Universitat Autònoma de Barcelona

Identification and characterization of novel latency-reversing agents to clear HIV-1 viral reservoir

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I per tal que en quedi constància, signen aquest document a Badalona,

10 de Setembre de 2019.

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A la meva família i a en Guille,

Per haver-me aguantat durant tots aquests anys

SUMMARY

Current antiretroviral therapy has changed the perspective of HIV-1 infection from a lethal illness to a chronic disease. However, the HIV-1 latent reservoir is a major hurdle to achieve a cure for HIV-1. The “shock and kill” strategy is based on inducing viral transcription of latent HIV-1 provirus followed by the selective killing of reactivated cells. Although several latency-reversing agents (LRAs) have been identified and tested, none of them has been able to efficiently eradicate the HIV-1 latent reservoir. Based on the need of novel agents and strategies to efficiently clear the latent reservoir, we evaluated compounds developed as modulators of the innate immune response or designed to modulate the cell cycle progression as novel agents able to purge the viral reservoir.

The study of innate immune modulators as agents able to clear the HIV-1 reservoir might represent an alternative due to its intrinsic functions, i. e., protection and clearance of infections. The innate immune regulator acitretin, an FDA-approved compound for psoriasis, has been proposed to induce HIV-1 reactivation and selective killing of the infected cells. However, the effect of acitretin on HIV-1 reactivation was negligible in the vast majority of models tested, albeit activation of RIG-I pathway was detected and a mild induction of viral reactivation was observed in a non-clonal T cell model. Moreover, acitretin treatment did not induce the selective killing of the infected cells.

Anti-cancer compounds have also been proposed as candidate therapies targeting the latent reservoir, mainly due to the ability of certain agents to modify gene transcription or to promote cell apoptosis. The assessment of the HIV-1 reactivation potential of an anti-cancer compound library reported several molecular targets whose inhibition promoted HIV-1 latency reversal, including the histone deacetylases (HDAC), Janus kinases (JAK), IκB kinases (IKKs) and heat shock proteins (HSPs). Among the new identified LRAs, Aurora kinases inhibitors (AURKi) represented the largest family of compounds not previously described as LRA that significantly and consistently showed HIV-1 reactivation capacity. AURKi were able to enhance the HDACi-mediated reactivation, suggesting that AURKi are able to target a distinct set of integrated provirus than that reactivated by the well-described HDAC inhibitors. Interestingly, AURKi

restricted acute HIV-1 infection, suggesting a dual role for these compounds on HIV-1 infection.

Midostaurin, a multi-kinase inhibitor approved for leukemia treatment, was also identified as an LRA. Midostaurin induced HIV-1 latency reactivation, either alone or in combination with other LRAs, consistent with previous reports that associated this activity with the activation of the innate immune NF-κB pathway. Moreover, we also observed a non-yet-reported and SAMHD1-dependent inhibitory effect of HIV-1 replication in primary cells.

The enhanced capacity to promote HIV-1 reactivation of AURKi and midostaurin in combination with other LRAs supports the idea that different agents are needed to reactivate all latent provirus, presenting different specificities towards HIV-1 provirus reactivation depending on its integration site in the host genome. Furthermore, these observations also raise concerns on the models used to study HIV-1 latency, as clonal models might not be suitable due to the lack of heterogeneity in proviral insertion site, characteristic of non-clonal models. Altogether, our results suggest that modulation of innate immunity and cell cycle may be taken into account for the design of future LRAs for the “shock and kill” strategy; however, further research is still necessary before it can lead to an HIV-1 cure.

RESUMEN

La terapia antirretroviral ha cambiado la perspectiva sobre la infección por VIH-1 de enfermedad letal a crónica. Aun así, el reservorio latente del VIH-1 es una de las mayores barreras para lograr una cura. La estrategia "shock and kill" se basa en inducir la transcripción viral del provirus latente del VIH-1 seguido de la muerte selectiva de las células reactivadas. Aunque muchos agentes reversores de la latencia (LRAs) han sido identificados y testados, ninguno ha logrado erradicar eficazmente dicho reservorio. Dada la necesidad de nuevos agentes y estrategias capaces de eliminar el reservorio latente, hemos propuesto moduladores de la respuesta inmune innata o del ciclo celular para dicho fin.

El estudio de moduladores de la inmunidad innata puede representar una alternativa dadas sus funciones intrínsecas, i. e., protección y eliminación de infecciones. Acitretina, regulador de la inmunidad innata aprobado contra la psoriasis, ha sido propuesto como inductor de la reactivación del VIH-1 y la muerte de las células infectadas. A pesar de ello, el efecto de acitretina en la reactivación fue muy modesto en la mayoría de modelos celulares que testamos, aunque se detectó la activación de la vía de RIG-I, y una ligera inducción de la reactivación viral en un modelo no clonal de células T. Además, acitretina tampoco promovió la eliminación de las células infectadas.

Los compuestos anti-cáncer también han sido propuestos como estrategia contra el reservorio, debido a la habilidad de ciertos agentes para modificar la transcripción génica o promover la apoptosis. El cribado de una librería de compuestos anti-cancer reportó varias dianas cuya inhibición reactivó el VIH-1, incluyendo histona deacetilasas (HDACs), Janus quinasas (JAKs), IκB quinasas (IKKs) y proteínas "heat-shock" (HSPs). Entre los nuevos LRAs identificados, los inhibidores de Aurora kinasas (AURKi) representaron la mayor familia de compuestos, no descritos como LRAs, que mostraron capacidad de reactivación del VIH-1 de forma significativa y consistente. Los AURKi mejoraron la reactivación mediada por los HDACi, sugiriendo la habilidad para reactivar distintos provirus insertados. Curiosamente, AURKi restringió la replicación aguda del VIH-1, insinuando un papel dual para dichos compuestos en la infección por VIH-1.

Midostaurin, un inhibidor de multi-quinasas aprobado contra la leucemia, también se identificó como LRA. Midostaurin reactivó el VIH-1, tanto por si solo como en combinación con otros LRAs, corroborando previos reportes que asociaron esa actividad con la activación de la vía de NF-κB. Además, también se observó una inhibición de la infección aguda del VIH-1 en células primarias dependiente de SAMHD1 no descrita.

El hecho de que los AURKi y midostaurina mejoren la reactivación del VIH-1 en combinación con otros LRAs, corrobora la idea de que distintos compuestos pueden ser necesarios para reactivar todos los provirus integrados, presentando así distintas especificidades para la reactivación del provirus que dependan de su lugar de integración en el genoma. Estas observaciones plantean dudas sobre los modelos usados para estudiar la latencia del VIH-1, pues los modelos clonales podrían ser inadecuados por la falta de heterogeneidad de lugares de integración. En conjunto, nuestros resultados sugieren que la modulación de la inmunidad innata y ciclo celular podrían incluirse en el desarrollo de futuros LRAs para la estrategia “shock and kill”; aun así, investigaciones adicionales siguen siendo necesarias con tal de avanzar hacia la cura del VIH-1.

RESUM

La teràpia antiretroviral actual ha canviat la perspectiva de la infecció pel VIH-1 de malaltia letal a crònica. Tanmateix, el reservori latent del VIH-1 és una de les majors barreres per aconseguir una cura. L'estratègia de "shock and kill" es basa en la inducció de la transcripció del provirus latent seguit de la mort selectiva de les cèl·lules reactivades. Tot i que s'han identificat i provat diversos agents reversors de la latència (LRAs), cap d'ells ha estat capaç d'eradicar el reservori latent del VIH-1. A partir de la necessitat de la identificació de nous agents i estratègies alternatives per a eliminar de forma eficaç el reservori viral latent, hem proposat moduladors de la resposta immune innata o de la progressió del cicle cel·lular amb aquesta mateixa finalitat.

L'estudi de moduladors de la resposta immune innata s'ha proposat com a estratègia alternativa degut a les funcions intrínseques del sistema immune innat, i e., protecció i eliminació d' infeccions. Acitretina, un regulador de la immunitat innata aprovat per al tractament de la psoriasi, s'ha proposat com a inductor de la reactivació del VIH-1 i mort selectiva de les cèl·lules infectades. Tot i això, l'efecte de l'acitretina en la reactivació del VIH-1 és molt modest en la gran majoria dels models que hem testat, si bé és capaç d'activar la via de senyalització depenent de RIG-I i es va observar una inducció lleu de la reactivació viral en models de cèl·lules T no clonals. A més, el tractament amb acitretina no va induir la mort selectiva de les cèl·lules infectades.

Els compostos anti-càncer s'han proposat també com estratègia per l'eliminació del reservori latent, principalment degut a la capacitat de modificar la transcripció gènica o de promoure l'apoptosi cel·lular. El cribratge d'una llibreria de compostos anti-càncer va identificar diverses dianes moleculars la inhibició de les quals va reactivar el VIH-1, incloent histona deacetilases (HDACs), Janus cinases (JAKs), IκB cinases (IKKs) i proteïnes "heat-shock" (HSP). Entre els nous LRA identificats, els inhibidors d'Aurora cinases (AURKi) representaven la família més nombrosa amb un potencial com a LRAs no descrit prèviament. A més a més de reactivar la latència viral, els AURKi són capaços de millorar la reactivació induïda per HDACi, el que suggereix que els AURKi són capaços de mobilitzar un conjunt diferent de provirus integrats, comparats als reactivats per HDACi.

Curiosament, els AURKi també presenten activitat antiviral en la infecció aguda per VIH-1, el que suggereix un paper doble per a aquests compostos en la infecció pel VIH-1.

Midostaurina, un inhibidor de la multi-quinasa aprovat per al tractament de la leucèmia, també es va identificar com a LRA. La midostaurina és capaç de revertir la latència del VIH-1, sola o en combinació amb altres LRAs, corroborant dades anteriors que associen aquesta activitat amb l'activació de la via de NF-κB. A més, també es va observar la inhibició de la infecció aguda del VIH-1 en cèl·lules primàries, el qual depèn de la presència de SAMHD1 i no ha estat prèviament reportat.

