CD4+ cells that also acted through enhanced oxidative stress (12). In this study, 5HN exhibited an EC_{50} of 0.5 μM and 50% cytotoxic concentration (CC_{50}) of 7.7 μM in primary cells, indicating an improved therapeutic range relative to anthralin. Like anthralin, this compound also induced weak expression of T cell activation markers, and proviral effects could be mitigated by co-treatment with antioxidant agents. Thus, further support of this latency reversal mechanism in primary cells and in LRA combinations may be better modelled using anthralin analogues with higher therapeutic indices.

Several aspects of our results warrant further study. Most notably, the limited efficacies and toxicities of KA and especially anthralin *in vitro* and *ex vivo* are likely to limit the potential of these agents as future clinical candidates. However, their ability to synergize with numerous LRAs representing different functional classes suggest that chemical derivatives which function like KA or anthralin, but harbor improved preclinical parameters, could eventually contribute to future LRA combination strategies which maximize LRA efficacy while minimizing off-target toxicities in humans.

Furthermore, prior to this latency modulation study of KA, the compound was reported by us to inhibit HIV-1 replication in PBMCs infected *in vitro*, with an EC_{50} of 4.3 μM (18). Our investigations therefore suggest that KA has dual effect on HIV-1 as it does not only possess latency reversal properties but it also blocks viral replication. The anti-HIV activity of KA is consistent with the reported antiviral properties of other anthraquinones which have been shown to particularly target HIV-1 reverse transcriptase, including activities of other analogues of anthraquinone which are inhibitory to the HIV-1 RT ribonuclease H (RNase H) activity (54-55). Taken together, our results support the search for novel latency modulating compounds and indeed antivirals from natural products in search for HIV-1 therapies.

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Chapter 6.
Conclusion

6.1. Summary

Combination antiretroviral therapy (cART) has remarkedly reduced the otherwise, deadly HIV/AIDS to a chronic, manageable health condition. cART successfully suppresses viremia to undetectable levels. However, cART does not eradicate the inducible, replication-competent provirus that is incorporated in the long-lived cellular reservoirs, including CD4+ T cells (1-3). The integrated viral genome can reactivate anytime upon treatment interruption, as such HIV-infected people have to remain enrolled in lifelong antiretroviral therapy (1-3). Lifelong ART is problematic because it is associated with long-term toxicities, heightened risks to chronic inflammation and other non-AIDS health disorders (4-7). HIV-1 drug resistance, which has been observed and reported in different classes of ARVs is also a cause for concern (8-13). Thus, novel therapeutic strategies are needed to target and purge the latent reservoir that remains a barrier to HIV cure. Chapter 1, reflected on the some of the strategies that are currently being pursued to achieve both functional and sterile HIV cure. These strategies include: Immunotherapy, Gene modification and cell therapy Early ART, 'Block-and-Lock' (Deep latency), and 'Shock-and-Kill' (Latency Reversal) (14-15).

This thesis is premised and focused on the 'shock-and-kill' approach (16-18). As discussed in Chapter 1, different classes of latency reversing agents (LRAs) have been identified. The first generation of LRAs included Histone deacetylase inhibitors (HDACis) Protein kinase C (PKC) activators, Histone methyltransferase inhibitors (HMTis), DNA methyltransferase inhibitors (DMTis) and BET bromodomain Inhibitors (19-21). Second generation LRAs include SMAC mimetics, Programmed cell death protein-1 (PD-1) inhibitors, Toll-like receptors (TLRs) agonists and IL15 super-agonists (22-25). Despite decades of research on the 'shock-and-kill' strategy, to date, no LRA or LRA combination has consistently reduced the viral reservoir, as such there is currently no licenced LRA. This underscores the need for new LRAs and LRA combinations to overcome the limitations associated with the current latency modulating agents (26-30). Furthermore, LRAs with new distinct mechanisms for HIV latency modulation could also work to

enhance the efficacy and potency of current library of LRAs, some which are in clinical trials, whose latency reversal activity has been lacking the 'kill' component (26-30).

Natural sources such as marine products and plant-based traditional medicines, with their structural diversity provide a rich reservoir for both new antivirals and LRAs with distinct mechanisms for HIV proviral reactivation. To highlight the HIV therapeutic potential of traditional medicinal plants, I wrote a systematic review (Chapter 2) involving a total of nineteen (19) African medicinal plants with documented anti-HIV profiles, including latency modulation properties and are supported by detailed laboratory data in the literature. We observed from this systematic review that medicinal plants-derivatives reported to have anti-HIV bioactivities have yet to be tested *in vivo* or in human clinical trials and would also benefit from additional documentation as part of supporting reverse pharmacology-based efforts.

There is also need to continue exploring traditional medicinal plants to search for more novel LRAs with distinct mechanisms to improve the 'shock-and-kill' therapeutic strategy. To this end, in Chapter 3 using the 'reverse pharmacology' approach I conducted an ethnopharmacological survey to document plants used for HIV/AIDS and related diseases management by the BaKalanga Peoples of the Tutume-sub district in Central Botswana. This ethnobotanical survey identified 83 medicinal plants used for HIV/AIDS in this region and 38 (45.8%) of these plants have not been previously assessed for HIV-1 specific or related bioactivities linked to their ethnomedical use as reported in our survey.

Chapter 4 identifies and characterizes 3 pure compounds from a marine natural product library which reversed latent HIV-1 provirus: psammaplin A (HDACi), aplysiatoxin, and debromoaplysiatoxin (PKC activators). These new LRAs induce proviral expression in J-Lat cell lines (J-Lat 9.2, 8.4 and 10.6 cells) and primary human cells. The identification of these novel latency modulating compounds, adds to the repertoire of LRAs that can inform the "shock-and-kill"-based strategies to purge HIV-1 reservoir.

In Chapter 5, I describe the discovery and molecular analysis of novel LRAs, the African natural product Knipholone anthrone (KA) and its analogue, anthralin (dithranol), that enhance HIV latency reversal activities of established LRAs. These were identified from the screening of 216 compounds from the pan-African Natural Products Library (pANAPL). Our investigation revealed that KA and anthralin reversed HIV latency in multiple *in vitro*

cell models including J-Lat 9.2, 8.4, 10.6 cells and OM-10.1 cells at at low micromolar concentrations. Using PKC inhibition (GÖ-6983) and HDAC-Glo I/II Assays, I demonstrated that KA and anthralin do not function as PKC activators or HDAC inhibitors, respectively. I further showed that KA and anthralin are differentially regulated by oxidative stress and metal ions and induce gene expression profiles distinct from established LRAs *in vitro* in J-Lat 10.6 cells. I described KA's LRA properties *ex vivo*, and showed that KA induces both HIV RNA and protein in primary cells from HIV-infected donors. We further showed KA has dual effect on HIV-1 as it also blocks viral replication, in addition to its latency reversal profile.