El fet de que les AURKi i midostaurina millorin la reactivació del VIH-1 en combinació amb altres LRA, recolza la idea que calen diferents agents per reactivar tots els provirus latents integrats, presentant els diferents agents diferents especificitats per a la reactivació del provirus en funció del seu lloc d'integració en el genoma. A més, planteja dubtes sobre els models fet servir en l'estudi de la latència del VIH-1, donat que models clonals poden no ser adequats per la falta d'heterogeneïtat en els llocs d'integració al genoma de la cèl·lula hoste. En conjunt, els nostres resultats suggereixen que la modulació de l'immunitat innata i del cicle cel·lular podrien incluirse per al desenvolupament de futurs LRAs per a l'estratègia de "shock and kill"; tanmateix, és necessari continuar amb el seu estudi per tal d'avançar cap a la cura de la infecció pel VIH-1.

ABBREVIATIONS

7AAD	7-aminoactinomycin D
ACI	Acitretin
AIDS	Acquired immunodeficiency syndrome
Akt	Protein kinase B
AP-1	Activator protein 1
APC	Antigen-presenting cell
APOBEC3	Apolipoprotein-B mRNA-editing catalytic polypeptide-like-3
ART	Antiretroviral therapy
AURK	Aurora kinase
AZT	Zidovudine
Bcl-2	B-cell lymphoma 2
BET	Bromodomain and extraterminal
bNAb	Broadly neutralizing antibody
CA	Capsid
CAR	Chimeric antigen receptor
CC50	50% cytotoxic concentration, or the concentration needed to induce 50% cell death
CCR5	C-C chemokine receptor 5
CCR5Δ32	CCR5 with a 32-bp deletion
CD	Cluster of differentiation
CDC	Center for disease control
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CDR	Cytosolic DNA receptors
cGAS	Cyclic GMP-AMP synthase
Chk	Checkpoint kinase
CNS	Central nervous system
CPC	Chromosomal passenger complex
CRISP/Cas9	clustered regularly interspaced short palindromic repeats/CRISP-associated protein nuclease-9
CTL	Cytotoxic T lymphocytes
CXCR4	C-X-C chemokine receptor type 4
DC	Dendritic cell
DDR	DNA damage response
DHFR	Dihydrofolate reductase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	Deoxynucleoside triphosphate

dsDNA	Double stranded DNA
EC50	50% effective concentration, or the concentration needed to induce the half-maximal response
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FDC	Follicular dendritic cell
FLT3	FMS-like tyrosine kinase 3
GALT	Gut associated lymphoid tissue
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GFR	Growth factor receptor
GIT	Gastrointestinal tract
GTP	Guanosine triphosphate
HDAC	Histone deacetylase
HIV	Human immunodeficiency virus
HSCT	Hematopoietic stem cell transplantation
HSP	Heat shock protein
IFI16	Interferon inducible protein 16
IFN	Interferon
IgG	Immunoglobulin G
IKK	IκB kinase
IL	Interleukin
IN	Integrase
INF	Infected
INSTI	Integrase strand transfer inhibitor
IRES	Internal ribosome entry site
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
IκBα	Nuclear factor κ-light-chain-enhancer of activated B cells inhibitor α
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LOD	Limit of detection
LRA	Latency-reversing agent
LTR	Long terminal repeat
MA	Matrix
MAVS	Mitochondrial antiviral signaling
M-CSF	Macrophage colony-stimulating factor

MDA-5	Melanoma differentiation-associated protein 5
MDM	Monocyte-derived macrophages
MHC-II	Major histocompatibility complex class II
MID	Midostaurin
mRNA	Messenger RNA
NC	Nucleocapsid
ND	No drug
Nef	Negative regulatory factor
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
NK	Natural killer (cell)
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NPC	Nuclear pore complex
NRTI	Nucleoside reverse transcriptase inhibitor
NVP	Nevirapine
ORF	Open reading frame
PAI	Post-attachment inhibitor
PAMP	Pathogen-associated molecular pattern
PARP	Poly(ADP-ribose) polymerase
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCP	*Pneumocystis carinii* pneumonia
PCR	Polymerase chain reaction
PD	Palbociclib
PHA	Phytohemagglutinin
PI	Protease inhibitor
PI3K	Phosphoinositide 3-kinase
PIC	Pre-integration complex
PKC	Protein kinase C
PLK	Polo-like kinase
PNB	Panobinostat
PR	Protease
pRB	Retinoblastoma protein
PRR	Pattern recognition receptor
P-TEFb	Positive transcription elongation factor b
PTM	Posttranslational modification
PVDF	Polyvinylidene difluoride
qPCR	quantitative real-time PCR
RAL	Raltegravir
Rev	Regulator of expression of virion proteins
RIG-I	Retinoic acid-inducible gene I

RISC	RNA-induced silencing complex
RLR	RIG-like receptor
RNA	Ribonucleic acid
RNAi	RNA interference
RNAPII	RNA polymerase II
RNaseH	Ribonuclease H
RNR2	Ribonucleotide reductase subunit R2
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase PCR
SAMHD1	Sterile alpha motif (SAM) histidine-aspartic (HD) domain protein 1
SD	Standard deviation
SMAC	Second mitochondria-derived activator of caspases
Sp1	Specificity protein 1
ssRNA	Single stranded RNA
STAT1	Signal transducer and activator of transcription 1
SU	Surface protein
TALEN	Transcription activator-like effector nuclease
TAR	Trans-activation response
Tat	Trans-activator of transcription
Tfh	T follicular helper (cell)
TGF-β1	Transforming growth factor β1
TGS	Transcriptional gene silencing
TI	Transcriptional interference
TLR	Toll-like receptor
TM	Transmembrane protein
TPA	Tetradecanoyl phorbol acetate, PMA
TRIM	Tripartite motif-containing protein
UN	Untreated
UNAIDS	Joint United Nations Programme on HIV/AIDS
Vif	Viral infectivity factor
VL	Viral load
VLP	Viral-like particles
VOR	Vorinostat, SAHA
Vpr	Viral protein R
Vpu	Viral protein U
Vpx	Viral protein X
VSV-G	Vesicular stomatitis virus G protein
ZFN	Zinc fingers nuclease

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INTRODUCTION

1. HISTORY OF THE AIDS EPIDEMIC

On June 5, 1981, the Center for Disease Control (CDC) of the USA published an article describing the case of five young men with *Pneumocystis carinii* pneumonia (PCP), a rare lung infection, in Los Angeles[1]. This would become the first reported case of what in 1982 was named AIDS (Acquired Immunodeficiency Syndrome)[2]. In 1983, researchers of the Institute Pasteur described a novel retrovirus that was proposed to be the causative agent of AIDS[3]. A year later, two independent groups confirmed the relationship between AIDS and the virus[4,5]. However, it was not until 1986 that the virus, known until then as lymphadenopathy-associated virus (LAV), human T cell leukemia virus type III (HTLV-III) or AIDS-associated retrovirus, was officially renamed as human immunodeficiency virus (HIV) by the International Committee on the Taxonomy of Viruses[6].

Almost forty years have passed since the first reported cases in Los Angeles, and more than thirty since the approval of zidovudine (AZT), the first antiretroviral compound approved by the Food and Drug Administration (FDA) to treat HIV [7]. However, according to the Joint United Nations Programme on HIV/AIDS (UNAIDS), there were approximately 37.9 (32.7 – 44.0) million people living with HIV in 2018. This data follows a steadily increase observed since 1990 as a direct consequence of the more people receiving the life-saving antiretroviral therapy (ART), which is currently around 62% (47 – 74%) of all people living with HIV. Furthermore, the number of new infections has decreased from 2.9 (2.3 –3.8) million in 1997 to 1.7 (1.4 – 2.3) million in 2018 as well as the number of AIDS-related deaths, which reduced from 1.7 (1.3 – 2.4) million in 2004 to 0.77 (0.57 – 1.1) million in 2018. Although thanks to the implementation of ART, the rates of AIDS-related deaths, HIV new infections and HIV morbidity have decreased, the AIDS epidemic is still one of the most serious health challenges of the world[8].

2. HIV. THE VIRUS

HIV is a virus from the family of the *Retroviridae*, member of the genus *Lentivirus*. According to its origin and the organization of its genome, HIV can be divided into two

subtypes: HIV-1 and HIV-2. Even though both subtypes are able to cause AIDS, HIV-1 is the main contributor to the epidemic, due to its higher virulence and worldwide distribution. In comparison, HIV-2 appears to have a slower infection course and its presence is primarily limited to Central and West Africa[9–12].

2.1 GENOME

HIV-1 is a virus with two identical copies of single-stranded RNA (ssRNA). The genome of the provirus, known as the proviral DNA generated from reverse transcription of the viral RNA, is about 9.8 kb long and has overlapping open reading frames that encode for several genes, including the three structural genes: *gag*, *env* and *pol*. *Gag* encodes for the precursor of p24 (CA, capsid), p17 (MA, matrix), p7 (NC, nucleocapsid) and p6 proteins, before being processed by the viral protease. *Env* encodes for the gp160 precursor that, after being processed, becomes the envelope glycoproteins gp120 (SU, surface protein) and gp41 (TM, transmembrane protein). *Pol* encodes for a precursor of the viral enzymes PR (protease), RT (reverse transcriptase) and IN (integrase). Besides the structural proteins, the HIV-1 genome also encodes for two essential regulatory proteins: Tat, that enhances viral transcription initiation and elongation, and Rev, which allows the export of unspliced viral mRNA from the nucleus to the cytoplasm. Additionally, HIV-1 encodes for four non-essential accessory genes (*vpr*, *vif*, *vpu* and *nef*)[12–15].