In conclusion, it is my well-considered view that my thesis has added to the body of scientific knowledge regarding HIV-1 latency modulation. My thesis has discovered and characterized five (5) novel LRAs: psammaplin A, aplysiatoxin, debromoaplysiatoxin, anthralin and knipholone anthrone. Not only has this discovery added to the repertoire of LRAs but the investigation of these latency modulating compounds has given some insights into unique mechanisms of provirus reactivation and viral production, and this may in turn inform future HIV cure research. For example, chemical derivatives which function like KA, but with improved preclinical parameters, could ultimately add to future LRA combination strategies by increasing their efficacies while reducing off-target toxicities.

Furthermore, that the identified LRAs trace their origin from natural sources evidently demonstrates that natural products are a promising source of new latency modulating agents. Thus, our findings further give credence to the efforts of searching novel HIV-1 latency modulators from marine products and plant-based traditional medicines. To this end, my thesis describes an ethnobotanical survey that identifies previously unreported 38 medicinal plants used for HIV/AIDS management by the BaKalanga Peoples of the Tutume subdistrict in Central Botswana. These medicinal plants will be prioritized for investigation to assess their HIV bioactivities, including their latency modulation properties. It is anticipated that these traditional medicinal plants would be an avenue to search for novel LRAs with distinct mechanisms that will advance the "shock-and-kill"-based strategies.

My thesis is also an example of how interdisciplinary research, combining community-based and laboratory-based approaches, can be conducted in HIV research. At the policy level, my thesis will help advance the World Health Organization (WHO) Traditional

Medicine Strategy 2014–2023 that aims to assist countries to harness the potential contribution of TCM to health, wellness and people-centred health care (31). This strategy also seeks to achieve TCM regulation in order to promote its safe and effective use with an overall goal of integrating TM products, practitioners, and practice into health care systems wherever appropriate (31).

In overall, my thesis will contribute to HIV cure research and add to our understanding of HIV latency.

6.2. References

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Appendix A. Questionnaire for Traditional Herbalists

Hello, my name is……. I am a PhD student at Simon Fraser University in Vancouver, Canada. I am doing a research project on medicinal plants used by herbalists in Central Botswana. The purpose of my study is to develop a profile of medicinal plants commonly used by herbalists to treat different health conditions. The information collected with my study will be confidential. I hope that you will provide me with the information I need. [English]

Dumela rra/mma, leina lame ke…….Ke moithuti wa dithuto tse dikgolo tsa mmadikolo ko Vancouver, Canada. Ke dira patlisiso ka ditlhare tsa melemo tse di dirisiwang ke dingaka tsa Setswana mo Legare la Botswana. Maikaelelo a patlisisiso yame ke go tlhotlhomisa gore ke mefuta efe ya ditlhare ee dirisiwang ke dingaka go alafa malwetsi a a farologaneng. Sepe se re buisanang ka sone gone fa, ga se kitla se abelwana le ope. Ke solofela fa o tla abelana le nna tse tsotlhe ke di tlhokang mo putsolotsong e. [Setswana]

Dumilani, zina langu ndi……. Indi mwana we ikwele tji kulu, we zwi-diyo zwi kulu ku Vancouver, Canada. Ndo shakisisa ne kwe meti ye Ikalanga/Itswana mu ntunthu we pakati kwe Botswana. Mazwimisilo ye zwidiyo zwangu i ku shakisisa ne kwe miti ino shingisiwa ne ngaa'nga dze ikalanga/itswana ku lapa magwele a ka siyinasiyana. Zwa ndi no lebesana namwi ipapa a zwi nga budziwe/zibisiwe umwe nthu upoga. Ndo tola nditi mo ndi batsha ne tja ndi no mu kumbila mu buzwisiso iyeyi. [IKalanga]
Can we proceed?
A re ka tswelela?
A tinga dwilila?

Thank you.
Ke a leboga.
Nda boka.

SECTION A: Background of the respondent

Interviewee:……. Date:…./…/….

Mmotsoloswa Letsatsi

Mbuzwiwa *Zhuba*

A1. Gender/Bong/*Nlume kene Nkadzi*:

i) Male/Monna/*Nlume* [ ]

ii) Female/Mosadi/ *Nkadzi* [ ]

A2. Exact age in years:…… (Ask for Omang if the age is not known)

Dingwaga

Makole

A3.Ward:

Kgotla

Khuta…….

A4. Ethnicity:

Letso

Ludzi……

A5. Place of birth:

Lefelo la matsalo

Pa ma ka tshibamigwa……..

A6. Are you from (specific town/village in Central Botswana)?

i) Yes / Ee/*Ee* [ ] ii) No / Nnyaa/*Ae* [ ]

A o tlholega mo toropong/motse o mo legare la Botswana?

A mo zwangwa mu toropo/nzi I mu nthunthu iwoyu u pakati kwe Botswana?

A7. If not, where are you originally from? (Country, town, village)

Fa e se jalo, o tlholega kae?

A ku si jalo, mo dwa ngai?

……

A8. How long have you lived in (specific town/village in Central Botswana)?

O na le lebaka le lekae o nna mo legareng la Botswana?

Mu na libaka ngu ngapani mu gala mu ngathu iwoyu we pakati kwe Botswana?

.....

A9. Where did you acquire your skill/knowledge of medicinal plants? (Family tradition, in service, apprenticeship?)

O anyile kae kitso ya melemo ya setswana? (setso sa mo lwapeng, a o ne wa bereka ka fa tlase ga

motho o o nang le kitso ka melemo ya setswana)

Ma ka tola ngai luzibo gwe miti ye ikalanga/itswana? (tjilenje/tjipo tji munzi, kana ma ka diyiwa ne nthu wa ke be a na lizibo gwe miti i no lapa)

……..

……

A10.How long were you trained/in service?

O tsere lebaka le lekae o ithuta?

Ma ka tola tjibaka tji ngapani mu zwi diya?.....

A11.How long have you been a herbalist?

Ke sebaka se se kae o ntse o le moitseanape wa melemo ya setswana?

I tjibaka tji ngapani mu li nyambi ye bungaa'nga kene mu lapa?

…….yrs…….months

…….dingwaga….dikgwedi

…….makole…….mimwedzi

A12. Are you currently training somebody?

A go na le mongwe yo o mo rutuntshang mo sebakeng se?

A kuna wa mu no diya mutjabaka tjino?

……

SECTION B: Consultations

In the last section we discussed about your background. In this section I will be asking you about different conditions that patients bring to your attention and how you do your consultation.

Mo sephatong se se fitileng, re buisantse ka wena. Jaanong ke tla go botsa ka diemo tse di farologang tse balwetsi ba di tsisang ko go wena le gore o ba thusa jang?

Mu libazhe gwa pinda, ta lebesana ne kwenyu. Ngwenu ndo be ndi mu buzwa ne kwe zwimo zwa ka leyanaleyana zwe bagwele zwa ba no hisa ku muli, ne ku ti mo ba batsha tjini?

……
……
……

B1. What kind of patients come to you for consultation?

Ke mofuta ofe wa batho ba ba tlang dithusong ko go wena?

Bagwele bapi ba no ha ku muli bi shaka batshiwa?

i) Women / Basadi/ *Bakadzi* [ ]
ii) Men / Banna/ *Balume* [ ]
iii) Children / Bana/*Bhana* [ ]
iv) Both / Botlhe/*Bose* [ ]

B2. Are all your patients from (specific town/village in Central Botswana) ?

i) Yes / Ee / *Ee* [ ] ii) No / Nnyaa/ *Ae* [ ]

A balwetsi ba gago botlhe ba tswa mo toropong/motse o mo legare la Botswana?

A bagwele benyu bose ba no dwa mu toropo/nzi i mu nthunthu iwoyu u pakati kwe Botswana?

B3. If not, which villages do they come from?

Fa go sa nna jalo, ba tswa kwa metseng efe?

A ku si jalo, ba no dwa mu mizi ipi?

……

……

……

B4. How often do you consult your patients?

O alafa balwetsi ba gago ga kae?

Mo lapa/bhona bagwele benyu mu tjibaka tji ngapani?

i)	Daily / Ka letsatsi/ *zhuba ne zhuba*	[ ]
ii)	Weekly / Ka beke/ *Viki*	[ ]
iii)	Monthly / Ka kgwedi/ *Mwedzi*	[ ]

B5. On average, how many patients do you consult? (per day/per week/per month)

Fa o akanyetsa, ke balwetsi ba le kae ba o ba thusang? (Ka letsatsi/Ka beke/ Ka kgwedi)

A mu kumbulila, mo batsha bagwele ba ngana? *(Mu zhuba/Viki/ Mwedzi)*

……

.....

B6. Do you usually follow up your patients?

i) Yes / Ee /*Ee* [ ] **ii)** No/Nnyaa/*Ae* [ ]

A bontsi jwa nako o batlisisa ka botsogo jwa balwetsi ba gago?

A mutjibaka tji njinji, mo so shakisisisa (lekula) ne kwe tjimo tje bagwelu benyu?

B7. If yes, why?

Fa o dumela, ka goreng ?

A pa mu duma, ne kutini?

.....

B8. What different health conditions do you commonly treat?

Ke malwetsi afe a a farologaneng a gantsi o a alafang?

A mutjibaka tji njinji, aagwele api a ka leyanaleyana a mu lapa?

.....

B9. Please explain the symptoms associated with each condition.

Ke kopa gore o tlhalose dikai tsa malwetsi a o a buileng

Ndo mu kumbila mu ndi pe zwikai/zwilakidzilo zwe zwimu zwimwe ne zwimwe zwa ma leba ipapa

……

B10. Which groups mostly suffer from these conditions? (Women, men, children)

Ke batho bafe ba gantsi ba lwalang malwetsi a? (Basadi, banna, bana)

I bathu bapi ba no gwala magwele a mu no lapa? (Bakadzi, balume, bhana)

Condition (Seemo; *Tjimo*)	**Symptoms (Dikai; *Zwikayi*)**	**Group (Setlhopa; *Ithopa*)**

B12. How often do you see patients with conditions you have just described?

Ke ga kae o bona balwetsi ba ba nang le diemo tse o setseng o ditlhalositse?

Ndi ka ngana mu bhona bagwele ba na zwimo zwa ma leba?

……

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B13.Which of the conditions you have just described above are the most frequent ones?

Ke diemo dife tse o setseng o di tlhalositse tse di bonalang thata?

Izwimo zwipi mu na zwa ma leba, zwi no boneka ne tjilizanyo tji njiji?

……

B14. Which of the conditions you have just described above are rare?

Ke diemo dife tse o di tlhalositseng tse di sa diragaleng thata?

Mu zwimu zwa ma leba, ndi zwipi zwi no boneke ne tjilizanyo tji pasi?

Frequent conditions (Diemo tse diragalang thata; *Zwimo zwi no boneka ne tjilizanyo tji njiji*)	**Rare conditions (Diemo tse di sa diragaleng thata; *Zwimu zwi no boneka ne tjilizanyo tji pasi*)**

B15. How often are you seeing patients with the following symptoms?

O bona balwetsi ba dikai tse di latelang ga kae?

Ndi ka ngana mu bhona bagwele ba na zwikai zwi no tobela?

Symptom (Dikai; *Zwikai*)	**Often**	**From time to time**	**Rarely**
i) persistent cough (kgotlholo e e sa feleng) *(khogola kwa ka pindilila)*	[ ]	[ ]	[ ]
ii) cough and chest pains (kgotlholo le botlhoko mo mahatlheng) *(khogola ne ku panda kwe ifhuba)*	[ ]	[ ]	[ ]
iii) frequent fevers (go gotela kgapetsakgapetsa) *(ku pisa tjibaka ne tjibaka)*	[ ]	[ ]	[ ]
iv) diarrhoea (letshololo) *(Ku tihisiwa kwa ka phindilila)*	[ ]	[ ]	[ ]
v) weight loss	[ ]	[ ]	[ ]

(tlhofofalo ya mmele)
Ku lashikigwa kwe mbili)

vi) skin rashes [ ] [ ] [ ]
(bogwata jwa letlalo)
(*Dzuka kwe mbili)*

vii) a combination of two or more of
these symptoms [ ] [ ] [ ]
(go diragala ga dikai tse ka nako e le nngwe)
(Kulakidzika kwe zwikai izwezwi tjabaka tji ngo mpela)

SECTION C: Medicinal plants

In the last section, we discussed about different conditions people bring to your attention. In this section I will be asking you about the medicinal plants you use to treat the conditions we discussed earlier.