2.2 VIRAL PARTICLE

The HIV-1 viral particle, or virion, is a spherical structure with a diameter of about 100 nanometers (nm) (**Figure 1**). It has an envelope that consists of a lipid bilayer obtained from the plasma membrane of the infected cell after the budding process, and thus it can contain proteins from the host cell such as adhesion proteins. The envelope also contains the so-called “Env spikes”, heterodimer complexes consisting of three subunits of gp41 and three subunits of gp120, which are essential to bind to the target cell. Contrary to what was thought before, recent studies have claimed that each virion has around 14 Env spikes in its envelope[16] that cluster into one spot after the virion maturation[17]. A spherical outer matrix formed by p17 protein and anchored at the inner membrane of the lipid bilayer is right below the envelope, which is at the same time

surrounding the conical capsid formed by the p24 protein. This conical capsid is only present in mature virions, as their immature counterparts contain a spherical capsid formed by the unprocessed Gag precursor[18], that is cleaved by the PR located in the space between the matrix and the capsid during the maturation of the virion. After maturation, the conical capsid will contain, for each virion, the protein p6, the enzymes RT and IN, and two copies of the viral ssRNA directly associated with p7 through a zinc-finger domain of the latter[19,20].

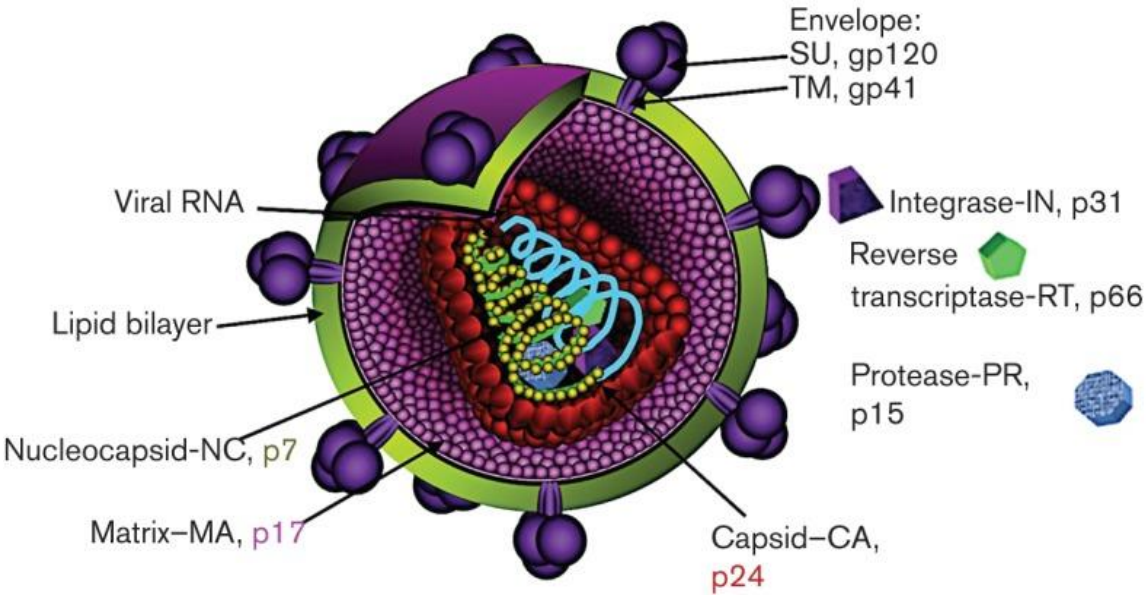

Figure 1. Structure of HIV-1 virion. Schematic representation of the structure of the mature infectious HIV-1 viral particle[21].

3. THE REPLICATION CYCLE OF HIV-1

Viruses are only able to replicate by infecting a host cell and taking advantage of the cellular machinery to produce new virions. However, as a retrovirus, HIV-1 is able to insert its own genome into the host DNA. The steps of the HIV-1 viral life cycle are described below (**Figure 2**).

3.1 ENTRY

In order to enter in the cell, HIV-1 needs to bind to cell surface proteins expressed in the target cell that serve as receptors for viral entry. The viral Env spikes, containing the glycoproteins gp41 and gp120, are responsible for the binding and fusion to the cell

membrane. Specifically, gp120 is able to bind the cellular cluster of differentiation 4 (CD4), a receptor for the major histocompatibility complex class II (MHC-II) molecule, that is expressed in circulating T-cells, as well as in T-cell precursors, but can also be found in other cells like monocytes and macrophages. Following CD4 binding, gp120 undergoes a structural change that allows its binding to either C-C chemokine receptor 5 (CCR5)[22] or C-X-C chemokine receptor type 4 (CXCR4)[23]. The virus specificity to one or another is what will define the viral tropism, being the R5-tropic viral strain able to bind to CCR5, whereas the X4-tropic viral strain binds to CXCR4. Dual tropic viral strains, i. e. able to bind both co-receptors, have also been described[24]. After the double binding of gp120, the N-terminal of gp41 containing a fusogenic hydrophobic peptide is able to interact with the cellular membrane, allowing the fusion of the viral and cellular membranes and releasing the viral capsid inside the cell[25–27].

3.2 UNCOATING, REVERSE TRANSCRIPTION AND NUCLEAR IMPORT

After the viral capsid is released into the cytoplasm, the virus proceeds to disassemble the p24-capsid, during the uncoating process. However, the exact mechanism underlying uncoating is still not well understood: it has been proposed to take place either early after entry near the cell membrane, at later stages remaining the capsid intact until reaching the nuclear membrane, or in an intermediate model where the capsid is gradually uncoated as it is transported towards the nucleus[28]. After viral uncoating (or before, depending on the model), the viral ssRNA is reverse transcribed by the viral enzyme RT into double-stranded (ds) DNA. In this process, the RT first creates a DNA-RNA hybrid by synthesizing the complementary DNA (cDNA) of the ssRNA, then the ribonuclease H (RNase H) site of the RT degrades the RNA and finally synthesizes the second DNA strand. During reverse transcription, the lack of proofreading function of the RT introduces a high number of errors, contributing to the generation of genomic mutations into the viral genome. These mutations promote high viral genetic variation that, under selective pressure like the presence of antiretrovirals, could allow the selection of resistant quasispecies. Once the viral dsDNA has been reverse transcribed, it interacts with several viral and host proteins, including IN, to form the pre-integration complex (PIC) that will be transported to the nucleus through the interaction of IN and

the nuclear pore complex (NPC). The formation of the PIC and subsequent interaction with the NPC is responsible for the ability of HIV-1 to infect non-dividing cells, as it does not require the disruption of the nuclear envelope to enter the nucleus[28–31].

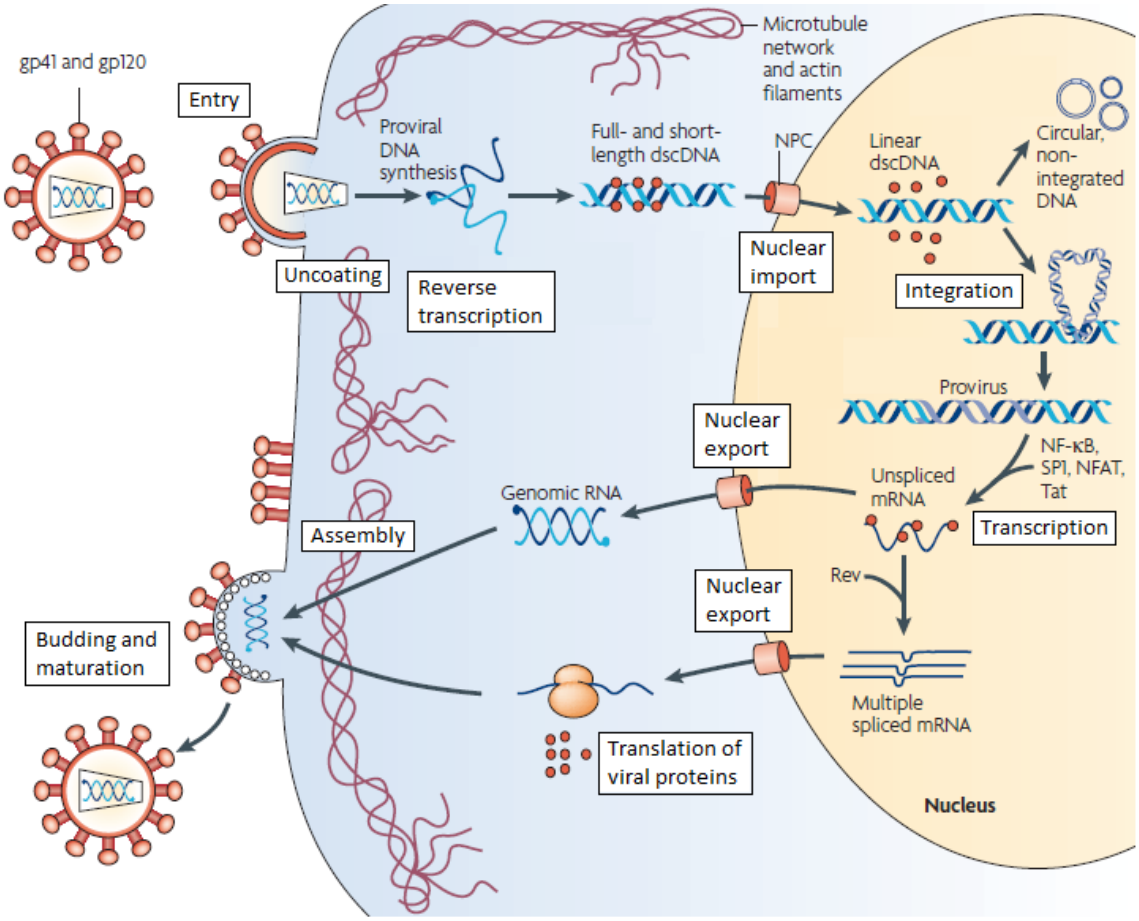

Figure 2. The HIV-1 life cycle. After binding to the cell surface receptors, HIV-1 is released inside the cell where its RNA is reverse transcribed into DNA. Afterwards, the viral DNA is imported to the nucleus, where it can remain in a non-integrated form or be integrated into the cell genome by the integrase. Once in the nucleus, the virus can either remain silent or be actively transcribed. In the latter scenario, new copies of viral RNA are transcribed and exported outside the nucleus to be translated into the precursors of the viral proteins. Then, the proteins are packaged together with two copies of the viral ssRNA and released outside the cell, where the viral protease will cleave the precursors to form the mature and infectious virion (Modified figure from [32]).