Mo sephatong se se fitileng re buisantse ka diemo tse di farologaneng tse batho ba di tsisang ko go wena. Jaanong ke tla go botsa ka melemo e o e dirisang go alafa malwetsi a re buisantseng ka one pele.

Mu libazhe gwa pinda, ta lebesana ne kwe zwimo zwa ka leyanaleyana zwe bagwele zwa ba no hisa ku muli. Ngwenu ndo be ndi mu buzwisisa ne kwe miti ya mu no shingisisa ku lapa magwele a ta lebesana ne kwawo khantile.

C1. How are you able to tell if the medicinal plant is fresh enough to produce good medicine? (Maturity, smell, leaf colour, taste)

O lemoga jang gore setlhare sa molemo se santse se na le boleng jwa go ka dira molemo?

(go gola, monko, mmala wa letlhare, tatso mo ganong)

Mo bhona tjini nti u no shingisiwa ku lapa u tji na bukhoni gwe lapa? (Ku kula kwawo, ku nuhwa kwawo, mbhala we hani le nti, ku ngatha ne nlomo ku ti mu u whe mu lulimi)

……..……

……..……

……..……

……..……

C2. How do you generally store your medicine? (Dry, liquid foam, powder)

Gantsi o baya molemo wa gago o ntse jang? (O omisitswe, o le mo metsing, o sidilwe)

Kanjinji mo bhiga nti wenyu u li mu tjimo tja ka tjini? (Wa ka omisiwa, U li mu vhula, u li gwisa)

……..……
……..……
……..……
……..……

C3. Where do you store your medicine?

O baya melemo ya gago kae?

Mo bhiga miti yenyu ngai?

……..……
……..……
……...……
……..……

C4. Which medicinal plants do you use to treat different health conditions you mentioned earlier?

Ke ditlhare tsa molemo dife tse o di dirisang go alafa malwetsi a a farologanyeng a o setseng a nankotse?

Mo shingisisa miti ipi ku lapa magwele a ka siyanasiyana a ma leba ne kwawo pezhugwi?

……..

C5. Do you use a single medicinal plant to treat each condition or a combination of plants?

A o dirisa setlhare se le sengwe go alafa bolwetsi kana o dirisa ditlhare di tlhakane?

A mo shingisisa nti u ngompela ku lapa bugwele kana mo shanganya miti minjini ku lapa bugwele gu ngompele.

……

……

C6. If a combination of medicinal plants is used, please explain the different plants used.

Fa go dirisiwa ditlhare se tlhakaneng, ke kopa o tlhalose gore ke dife.

A pa mu shingisa miti ya ka shanganyiwa, ndo kumbila mu tjenesese ku ti ndi ipi miti iyeyo.

……

C7. Which part(s) of the medicinal plant do you use? (roots, leaves, bark?)

Ke dikarolo dife tsa setlhare tse o di dirisang (medi, matlhare, lekwati?)

Mo shingisa zwipithu zwipi zwe nti ku lapa? (Midzi, mahani, gwati?)

……

C8. Could you please explain to me how you prepare the medicine.

Ke kopa gore o ntlhalosetse ka fa o dirang molemo ka teng.

Ndo kumbila mu ndi tjenesele kuti mo thama miti yenyu tjini?

……..

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.....

C9. In what quantity is the medicine taken? (Dosage)

Molemo o o tsewa ka sekale se sekae?

Nti iwoyu wa mu no pa ngwele u no togwa ne tjilizanyo tji ngapani?

……

C10. How should the medicine be taken? (Inhaled, orally)

Molemo o o dirisiwa jang? (o a aramelwa, o a nowa)

Nti iwoyu u no togwa/shingisiwa tjini? (U no arhamegwa, u no mwihwa)

……

Health condition (Seemo sa botsogo) ***(Tjimu tje mamuko)***	**Medicinal plants (Ditlhare tsa melemo)** ***(Miti ya mu no tola miti mu ili)***	**Part(s) used (Dikarolo tse di dirisiwang)** ***(Zwipithu zwe nti zwi no shingisisiwa)***	**Mode of preparation (Ka fa molemo o dirwang ka teng)** ***(Zila ye ku thama nti)***	**Dosage & method of administration (Sekale le ka fa molemo o dirisiwang ka teng)** **(*Tjilizanyo ne zila ye ku pa ngwele nti)***

Persistent cough (Kgotlholo e e sa feleng) *(khogola kwa ka pindilila)* Cough and chest pains (kgotlholo le botlhoko mo mahatlheng) *(khogola ne ku panda kwe ifhuba)* Frequent fevers (Go gotela kgapetsakgapetsa)				

(ku pisa tjibaka ne tjibaka) Diarrhoea(Letshololo) *(Ku tihisiwa kwa ka phindilila)*				

Health condition (Seemo sa botsogo) ***(Tjimu tje mamuko)***	**Medicinal plants (Ditlhare tsa melemo)** ***(Miti ya mu no tola miti mu ili)***	**Part(s) used (Dikarolo tse di dirisiwang)** ***(Zwipithu zwe nti zwi no shingisisiwa)***	**Mode of preparation (Ka fa molemo o dirwang ka teng)** ***(Zila ye ku thama nti)***	**Dosage & method of administration (Sekale le ka fa molemo o dirisiwang ka teng)** ***(Tjilizanyo ne zila ye ku pa ngwele nti)***
Weight loss (Go tlhofofala ga mmele) *(Ku lashikigwa kwe mbili)* Skin rashes (Bogwata jwa letlalo) *(Dzuka kwe mbili)*				

Combination of the above-mentioned symptoms (Go diragala ga dikai tse di nankotsweng fa godimo ka nako e le nngwe) *(Ku lakidzika kwe zwikai izwezwi tjabaka tji ngo mpela)*				

Health condition (Seemo sa botsogo) ***(Tjimu tje mamuko)***	**Medicinal plants (Ditlhare tsa melemo)** ***(Miti ya mu no tola miti mu ili)***	**Part(s) used (Dikarolo tse di dirisiwang)** ***(Zwipithu zwe nti zwi no shingisisiwa)***	**Mode of preparation (Ka fa molemo o dirwang ka teng)** ***(Zila ye ku thama nti)***	**Dosage & method of administration (Sekale le ka fa molemo o dirisiwang ka teng)** ***(Tjilizanyo ne zila ye ku pa ngwele nti)***
Others (Tse dingwe) *(Zwimu zwimwe)*				

C11. Can any other medicinal plant(s) be substituted when the above-mentioned plants are not available?

A go na le ditlhare dingwe tsa molemo tse di ka dirisiwang fa tse di nankotsweng di
seyo?
A kuna miti imwe i nga shingisiwa a pa ya ma leba pezhugwi i sipo?

……
……
……
……
……

C12. Where do you obtain your medicinal plants? (buy, collect, grow?)

O tsaya kae ditlhare tsa molemo? (reka, kgetla, lema?)
Mo tola ngai miti ya mu no shingisa ku lapa? (tenga, mu shango, lima?)

……
……..
……

C13. If you collect plants yourself where do you collect your medicinal plants?

Fa e le gore wa di kgetla, o di kgetla kae?
A pa i li kuti mo tola miti mu shango, mu i tola ngai?

Around Central Botswana/Legare la Botswana/ *Nthuthu u pakati kwe Botswana* [ ]
Somewhere else/Go sele/ *Ntome* [ ]

C14. Are these medicinal plants easily available?

A ditlhare tse di motlhofo go bonala?
A miti iyeyi i no boneka ku sa ka sima?

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……

……

……

C15. Are the medicinal plants you mentioned seasonal?

A di bonala mo pakeng e e rileng fela?

A miti iyeyo i no bhomwa ku enda ne tjibaka?

……

……

……

……

C16. Are these medicinal plants becoming scarce?

A ditlhare tse di a nyelela?

A miti i pejo ne pela mu shango?

……

……

……

C17. If so, what do you think contribute to the scarcity?

Fa go ntse jalo, di nyeletswa ke eng?

A pa kwa ka jalo, i no pedziwa neni?

……

……

……

SECTION D: Healing process (Tsela ya kalafi) (Gwendo gwe ku lapa)

In the previous section, we discussed about different medicinal plants used to treat different conditions. In this section I will ask you on how the medicine gets to work for a patient to be healed.

Mo sephatong se se fetileng re buisantse ka ditlhare tsa melemo tse di farologanyeng. Jaanong ke tla go botsisisa ka gore molemo o bereka jang gore molwetsi a fole.

Mu libazhe gwa pinda, ta lebesana ne kwe miti ya mu no shingisisa ku lapa magwele a ka siyanasiyana. Mu bhemu ileli, ndo mu buzwa ne kuti nti wenyu u no shinga tjini kuti ngwele a pole.

D1. For each condition mentioned above, what is the effect which is to be expected of the plant treatment regimen and/or plant?

Mo bolwetsing/seemong sengwe le sengwe se o se nankotseng, go solofelwa gore molemo o ame molwetse jang?

Mu bugwele/tjimo tjimwe ne tjimwe tja ma leba ne kwatjo, mo sulufela ku ti nti u ame ngwele tjini kuyi wa lapa?

D2. For each condition you have mentioned, how long does it take for the patient to get healed?

Mo bolwetsing/seemong sengwe le sengwe se o se nankotseng, go tsaya sebaka se se kae gore molwetsi a fole?

Mu bugwele/tjimo tjimwe ne tjimwe tja ma leba ne kwatjo, ko tola libaka gu ngapani kuti ngwele a pole?

……

……

……

D3. How do you follow up patients to observe whether treatment is successful?

O latedisa jang balwetsi go tlhomamisa fa kalafi e atlegile?

Mo sala bagwele shule tjini kuti mu bhone ku ti lapa kwenyu kwa khonekala?

……

……

D4. For how long should the medicine be used before it loses its potency?

Ke sebaka se se kae se molemo o tshwanetseng wa dirisiwa pele fa o ka latlhegelwa ke

boleng jwa one?

Nti wenyu uno tola tjibaka tji ngapani kuti u lashikigwe ne simba le ku podza?

……

……

……

D5. Are there any side effects of the medicine? (Dizziness, vomiting?)

Please explain

A go na le ditlamorago tsa molemo o? (Sedidi, go kgwa)

A miti ya mu no shingisa i na zwi mu wunde, zwi nga gwadza ngwele? (Dzungu, Ku thwaa)

……

……

……

……

D6. What treatment do you give to the patients if there are any side effects?

Ke kalafi efe e o e fang balwetsi fa go na le ditlamorago?

A pa kuna zwi wunde mu nti wa ma pa ngwele, zwi nga gwalisa, mo mpa nti upi kakale kene mo nlapa tjini?

……

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……

D7. How do you know if your patient(s) adhere to the treatment?

O itse jang gore balwetsi ba gago ba sala morago dikaelo tsa molemo?

Mo ziba tjini kuti bagwele benyu ba no tobela ndailo ye shingisa nti zhubuyanana?

……
……
……

D8. What do you do if your patients do not adhere to the treatment?

O dira jang fa balwetsi ba gago basa sale morago dikaelo tse?

Mo thama tjini a pa bagwele benyu ba singa tobele ndailo ye shingisa zhubuyanana?

……
……
……

Health condition (Seemo sa botsogo) ***(Tjimu tje mamuko)***	**Medicinal plants (Ditlhare tsa melemo)** ***(Miti ya mu no tola miti mu ili)***	**Healing time(Nako ya pholo)** ***(Tjibaka tje ku pola)***	**Maximal use before medicine looses its potency(Sebaka sa tiriso ya molemo pele fa o latlhegelwa ke boleng)** ***(Tjibaka tje ku shingisa nti u sathu u ka lashikigwa ne simba le ku podza)***	**Side effects(Ditlamorago)** ***(Zwi mu wunde)***
Persistent cough (Kgotlholo e e sa feleng) *(khogola kwa ka pindilila)* Cough and chest pains(kgotlholo le botlhoko mo mahatlheng)				

(khogola ne ku panda kwe ifhuba) Frequent fevers (Go gotela kgapetsakgapetsa) *(ku pisa tjibaka ne tjibaka)* Diarrhoea(Letshololo) *(Ku tihisiwa kwa ka phindilila)*				

Health condition (Seemo sa botsogo) ***(Tjimu tje mamuko)***	**Medicinal plants (Ditlhare tsa melemo)** ***(Miti ya mu no tola miti mu ili)***	**Healing time(Nako ya pholo)** ***(Tjibaka tje ku pola)***	**Maximal use before medicine looses its potency(Sebaka sa tiriso ya molemo pele fa o latlhegelwa ke boleng)** ***(Tjibaka tje ku shingisa nti u sathu u ka lashikigwa ne simba le ku podza)***	**Side effects(Ditlamorago)** ***(Zwi mu wunde)***
Weight loss (Go tlhofofala ga mmele) *(Ku lashikigwa kwe mbili)*				