3.3 INTEGRATION

A hallmark of retroviruses and a key step in the HIV-1 replication cycle that enables viral persistence is the integration of the HIV-1 DNA into the host genome. Integration is a multistep process that involves both viral and host factors resulting in a stable and

irreversible positioning of the provirus within the host cell. Integration does not require the viral DNA to be replication competent or even full length, as integration process may proceed with highly deleted genomes. Once the PIC has been imported to the nucleus, the viral IN mediates the integration of the viral DNA by the recognition of specific sequences located into the long terminal repeat (LTR) regions of the dsDNA, generated during the reverse transcription. The integration of the viral DNA can take place in different locations all across the cellular genome, even in sites with low transcriptional activity, where the provirus may remain in a silent/latent state[12,29,33,34]. However, the choice of location of the retrovirus integration site within the host genome is not entirely random, in fact, it has been shown that HIV-1 favors the integration within active genes[35,36].

Upon integration, the HIV-1 provirus persists for the life of the cell, which can last many years, undergo clonal expansion and/or produce replication competent HIV-1. Whereas the mechanism of integration has been well studied from a catalytic point of view (reviewed in[37]), it remains unknown how integration site selection and transcription are linked. Integration of HIV-1 into long-lived cells represents an intrinsic characteristic that is central to HIV-1 persistence and therefore a major barrier to an HIV-1 cure or control strategy[38].

Proviral DNA can also be found in the nucleus as linear or circular unintegrated viral DNA[39]. In the latter case, circular unintegrated provirus can be generated either by the direct union of the ends of the linear provirus, generating a circular provirus with two LTRs (2-LTR circle), or by the homologous recombination of the two viral LTRs, generating a circular provirus with only one LTR (1-LTR circle). Circular forms are not replicated as the cell divides, are diluted out upon cell replication and do not contribute to ongoing viral replication.

3.4 TRANSCRIPTION, NUCLEAR EXPORT AND TRANSLATION

After the HIV-1 provirus is integrated into the host genome, it depends on host RNA polymerase II (RNAPII) for transcription, a critical step in the viral life cycle as viral mRNA is both the template for the synthesis of viral proteins and the genome for progeny viruses. However, the HIV-1 provirus also depends on both cellular and viral factors for

the transcription of its genome aside from the RNAPII, contrary to other viruses that depend entirely on host cell machinery for synthesizing new virions. Robust transcription assures that sufficient mRNA and genomic RNA are produced for efficient virus assembly and infectivity. Repression of HIV transcription leads to the establishment of HIV-1 latency.

The dominant HIV-1 transcriptional regulatory element is the 5' LTR. The HIV-1 LTR is divided into four functional elements: the trans-activation response (TAR) element, the promoter, the enhancer and the modulatory regulatory element. The promoter, enhancer and modulatory elements recruit a plethora of host transcription factors, such as AP-1, Sp1 and NF-κB, which function as activators, repressors or adapter proteins (reviewed in[40,41]).

The RNAPII-mediated transcription can be subdivided into several interconnected stages that include pre-initiation, initiation, promoter clearance, elongation and termination[42]. First, cellular transcription factors are recruited to LTR elements and initiation complex forms at the transcriptional start site. At this point, consequence of a condensed chromatin structure, transcriptional elongation is impeded and RNAPII processes a short distance downstream from the transcriptional start site. Histone deacetylases (HDACs) recruitment to the paused complex reinforces a transcriptionally repressed chromatin state. During this early transcription phase, small mRNAs are transcribed and exported to the cytoplasm to be translated as the regulatory proteins Rev or Tat. Tat dramatically enhances the efficiency of RNAPII elongation by directly interacting with the TAR element, a stem-loop structure located at the 5' end of all nascent viral transcripts and synthesized by RNAPII just after the initiation of transcription[43,44]. RNAPII elongation complex is released from the transcriptional pause by the recruitment of positive transcription elongation factor b (P-TEFb). P-TEFb is a heterodimer composed of the cyclin dependent kinase 9 (CDK9) and its regulatory cyclin T1 and, once positioned next to the RNAPII, mediates distinct phosphorylation events that finally lead to the release of RNAPII from promoter-proximal pausing and the production of full length HIV-1 transcript[45,46]. Finally, the recruitment of chromatin remodeling machinery facilitates acetylation of histones, which displaces the blocking nucleosomes and supports

transcription elongation. In the late transcription phase, long unspliced and single-spliced RNAs are generated, which will be exported to the cytoplasm either as viral ssRNA or as the mRNA coding for the precursors of the structural proteins of the virion[41].

3.5 ASSEMBLY, BUDDING AND MATURATION

After translation, the immature precursors Env and Gag are transported independently to certain regions of the plasmatic membrane by palmitoylation and myristoylation signals[47,48]. As an integral membrane protein, Env is integrated into the endoplasmic reticulum (ER) while it is being translated and delivered to the outer face of the plasmatic membrane through vesicular transport. During this process, the Env precursor is processed by the cellular protease furin into the two subunits of the Env spike, gp41 and gp120. Meanwhile, Gag associates to the inner face of the cell membrane and multimerizes with itself to start forming the spherical and immature capsid around the two copies of viral ssRNA and the enzymes RT, IN and PR. After the assembly of the viral components, the immature virion will be released from the plasma membrane by the cellular endosomal sorting complexes required for transport (ESCRT) machinery. It is during the budding process that the virion may incorporate cellular proteins from the plasmatic membrane to its viral membrane. Finally, after the activation of the viral protease, the PR will recognize and cleave the Gag precursor at specific cleavage sites, a process that will mediate the capsid structural change to its conical and mature structure **(Figure 3).** Once the process of maturation is completed, the new mature virion will be able to infect other target cells[49,50].

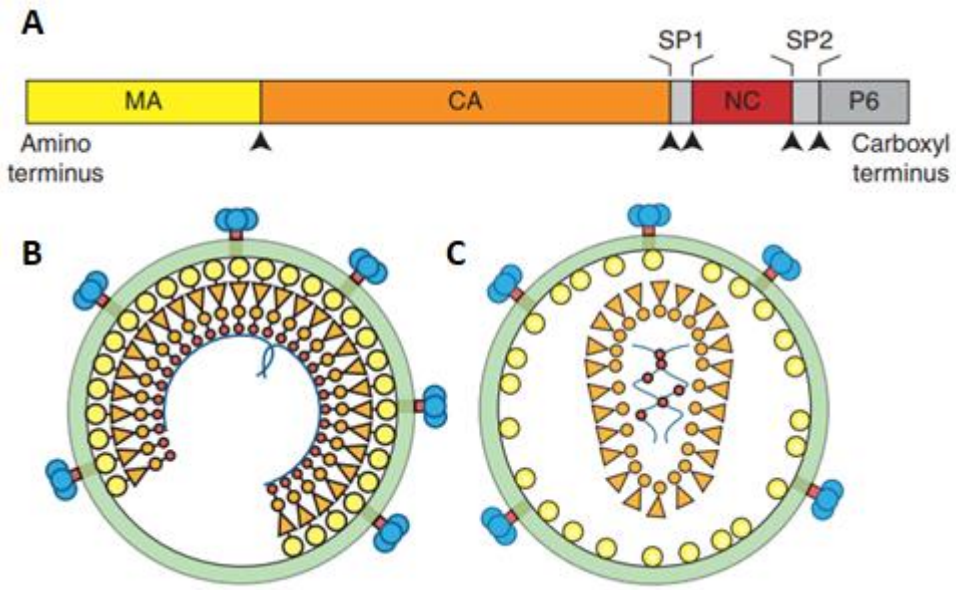

Figure 3. Gag cleavage and HIV-1 virion maturation. (A) Structure of the HIV-1 Gag precursor. Arrows indicate the PR cleavage sites. (B, C) Models of the immature (B) and mature (C) HIV-1 virion (Modified figure from [49]).

4. INNATE IMMUNITY AGAINST HIV-1 INFECTION

The immune system protects the host from infections by either eliminating the invading pathogens or by reducing the negative impact of infections on host fitness[51]. The immune system comprises molecules, cells, tissues and organs which serve as the host defense against pathogens. This defense can be divided into two components, the innate and adaptive immunity, each one with their own functions and roles, although they interact and collaborate in order to generate a global immune response.

To be able to infect a cell and start its viral cycle, HIV-1 has to bypass the body's first line of defense: the innate immune system. Unlike the adaptive immunity, which constitutes a specific response generated against pathogens that have already been recognized by the immune system, innate immunity relies mainly on the ability to recognize pathogen-associated molecular patterns (PAMPs), evolutionary conserved motifs common to large

classes of infectious agents but often absent in eukaryotic organisms, and develop a response against them.

The innate immune system consists of physical barriers (epithelia of skin, gastrointestinal, respiratory and genitourinary tracts), antimicrobial peptides and proteins, humoral components (the complement system and opsonins) and cellular components with phagocytic and antigen presenting properties (mainly monocytes/macrophages, dendritic cells (DCs) and natural killer (NK) cells). Macrophages and dendritic cells express pattern recognition receptors (PRRs) that respond to PAMPs.

Innate immunity is initiated within hours after HIV-1 infection and provides a rapid array of defenses, whereas the antigen-specific adaptive immune responses are induced during the first weeks after infection. Furthermore, selected innate responses, such as the activation of DCs, initiation of antigen processing, migration to lymph nodes, up-regulation of co-stimulatory molecules and the composition of the early cytokine profiles to shape downstream responses, are the essential initial steps in the induction of adaptive immunity. Although there is a detailed understanding of innate immune general functions during acute primary viral infections, much remains to be learned about how the innate and adaptive arms interact under conditions of sustained viral burdens.

Defects in innate immunity are associated with invasive, life-threatening infections; however, inappropriate activation of the innate immune system can lead to autoimmune diseases.