Skin rashes (Bogwata jwa letlalo) *(Dzuka kwe mbili)*				
Health condition (Seemo sa botsogo) ***(Tjimu tje mamuko)***	**Medicinal plants (Ditlhare tsa melemo)** ***(Miti ya mu no tola miti mu ili)***	**Healing time (Nako ya pholo)** ***(Tjibaka tje ku pola)***	**Maximal use before medicine looses its potency (Sebaka sa tiriso ya molemo pele fa o latlhegelwa ke boleng)** ***(Tjibaka tje ku shingisa nti u sathu u ka lashikigwa ne simba le ku podza)***	**Side effects(Ditlamorago)** ***(Zwi mu wunde)***

Others (Tse dingwe) *(Zwimu zwimwe)*				

SECTION E: Specific conditions

In this section I want to find whether there is a working relationship between you as a herbalist and other medical doctors in the hospital setting and whether a cooperation with scientists can help in addressing some problems you face as a herbalist.

Mo sephatong se ke batla go itse gore a go na le tirisano mmogo magareng gago o le ngaka ya setswana le dingaka tse dingwe tsa sekgoa ko kokelong, le gore a tirisano mmogo le baitseanape ba tsa maranyane e ka thusa mo go rarabololeng mathata a a go lebaneng o le ngaka ya setswana.

Mu na itjino tjibaka, ndo shaka ziba kuti a kuna shingidzano pakati kwenyu ne dzinghanga dze ikhuwa mu zwipatela, nanga ne ku ti a ku shingidzana ne be maranyane kunga mu batsha ku kunda mata'thaa a ka mu lingisisana mu li nga'nga ye itswana/ikalanga?

E1. Are there any conditions which you as a herbalist believe you can treat better?

A go na le malwetsi/diemo dingwe o le ngaka ya setswana tse o dumelang o ka di alafa

botoka?

A kuna zwimo/magwele mangwe a mu no dumila, mu li ngaa'nga ye itswana/ikalanga

kuti mu ngaa a lapa butuka?

……

E2. If yes, what are these conditions?

Fa o dumela, ke dife diemo

tse?

A pa mu duma, ndi zwipi zwimo izwezwi?

.....

E3. What makes you think that you are competent to treat these conditions?

Ke eng se se dirang gore o akanye gore o na le bokgoni mo go alafeng diemo tse?

Ini tji no thama kuti mu kumbule kuti mu na bukhoni/bunyambi gwe ku lapa zwimo izwezwi?

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……

E4. Are there any conditions which you think the medical doctors can treat better?

A go na le diemo tse o akanyang gore ba bongaka jwa sekgoa ba ka di alafa botoka?

A kuna zwimo/magwele mangwe a mu no dumila, nga'nga dze ikhuwa dzinga ngaa a lapa butuka?

.....

E5. If yes, what are these conditions?

Fa o dumela, ke dife?

A pa mu duma, ndi zwipi zwimo izwezwi?

.....

E6. Why do you think the medical doctors are in a better position to treat these conditions?

Ke eng o akanya gore bongaka jwa sekgoa bo mo seemong se se botoka go ka alafa diemo tse?

Ne kutini mu kumbula kuti nga'nga dze ikhuwa dzinga ngaa lapa zwimu izwezwi butuka?

……
……

……

……

……

……

…

E7. Have you ever referred patients to a hospital?

A o kile wa fetisetsa balwetsi ko kokelong?

A ma ka tongo pindisila bagwele ku ipatela?

.....

E8. If yes, how many patients did you refer to a hospital in the past 12 months?

Fa o dumela, ke ba le kae ba o ba fetiseditseng kwa kokelong mo dikgweding di le lesome le bobedi tse di fetileng?

A pa mu duma, bagwele bangana ba ma ka tongo pindisila ku ipatela mu tjibaka tja ka pinda, tje mimwedzi i gumi li na bubili?

……
……

E9. Have you ever received self-referred patients who have been to a hospital?

A o kile wa amogela balwetsi bangwe ba ba itsisitseng kwa go wena mme ba tswa kwa
kokelong?

A ma ka to ngo amutjila bagwele ba zwi hisa ku muli bi dhwa ku ipatela?

.....

E10. If yes, which reasons for self-referral did patients state?

Fa o dumela, ke mabaka afe a ba a boletseng go bo ba itsisitse ko go wena?

A pa mu duma, mabaka api a ba ka mu pa kubo ba zwi hisa ku muli?

E11. If yes, how many self-referred patients have you received in the past 12 months?

Fa o dumela, ke balwetsi ba le kae ba o ba amogetseng mo dikweding di le lesome le
bobedi tse di fetileng?

A pa mu duma, bagwele ba ngana ba ma ka amutjila mu tjibaka tja ka pinda, tje mimwedzi i gumi li na bubili?

……

E12. Do you ask what kind of conventional medicines patients have received?

A o botsolotsa balwetsi ka tiriso ya melemo e ba e filweng ko
dikokelong?

A mo ba buzwisisa ne kwe miti ya ba piwa ku ipatela?

……

E13. Do you advise patients not to take conventional medicine and herbal medicine at the same time?

A o gakolola balwetsi gore ba seka ba tlhakanya melemo ya setso le ya kwa dikokelong?

A mo so kumbuludza bagwele benyu kuti ba si to ngo shanganya miti ye ikhuwa ne ye itswana/ikalanga?

……

……

E14. Do you think the cooperation between herbalists and doctors should be better?

A o akanya gore tirisanyo mmogo magareng ga dingaka tsa setswana le tsa sekgowa e

tshwanetse ya tokafadiwa?

A mo kumbula ku ti shingidzanyo makati kwe dzi-ngaa'nga dze itswana/ikalanga ne dze

ikhuwa inga thabulugwa?

.....

E15. What do you think should be done to improve this cooperation?

O akanya gore go ka dirwa jang go tlokafatsa tirisano mmogo e?

Mo kumbula kuti inga thabulugwa tjini?

……

E16. Are you cooperating with other traditional doctors?

A o na le ditirisanyo le dingaka tse dingwe tsa setso?

A mo shingidzana ne dzi-ngaa'nga dzimwe dze itswana/ikalanga?

……..

E17. Are you referring patients to other traditional doctors?

A o atle o romele balwetsi ko dingakeng tse dingwe tsa setso?

A mo so tumila bagwele ku dzi-ngaa'nga dzimwe dze itswana/ikalanga?

……

……

E18. Are there any problems you experience as a herbalist (Supply, storage, conservation, scarcity of plants) where a scientist might be helpful?

A go na le mathata a a go amang o le ngaka ya Setswana (go bona melemo, peo, tshomarelo, tlhokafalo ya ditlhare) a moitseanape wa tsa maranyane a ka go thusang teng?

A ku na buthata gwa mu no shangana nago mu li ngaa'nga ye itswana/ikalanga (ku bhona miti, ku i biga, ku bigidzila, ku sheteka kwayo), gu nga batshiwa ne nyambi ye maranyani?

……..

……..

…….

E19. Do you think it would be good to cultivate plants to secure a constant supply?

A o akanya fa go siame go ka lema ditlhare go netefatsa gore di nna di le teng?

A mo kumbula ku ti ko be kwa ka lulwama/naka ku lima miti iyeyi i no lapa ku batsha ku ti i sitongo sheteka, i be i lipo mu misi yose a mu i shaka?

E20. If, yes, which ones?

Fa o dumela, ke dife?

A pa mu duma, ndi ipi miti iyeyo?

.....

Condition treated better by herbalists (Diemo tse di alafiwang botoka ke dingaka tsa setswana) (*Zwimo zwi nga lapiwa butuka ne nga'nga dze itswana/ikalanga?)*	**Conditions treated better by the hospital (Diemo tse di alafiwang botoka ke ba kokelo) (***Zwimo zwi nga lapiwa butuka ne nga'nga dze ikhuwa dzinga?)*

SECTION F

In this section I will ask you about medicinal plants used to treat specific conditions. I will also collect samples of these plants to analyze in the laboratory whether they are effective against infections (viruses, bacteria, parasites etc.) and whether they strengthen the immune system.

Mo sephatong se ke tla go botsisisa ka ditlhare tsa melemo tse di dirisiwang go alafa diemo tse di rileng. Ke tla tsaya bonyennyane jwa ditlhare tse go ya go di sekaseka ka tsa maranyane go bona gore a di ka bereka kgatlhanong le megare le gore a di tiisa mmele gore o itshireletse.

Mu libazhe igogu, ndo mu buzwisisa ne kwe miti i no shingisiwa ku lapa magwele/zwimo zwa ndi no mu tjenesela izo. Ndo tola butikinini gwe miti iyeyi, ndibe ndi no zwi thathubha ndi shingisa bunyambi gwedu gwe maranyani — ndi bhone kuti a zwi nga bulaya/kunda zwikonyana zwe magwele, nanga ne kuti zwi nga simisa masole e mbili.

F1. Which medicinal plants are used to treat the below mentioned conditions?

Ke ditlhare tsa melemo dife tse di dirisiwang go alafa diemo/malwetsi a a nankotsweng fa

tlase?

I miti ipi i no shingisiwa ku lapa zwimu/magwele a legwa kusi ipapa?

i) Tuberculosis/Kgotlholo e tona/ *Khogola ku kulu*

ii) Malaria/Letshoroma/ *Ku pisa ku no yi Malaria*

iii) Skin conditions related to HIV/AIDS/

Malwetsi a dikobo a a amanang le bolwetsi jwa HIV/AIDS

Magwele e guguta le mbili a no amana ne phamukati/zhibizhibi le HIV/AIDS?

- Thrush/Bogwata mo dikarolong tsa senna kana sesadi/*ku dzuka kwe bulume kene bukadzi*
- Herpes/Herpise/*Malonda e bulume kene bukadzi, Herpisi ne ikhuwa*

F2. How do you know if a patient is HIV positive? (The symptoms)

O itse jang gore molwetsi o na le mogare wa HIV (Dikai)

Mo zibha tjini ku ti n'gwele u na gonyana le phamukati/zhibizhibi linoyi HIV/AIDS

……
……
……
……

F3. Do you usually ask patients about their HIV status?

A o atle o botsolotse balwetsi ka seemo sa bone sa mogare wa HIV?

A mo so buzwa bagwele ne kwe tjimo tjabo tje phamukati/zhibizhibi linoyi HIV/AIDS

…….

F4. How do you know if a patient has tuberculosis or malaria? (The symptoms?)

O itse jang gore molwetsi o na le mogare wa letshoroma kana kgothlolo e tona (Dikai)

Mo zibha tjini kuti n'gwele u na bugwele gwe 'Khogola ku kulu, TB' kene 'ku pisa ku no yi

Malaria'

……..
……..

F5. Collect a sample of medicinal plants for laboratory analysis.

Tsaya bonyennyane jwa ditlhare tsa melemo go di sekaseka ka tsa maranyane.

Tola butikinini gwe miti i no lapa ku no thathubha ne bunyambi gwe maranyani

Section G

We have come to the end of the interview. Is there anything you want to ask us or is there anything which you would like to comment on, which was maybe not covered in the questionnaire?

Re gorogile ko bokhutlong jwa potsolotso ya rona. A go na le sengwe se o batlang go se re botsa, kana, sengwe fela se o batlang go akgela ka sone — se re ka tswang re sa se bua mo potsolotsong e?

Ta tji swika ku bupelo gwe buzwisiso yedu. A kuna tja mu no shaka ku ti buzwa ne kwatjo, kana tjimwe koga tja mu nga shaka lashila ne kwatjo — tja ti nga dhwa ti sa tji leba mu buzwisiso iyeyi?

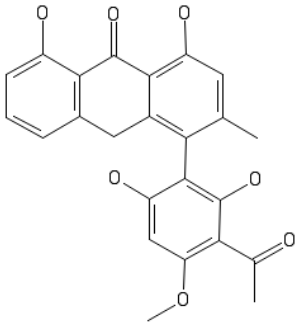
Thank you!

Re lebogile!

Ta boka!

Appendix B. Supplemental Figure

Supplemental Figure 1. Structures of all anthrones assessed for in vitro HIV latency reversal.