4.1 PATTERN RECOGNITION RECEPTORS

Viruses are detected by the innate immune system primarily by recognition of viral nucleic acids. Recognition of this PAMPs allows the innate immune system to respond immediately[52,53]. The molecules responsible for the recognition of PAMPs are the PRRs, like the Toll-like receptors (TLRs) that are expressed in the plasmatic and endosomal membranes, or the retinoid acid-inducible gene (RIG)-like receptors (RLRs) and cytosolic

DNA receptors (CDRs) that are able to recognize nucleic acids in the cytoplasm (**Figure 4**).

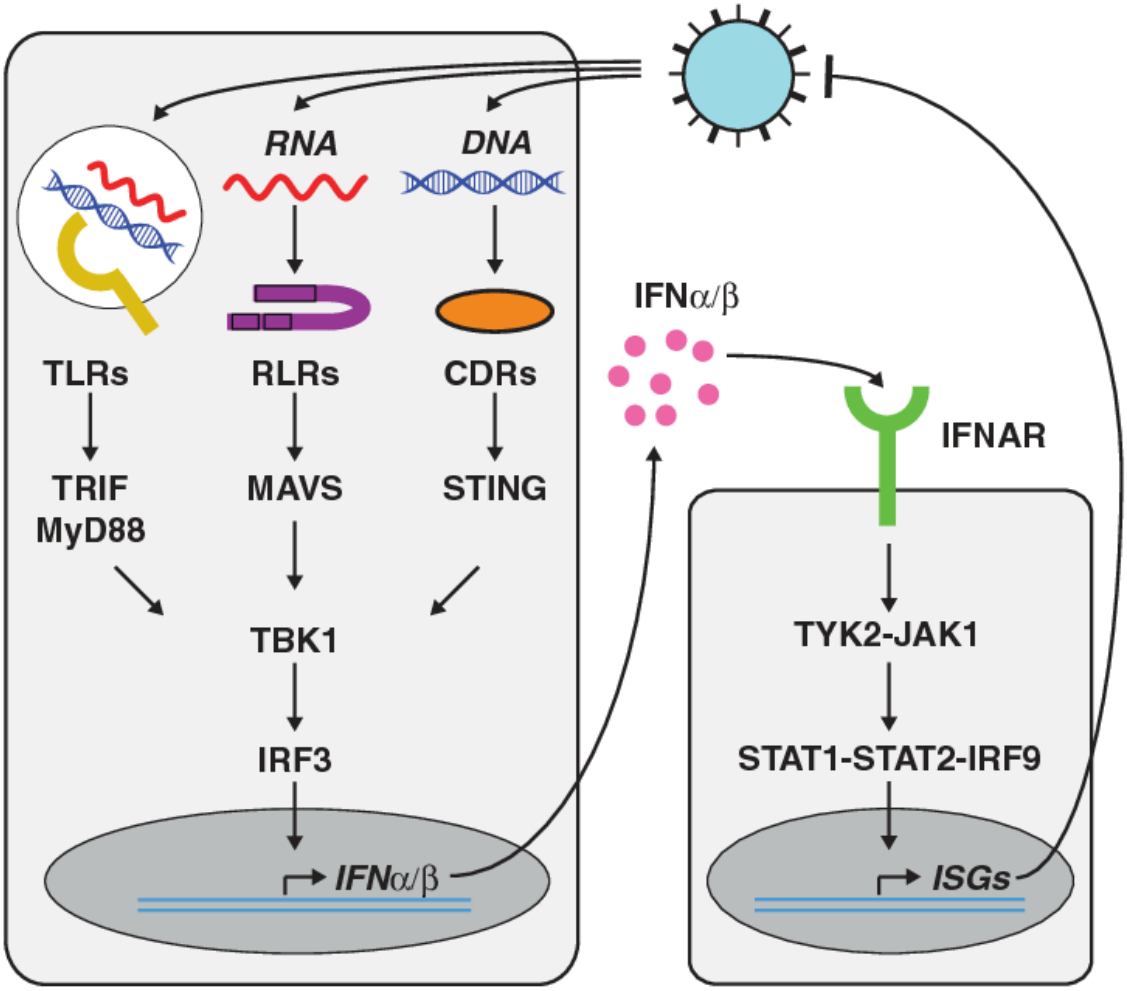

Figure 4. Innate immune sensing of viruses. Innate immune system is able to recognize viral pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs), including RLRs like RIG-I and CDRs like cGAS. Recognition by PRRs leads to the activation of innate immune pathways following the production of type I interferon (IFN), which in turn enhances the transcription of interferon-stimulated genes (ISGs) and host restriction factors that counteract or restrict viral replication, and induces the apoptosis of the infected cells (Figure modified from[54]).

The concerted actions of PRR signaling, specific viral-restriction factors, innate immune cells, innate-adaptive immune crosstalk and viral evasion strategies determine the outcome of HIV-1 infection and immune responses (reviewed in[55]). For HIV-1 infection, pathogen sensing and innate immune induction typically occur in CD4+ cells, including innate immune cells and T cells. Virus-host interactions at mucosal sites of virus

exposure and in lymphoid tissues mediate innate immune activation to determine outcomes of immune responses, virus control, inflammation and immune pathology, including the death of CD4+ T cells. Several host proteins have been identified as PRRs for HIV-1 PAMPs, including various TLRs and RLR. Each likely has a role in inducing, amplifying or differentiating the innate immune response and immune activation to HIV-1 (reviewed in[56]). Studies of the infection and replication cycle of HIV showed that HIV-1 infection is sensed in infected cells through the recognition of viral reverse transcriptase products early in the viral replication cycle by at least two additional intracellular PRRs, interferon inducible protein 16 (IFI16) and cyclic GMP-AMP synthase (cGAS) and that HIV-1 replication is highly sensitive to restriction by innate immune actions of the host cells[57,58].

PRR signaling activates downstream pathways that end up with the induction of inflammatory cytokines and type I interferon (IFN I), a process mediated by the transcription factors IRF3, IRF7 and NF-κB. IFN signaling leads to the induction of hundreds of interferon-stimulated genes (ISGs), many with antiviral properties[59] or even able to induce the death of the affected cells[60–62]. Among the antiviral proteins induced as a consequence of innate immune activation are the so-called restriction factors, such as APOBEC3, TRIM5α, SAMHD1 and tetherin, which limit HIV-1 replication and spread. Restriction factors have been the focus of intensive research in the HIV-1 field (reviewed in [63–65]) due to their intrinsic antiviral properties and the link with innate immunity.

In summary, it has been shown that the innate immune pathway is able to respond to and modulate HIV-1 infection. Therefore, in recent years, therapeutic strategies involving the activation of pathways like retinoic acid-inducible gene I (RIG-I)[66] or TLRs[67] by chemical compounds have been proposed as a way to enhance the specific clearance of HIV-1 infected cells.

4.2 HOST RESTRICTION FACTORS

Host restriction factors are cellular factors that counteract or restrict viral replication, including HIV-1[65]. For a host protein to be considered a restriction factor, it must present at least the following characteristics[68]: (1) it directly decreases viral infectivity, (2) virus have developed a mechanism to counteract its function, (3) it shows evolutionary

signatures of positive genetic selection, to escape the counteracting mechanism, and (4) its expression is linked to the innate immune response. Currently, several HIV-1 restriction factors have been described, albeit the detail on the description of their mode of action and the specific contribution to HIV pathogenesis differs between them[63–65]. One of the restriction factors that has been the focus of intensive research in the last years is the sterile alpha motif (SAM) histidine-aspartic (HD) domain protein 1 (SAMHD1) (**Figure 5**).

SAMHD1 is a deoxynucleotide triphosphohydrolase that converts deoxynucleoside triphosphates (dNTPs) to deoxynucleosides and inorganic triphosphate, controlling the size of the intracellular dNTP pool. The phosphohydrolase activity of SAMHD1 is only active in its homotetrameric form[69], in a nucleotide-dependent manner. SAMHD1 monomer contains two allosteric sites that bind nucleotides, AL1 and AL2. Nucleotide binding in AL1 and AL2 stabilizes the dimerization and tetramerization, respectively, with AL1 having a preference for GTP or dGTP[70,71] while AL2 is able to bind the four dNTPs, exhibiting preference for dATP[72]. Albeit SAMHD1 is ubiquitously expressed, the requirement of dNTPs differs significantly depending on the cell cycle, an observation that prompted the discovery of SAMHD1 regulation through post-transcriptional mechanisms. In fact, phosphorylation and inactivation of SAMHD1 by cell cycle cyclin-dependent kinases (CDK) has been described[73–75]; however, how the phosphorylation regulates SAMHD1 activity is still under debate[76,77].

During HIV-1 infection, viral RNA is reverse transcribed into cDNA, a process that is dependent on the availability of dNTPs that is, in turn, controlled by SAMHD1 dNTPase activity. As a result of that, SAMHD1 has been proposed as a HIV-1 restriction factor in non-cycling cells, including myeloid cells[78] and resting CD4+ T cells[79,80] by depleting the cellular dNTP levels, inhibiting HIV-1 reverse transcription and, consequently restricting HIV-1 infection. This mechanism is supported by the fact that the addition of exogenous dNTPs is able to bypass the SAMHD1-dependent HIV-1 restriction[81]. Unlike HIV-1, HIV-2 has evolved a mechanism to escape of SAMHD1-dependent restriction using the accessory protein Vpx, and thus may be able to replicate in a more efficient way in myeloid and resting CD4+ T cells than HIV-1. Vpx interacts with SAMHD1 recruiting it to

the E3 ubiquitin ligase complex that finally leads to SAMHD1 degradation in a proteasome-dependent manner[78,82,83].

In addition to the role of SAMHD1 as a viral restriction factor, mutations in SAMHD1 have been linked to the auto-inflammatory disease Aicardi-Goutières syndrome (AGS), characterized by an IFN upregulation and an IFN-stimulated gene expression signature resembling a congenital infection[84]. This suggests a link between SAMHD1 and innate immunity, as SAMHD1 inhibits dNTP accumulation and its subsequent recognition by innate immune sensors which would, in turn, trigger IFN production and chronic inflammation[85]. More recently, novel functions have also been proposed[76], involving SAMHD1 in the DNA damage response (DDR)[86], the stress response[87] and the innate immune response through NF-κB and IFN pathways[88].

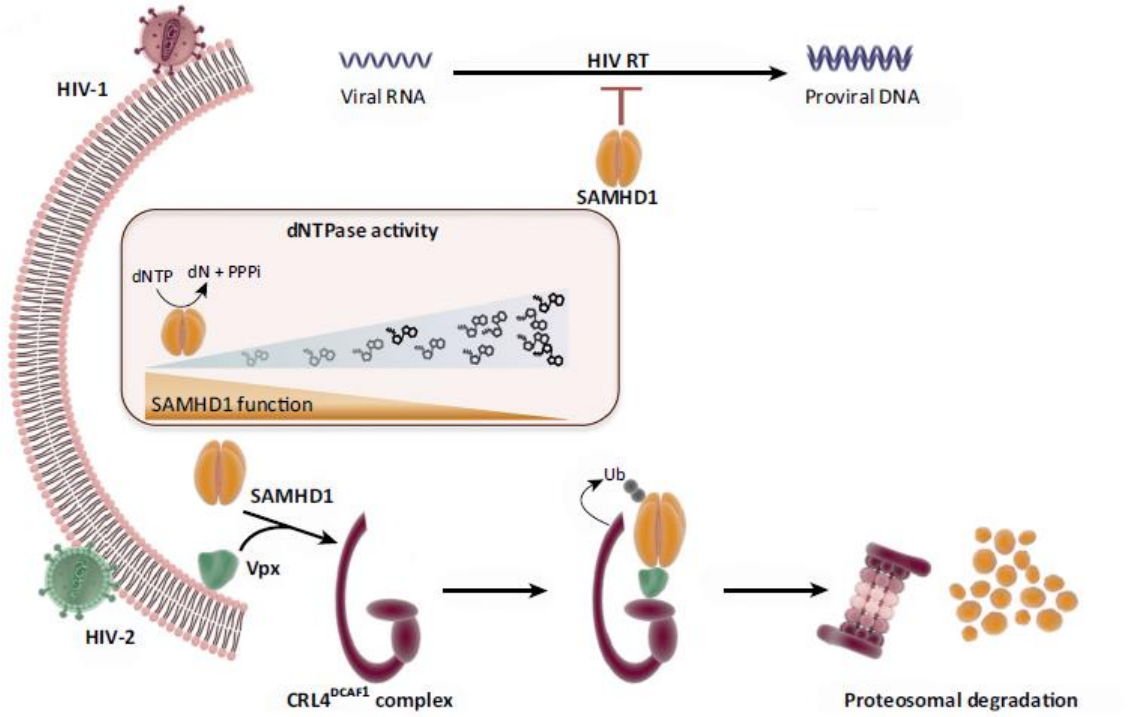

Figure 5. SAMHD1-mediated HIV-1 restriction. Proposed model for SAMHD1-dependent restriction of HIV-1. HIV-2 vpx promotes SAMHD1 degradation by recruiting it to the E3 ligase complex (CRL4DCAF1), inducing its ubiquitination and degradation by the proteasome (Modified figure from [77]).

5. PATHOGENESIS OF HIV-1 INFECTION

HIV-1 is not able to survive outside the bloodstream or the lymphatic fluid and, thus, requires the direct exposure of infected blood or fluids for an effective transmission. In the infection by sexual intercourse, the most common transmission route, HIV-1 has to cross the mucosal epithelium, a process that may involve distinct mechanisms such as transcytosis, infection of the epithelial cells or uptake by the Langerhans cells of the epithelium[89]. After crossing this physical barrier, HIV-1 is free to infect dendritic cells, CD4+ T cells or macrophages from the mucosa that will, in turn, spread the infection through the lymph nodes and, finally, to the bloodstream. The natural course of infection of HIV-1 can be classified in 3 stages: (A) Acute infection; (B) Chronic phase and (C) Acquired Immunodeficiency Syndrome (AIDS) (**Figure 6**).

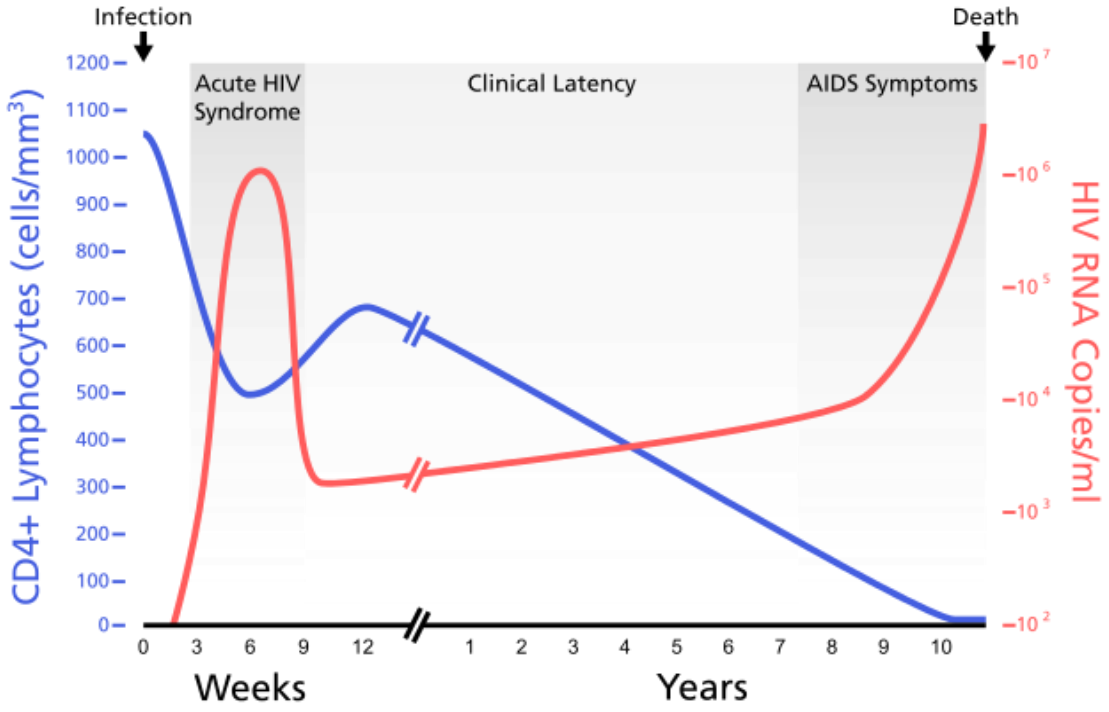

Figure 6. Typical course of HIV-1 infection. In the absence of treatment, HIV-1 infection can be classified in 3 phases: (1) Acute infection, characterized by a sudden increase on HIV-1 viremia and a decrease of the CD4+ T cell count; (2) Chronic phase, characterized by a decrease of HIV-1 viremia due to the host immune system, and a partial recovery of the CD4+ T cells at the beginning, it can last for years, though CD4+ T count will decrease steadily without treatment; and (3) AIDS phase, characterized by a low CD4+ T count, a raise in HIV-1 viremia and the apparition of opportunistic infections, which will ultimately lead to the death of the infected individual[90].

5.1 ACUTE INFECTION

After the initial exposure and transmission, the virus infects and replicates in the cells of the lymphatic tissue and lymph nodes[91] before reaching the bloodstream. Those infected cells can either actively replicate the virus, contributing to the dissemination of the infection, or establish a latent infection, constituting the first cells of the HIV-1 latent reservoir[92]. During this early infection, there is a gap of 10 to 12 days where the virus cannot be detected in the plasma, the eclipse phase[93], followed by a rapid increase of viral RNA in the blood (10^5 – 10^9 copies/mL). This increase in viremia is accompanied by a decrease of the immune CD4+ T cells, which is maintained until the body is able to generate an immune response to partially control the viremia.

During this phase, infected patients may present certain symptoms including fever, fatigue, rash, myalgia, lymphadenopathy and oral or genital ulcers. Although these symptoms might last for more than 10 days, the duration rarely exceeds 14 days[94].

5.2 CHRONIC PHASE

At the beginning of the chronic phase of HIV-1 infection (also called asymptomatic HIV-1 infection or clinical latency) there is a decrease in the viral load (VL) driven by the humoral and cellular immune responses, including the generation of antibodies and the activity of CD8+ cytotoxic T lymphocytes (CTL)[95,96]. The VL keeps decreasing until it reaches a steady level, the viral set point, usually within 2 months after the primary exposure to HIV-1[97]. The establishment of this viral set point is also accompanied by an increase of the CD4+ T cells, although at lower levels than those present before the infection.

During this asymptomatic infection, HIV-1 establishes a low but persistent infection, leading to a chronic HIV-associated systemic inflammation and immune activation. As the HIV-1 infection persists, peaks of viremia can be detected even without the presence of HIV-related symptoms. The persistent infection also leads to a steady decrease of the CD4+ T cell count, weakening the immune system, which marks the end of the clinical latency. At this point, viral replication and chronic activation of immune cells promote the destruction of the lymphoid tissue architecture, which leads to an increased viral

replication and diffusion, and the subsequent depletion of CD4+ T cells. There are some patients, however, that are able to control the infection, having a non-detectable viremia for years: the so-called "elite controllers"[98].

5.3 ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS)

The last stage of HIV-1 infection is characterized by extremely low levels of CD4+ T cells (<200 cells/µL) in blood and the dramatic increase of the VL. The CD4+ T cell count can even reach very low levels (up to 0 cells/µL), being the lowest count the CD4+ nadir. During this phase, the organism becomes susceptible to opportunistic infections by other viruses, bacteria, fungi and parasites, and tumors, which define the AIDS stage[12,99].

Typical progressors may reach the AIDS phase 8-10 years after HIV-1 primary infection in the absence of therapy. However, there are records of non-typical progressors who can reach this phase earlier or later[100].