Knipholone anthrone

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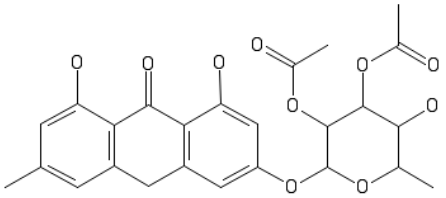
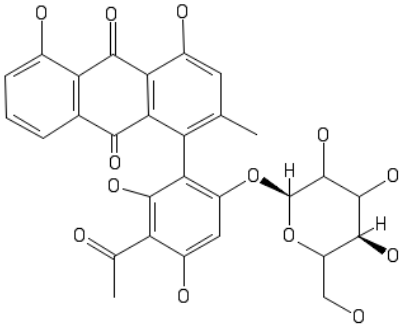
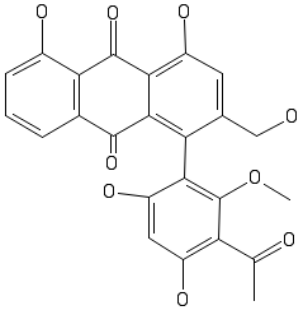
ıol]



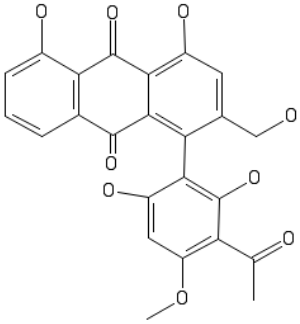
Prinoidin



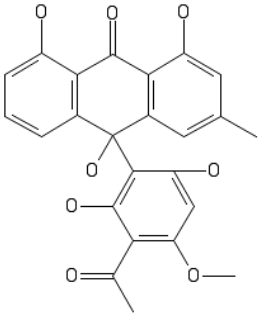
1-[3-acetyl-2,4-dihydroxy-6-(β-D-glucopyranosyloxy)phenyl]-4,5-dihydroxy-2-methyl-9,10-anthracenedione



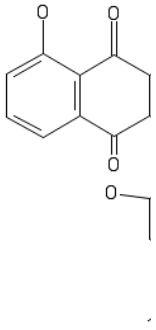
Gaboroquinone A



Gaboroquinone B



Foliosone



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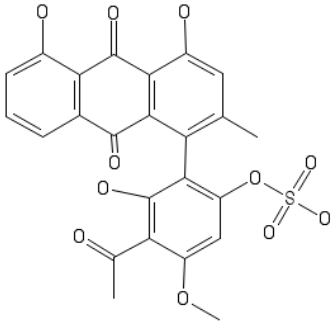
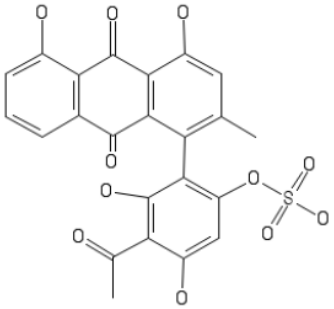
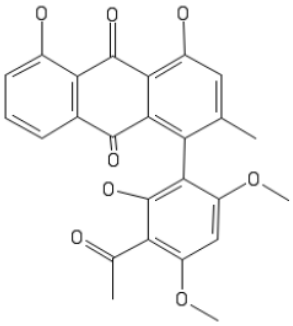


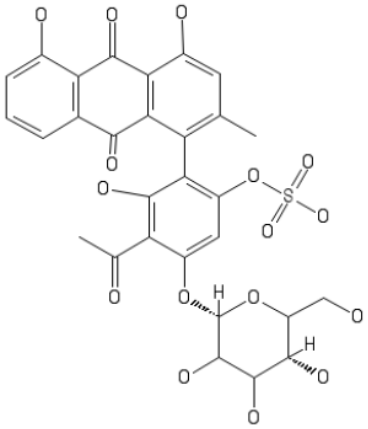
1-[3-acetyl-2-hydroxy-4-methoxy-6-(sulfooxy)phenyl]-4,5-dihydroxy-2-methyl-9,10-anthracenedione



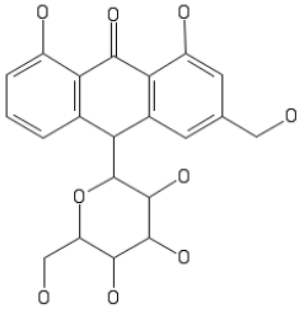
1-[3-acetyl-2,4-dihydroxy-6-(sulfooxy)phenyl]-4,5-dihydroxy-2-methyl-9,10-anthracenedione



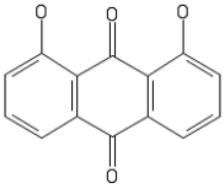
Knipholone 6'-methyl ether



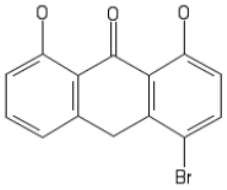
1-[3-acetyl-4-(β-D-glucopyranosyloxy)-2-hydroxy-6-(sulfooxy)phenyl]-4,5-dihydroxy-2-methyl-9,10-anthracenedione



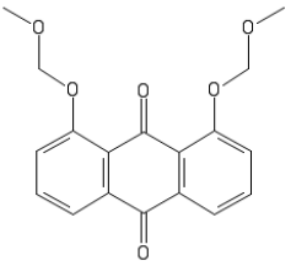
Aloin



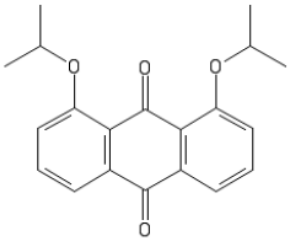
Danthron



4-Bromo-1,8-dihydroxy-10H-anthracen-9-one



1,8-Bis(methoxy)anthracene-9,10-dione



1,8-Diisopropoxyanthracene-9,10-dione

Appendix C. Supplemental Table

Supplemental Table 1. Absolute copies of HIV RNA per million cells, estimated number of cells assessed per qPCR reaction, and limit of detection of *ex vivo* studies in Figure 5.8, as determined by qPCR of viral RNA and 18S housekeeping gene and normalized to copy number standards.

Donor	Treatment	Absolute copies of HIV RNA per million cells	Total # of cells analyzed	Limit of detection
1	0.1% DMSO	< 76	13,124	76
	100 ng/mL PMA + 0.1 µg/mL ionomycin	< 50	20,075	50
	10 µM prostratin	< 54	18,687	54
	10 µM KA	8633	14,630	68
2	0.1% DMSO	626	27,953	36
	100 ng/mL PMA + 0.1 µg/mL ionomycin	1008	30,172	33
	10 µM prostratin	1181	35,139	28
	10 µM KA	846	28,836	35
3	0.1% DMSO	602	279,932	4
	100 ng/mL PMA + 0.1 µg/mL ionomycin	672	732,347	1
	10 µM prostratin	1514	308,599	3
	10 µM KA	617	400,645	2
Average ± SD	0.1% DMSO	435 ± 311		
	100 ng/mL PMA + 0.1 µg/mL ionomycin	576 ± 486		
	10 µM prostratin	916 ± 765		
	10 µM KA	3365 ± 4563		