5.4 PATHOGENESIS IN THE PRESENCE OF ANTIRETROVIRAL THERAPY

Since the FDA approval of the first antiretroviral compound, AZT[7], in 1987, advances in antiretroviral therapy (ART) have changed the perspective of HIV-1 infection from a lethal illness to a somewhat manageable chronic disease[101]. Nowadays, the use of combination therapy suppresses viral load below the limit of detection (LOD) (<50 copies of viral RNA/mL) following a four-phase decay[102,103] (**Figure 7**):

- Phase I: Initial dramatic decrease caused by the inhibition of free virus and the clearance of infected activated CD4+ T cells susceptible to viral cytopathic effects or host cytolytic effector mechanisms.
- Phase II: Less dramatic decrease than in phase I, result of the clearance of infected cells that are more resistant to cytopathic effects or apoptosis, or that have a longer half-life, like macrophages or partially activated CD4+ T cells.
- Phase III: An even slower decrease due to the clearance of cells with even longer half-lives. During this phase, viral RNA in blood is already below LOD.
- Phase IV: Final phase with stable levels of viremia below LOD. Ideally, this phase should last for an unlimited period of time.

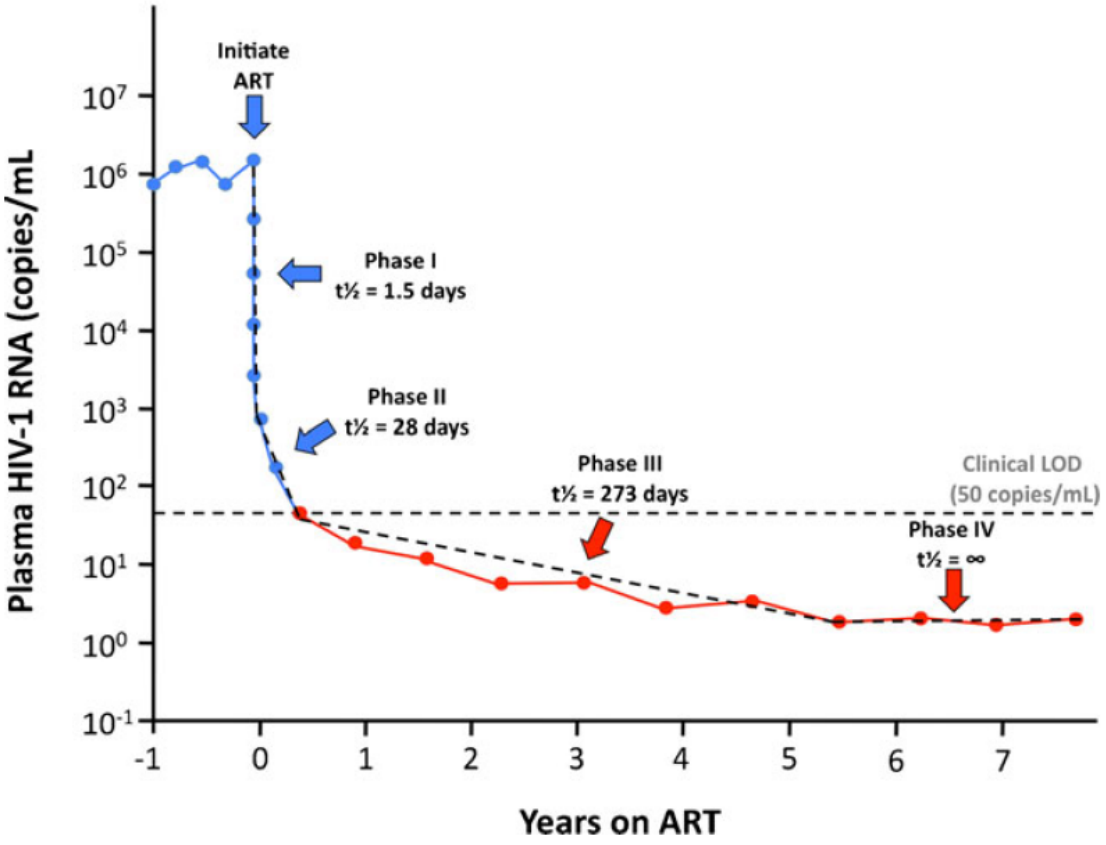

Figure 7. Four-phase decay of viremia in ART. Levels of HIV-1 RNA in the blood (copies/mL) after ART initiation. The decrease in viremia can be classified in four phases depending on the half-life of the infected cells that are being cleared[102].

This decreased replication allows the treated individuals to control viremia, delay disease progression, prevent transmission and partially recover the CD4+ T cell count indefinitely[104]. However, upon treatment failure, virus replication increases again and CD4+ T cell count plummets, as observed in the HIV-1 acute phase. Treatment failure is caused by the acquired resistance to treatment due to the high mutagenesis rate of the RT, resulting in the appearance of resistant quasispecies in the presence of ART[105]. In an effort to avoid this drug resistance, current combination therapy uses three antiretrovirals targeting at least two different steps of the viral cycle, thus, the chances of a virus to evolve and become resistant to the three drugs are decreased[106].

Depending on their molecular targets, current HIV-1 antiretrovirals can be classified into seven main classes:

- Nucleoside reverse transcriptase inhibitors (NRTIs)
- Non-nucleoside reverse transcriptase inhibitors (NNRTIs)
- Integrase strand transfer inhibitors (INSTIs)
- Protease inhibitors (PIs)
- Fusion inhibitors
- CCR5 antagonists
- Post-attachment inhibitors (PAIs)

Although current ART is able to successfully control viral replication, it is unable to target those cells in which HIV-1 remains silent, the HIV-1 latently infected cells[107]. Due to this incomplete clearance of the infection, HIV-1 is able to rebound after ART discontinuation, independently of the time spent under treatment. Therefore, in order to grant a near-normal lifestyle for the individuals in treatment, ART needs to be taken as a lifelong treatment, with the associated toxicities and adverse events that come along with it[108].

6. THE LATENT RESERVOIR

During infection, there is a population of long-lived cells that contain transcriptionally silenced replicating-competent virus. These cells constitute the HIV-1 latent reservoir, representing the main barrier to achieve a cure for HIV-1 infection, and one of the reasons behind the viral rebound after ART interruption[109–111]. HIV-1 latency can be classified into two categories depending on whether the proviral DNA has been integrated or not into the host genome[103,112–114]:

- Pre-insertion latency: This type of latency occurs when the HIV-1 viral cycle is stopped before the integration of the provirus into the host genome. Several authors[103,113] claim that this may happen due to unfinished reverse transcription, due to the lack of ATP for the nuclear import or due to the effect of host restriction factors. Upon T cell activation, this unintegrated DNA can integrate into the host genome and lead to a productive infection[115]. This type of latency does not seem to play a relevant role for HIV-1 persistence in T cells, as it is only

able to remain in there for a short period of time[113]; however, it may be relevant in non-dividing macrophages, where unintegrated DNA is able to last for longer periods[116].

- Post-insertion latency: viral cDNA integrates into the host genome; however, viral transcription is suppressed and therefore, the virus is not able to replicate and generate new virions. In post-insertion latency, the provirus is stable and its half-life is the same as that of the infected cell, being able to be duplicated along with the rest of the cell genome during mitosis, too. Given its stability, post-insertion latency seems to play a major role in HIV-1 persistence, and thus, in this dissertation, it is going to be referred to as latency from hereafter.

The HIV-1 latent reservoir is preferentially formed by HIV-1 target cells, i. e. CD4+ cells, and in particular CD4+ T cells. The first cells described as part of the HIV-1 latent reservoir were CD4+ resting T cells. CD4+ T naïve cells are long-lived cells at the earliest stage of differentiation that, after antigen presentation of pathogens like HIV-1, are able to proliferate and differentiate into T helper or T regulatory effector cells, transcriptional active cells that have a short half-life. Most of those effector T cells will die during the immune response, but some of them differentiate to long-lived and non-dividing memory T cells. If those cells are infected during their phase as T helper cells, reverting back to a resting stage as memory T cells would allow the silencing of any integrated provirus, inducing HIV-1 silencing and thus becoming part of the HIV-1 latent reservoir[117]. Resting memory T cells represent the best studied cellular subset of the HIV-1 latent reservoir and have been reported to account for the major part of the reservoir[118]. However, other subsets of T cells have also been reported to contribute to HIV-1 persistence, including T cells with stem-like properties (CD4+ T memory stem cells, T_{SCM})[119] and T follicular helper cells (Tfh)[120] either from the germinal centers or circulating in the blood (peripheral Tfh)[121]. In addition to CD4+ T cells, other cells including monocyte-derived macrophages and dendritic cells can also become part of the HIV-1 reservoir[122]. Macrophages are long-lived cells that are highly resistant to cytopathic effects and apoptosis, also the viral cycle in those cells is 6 times slower than in T cells, which makes the macrophages a long-lasting HIV-1 reservoir[112,123]. Dendritic cells and follicular dendritic cells (FDCs) have also been reported to contribute in HIV-1

persistence and propagation by capturing virus on their surface that, in the case of FDCs, can remain infectious for at least 9 months[117,124,125].

The CD4+ memory T cells reservoir is estimated to have a half-life of 44 months. Thus, the complete clearance of the HIV-1 latent reservoir under ART may take more than 60 years[126]. During this time, patients should go under an uninterrupted treatment, as HIV-1 can spontaneously become active in latently infected cells, promoting the generation of new virions that, in the absence of ART, will infect other cells and result in viral rebound. Thus, it is clear that the HIV-1 latent reservoir is a major hurdle to achieve a cure for HIV-1.

Organs and tissues also contribute in HIV-1 persistence through several proposed mechanisms that include being the source of cells containing latent HIV-1, allowing viral propagation due to suboptimal drug levels in these tissue reservoirs[127] and/or delaying the immune-mediated elimination of infected cells while acting as immune privileged sites[128,129]. Peripheral blood constitutes the most studied and understood anatomical reservoir, as it contains several memory T subsets, however other reservoirs also comprise the central nervous system (CNS), gut associated lymphoid tissue (GALT), lymphoid organs (lymph nodes, spleen, thymus and bone marrow), liver, lungs, kidneys, skin, adipose tissue and genital tract (reviewed in[130,131]).

Latency and the latent reservoir is thought to be established very early, during the acute phase of HIV-1 infection[132,133]. Several mechanisms may play a role in the establishment and persistence of latent provirus, including: (1) site and orientation of the integrated provirus, (2) chromatin organization and epigenetic regulation, (3) availability of cell transcription factors and viral proteins, (4) cellular transcriptional repressors and restriction factors and (5) RNAi and microRNAs.

Site and orientation of the integrated provirus: Contrary to what was previously thought, HIV-1 does not integrate in a random manner into the host genome. In fact, most of the integrated provirus are found in intronic regions of transcriptionally active genes[36,134], maybe due to the accessibility of regions with active transcriptional activity. As contradictory as it may seem, latent provirus can be found in these regions too, however, its transcription may be repressed due to transcriptional interference (TI) led

by cellular genes located in the vicinity of the proviral integration site. If the provirus is integrated in parallel and close to a cellular gene, the transcription of the latter from an upstream promoter may lead to the displacement of key transcription factors from HIV-1 promoter, repressing viral replication. However, if the provirus is integrated in the opposite direction to a cellular gene, divergent orientation between the two promoters may lead to a deficit in the recruitment of transcription factors for the HIV-1 promoter, while convergent orientation may lead to a premature termination of the transcription due to the collision of the two RNAPII complexes during elongation[32,103,112].

Chromatin organization and epigenetic regulation: To ensure an efficient storage of the genetic information, DNA is packaged within the chromatin in the nucleosomes, the chromatin structural units that contain DNA and histones. Chromatin condensation is able to regulate gene expression, allowing the access of the transcription factors into loose chromatin (euchromatin) and restricting the transcription in condensed areas (heterochromatin). A dynamic process is required to modulate the condensation state of the chromatin, and thus control the expression or repression of cellular and/or viral genes, a process that involves posttranslational modifications (PTMs) of different DNA-associated proteins, like histones. Histone modifications are reversible and include acetylation, methylation, phosphorylation, sumoylation, ADP-ribosylation and ubiquitination of the C- and N- terminal tails of the histones, although the most studied ones in the field of HIV-1 latency are the histone acetylation and methylation. In the case of the latter, the effect upon gene transcription may vary depending on the site of modification. On the other hand, histone hyperacetylation is usually associated with active transcription while histone hypoacetylation induced by histone deacetylases (HDACs) is associated with gene repression and HIV-1 latency[32,103,112,114,135].

Two nucleosomes, named nuc-0 and nuc-1, are known to be positioned in the HIV-1 5'LTR of latently infected cell lines, blocking HIV-1 transcription[136]. These nucleosomes are susceptible to PTMs. In fact, specific histone acetylation and remodelling of nuc-1 induced by HDAC inhibitors (HDACi) has been shown to induce the transcription of latent provirus[137], which may indicate that in HIV-1 latency nuc-1 is constitutively deacetylated.

Availability of cell transcription factors and viral proteins: 5'LTR serves as the HIV-1 promoter and is able to recruit cellular factors in order to initiate HIV-1 transcription. In activated cells, these factors are available as the cells need them for the transcription of cellular genes; however, in resting cells, those factors are sequestered in the cytoplasm, inhibiting transcription. Thus, HIV-1 provirus is able to undergo latency if an activated cell returns to a resting state after infection. Besides the cellular factors, HIV-1 transcription can also be modified by viral proteins like Tat, which is required for the elongation of viral transcripts and whose inhibition can lead to viral latency in a repressive chromatin environment.

Cellular transcriptional repressors and restriction factors: As HIV-1 requires the cellular machinery for its transcription, it is susceptible to the inhibition of cellular transcription factors. That is the case of the transcription factor NF-κB, which is able to bind the HIV-1 5'LTR, and its inhibitor, IκBα. IκBα sequesters the dimers of the transcription factor in the cytoplasm, leading to HIV-1 transcription inhibition and latency induction[138]. Additionally, host restriction factors are also able to induce HIV-1 latency by inhibiting the viral cycle. As an example, TRIM22 can lead to the suppression of HIV-1 transcription by preventing the binding of the transcription factor Sp1 to the HIV-1 promoter, and thus, induce HIV-1 latency[139].

RNAi and microRNAs: Additionally to the mechanisms noted above, HIV-1 transcription can also be regulated and silenced by cellular microRNAs (miRNAs) through the RNA-induced silencing complex (RISC). These miRNA are able to regulate gene expression by remodeling the chromatin structure[140], but are also able to directly target HIV-1 transcripts[141] and cell factors required for transcription[142]. The same viral genome is also able to produce viral interference RNA able to target viral RNAs, which would also induce latency[32,143].

Fundamental to bridging knowledge gaps towards HIV-1 eradication is an understanding of the establishment and maintenance of cellular reservoirs and their persistence.

7. THE HIV-1 CURE: CURRENT STRATEGIES

Current ART is not able to eradicate HIV-1 due to the presence of the latent reservoir, as discussed above. Furthermore, HIV-1+ patients need to adhere to a long-term ART that, even in the absence of treatment interruption, can lead to the appearance of drug resistant viruses with the subsequent viral rebound. Long-term ART is also linked to a persistent immune activation and inflammation[144] as well as to toxicities associated with the treatment. Therefore, there is a need to develop novel strategies to achieve an effective HIV-1 cure, defined as a treatment that should be able to induce a sustained remission of the virus after ART discontinuation. Depending on whether it involves a complete eradication of the virus or not, proposed strategies may aim for a (1) functional cure, or a (2) sterilizing cure.

7.1 FUNCTIONAL CURE

This category includes those strategies that aim for the control of HIV-1 replication in the absence of ART, while maintaining the VL under detection levels and a normal CD4+ T cell count. HIV-1 elite controllers, which can maintain viral RNA under detection levels in the absence of ART, are a proof of concept for this type of cure, and are currently being studied for the development of novel therapies. Examples of strategies that were or are being proposed in hopes of achieving a functional cure include:

Early treatment: It has been suggested that starting ART as early as possible could help to reduce the latent reservoir and allow a transient remission in the absence of ART. A clinical case supporting this idea was the “Mississippi baby”, a perinatally infected child that started ART at 30h after birth and underwent treatment until 18 months old. This patient remained undetectable for viral RNA in plasma for more than 2 years without ART, although the virus eventually rebounded. This clinical case, and others, provide evidence that early treatment can impact long-term progression, however, this treatment alone is not an effective cure[145–148].

ART intensification: During ART, there can be residual viral replication[149] due to suboptimal drug concentrations in tissues. In this regard, there have been studies to determine if the residual replication could be inhibited with therapy intensification.

Although it has been studied with several compounds, no conclusive evidence has been found so far[145,150–152].

Antibody-based strategies: There are several therapies involving antibodies proposed as anti-HIV strategies[145,146,153], including broadly neutralizing antibodies (bNAbs) targeting Env to prevent HIV-1 infection[154], nonneutralizing antibodies able to induce antibody-dependent cellular cytotoxicity (ADCC) in the infected cells[155], antibodies that potentiate cytotoxic CD8+ T cells[156], antibodies conjugated with toxins to directly eliminate infected cells[157], or antibodies targeting cellular proteins to limit the access of CD4+ T cells to gastrointestinal tract, where HIV-1 can target them[158]. Although the use of antibodies to treat HIV-1 seems promising, some challenges need to be overcome in order to represent a feasible strategy, including the short half-life and delivery of the antibodies, the manufacturing costs and, in the case of antibodies targeting viral epitopes, the generation of resistant quasispecies.

Block and lock: Contrary to the idea of eradicating the latent reservoir, the block and lock strategy is based on the use of latency-promoting agents (LPAs) to deep silence the latent proviruses as a mean to inhibit spontaneous reactivation and induction of the viral rebound. Strategies aiming to establish deep-latency include the inhibition of viral components that are essential for HIV-1 transcription, like Tat or TAR. In fact, several inhibitors have been studied including the Tat inhibitor dehydrocorticostatin (dCA), which was shown to inhibit HIV-1 reactivation from latently infected cells[159], and delayed by one week the viral rebound in an in vivo assay in combination with ART in a humanized mouse model[160]. Alternatives to Tat and TAR inhibition include the targeting of cell host factors like histone acetyltransferases (HATs) or histone demethylases (HDMs), which can either directly affect viral expression through histone modification or modulate the activity of transcription factors needed for HIV-1 transcription like Tat[161]. Although promising, disadvantages for the block and lock strategy include the rise of escape mutants upon the inhibition of viral components or the lack of specificity and potential toxicity for cell host factors inhibitors. Moreover, in order to achieve a functional cure, it is essential for LPAs to deep silence the whole HIV-1 latent reservoir,

including the infected cells from tissues where current ART is not able to penetrate in optimal doses, therefore further studies are still necessary[162,163].

7.2 STERILIZING CURE

Strategies aiming for a sterilizing cure are those whose objective is the global eradication of the virus in the organism, including the reservoirs. However, as the HIV-1 reservoir is not yet fully understood and current technology is not able to prove if the latent reservoir has been completely eradicated, it is difficult to demonstrate that those cure strategies are really sterilizing rather than functional. In fact, there are disagreements on whether the "Berlin patient" or gene editing strategies, described below, represent a functional or a sterilizing cure. Nevertheless, in this dissertation, those strategies that have a direct impact on the reservoir are considered sterilizing. Sterilizing strategies may include:

Allogeneic stem cell transplantation: The "Berlin patient" was the first case of apparent HIV-1 eradication and therefore, represented the first evidence to the scientific community that HIV-1 cure was possible. Timothy Ray Brown, who was diagnosed with both acute myeloid leukemia and HIV-1 infection, underwent an allogeneic hematopoietic stem cell transplantation (HSCT) in order to replace his immune system and treat the former disease. The donor, however, was screened for homozygosity of the CCR5Δ32 allele, which confers cell resistance towards R5-strains of HIV-1 for their inability to bind the CCR5 co-receptor. Three months after the transplantation, HIV-1 was no longer found in the patient's plasma and now, more than 10 years after the transplantation, HIV-1 levels are still undetectable in the absence of ART[164,165].

Despite the success of the "Berlin patient" case, other attempts to achieve HIV-1 eradication through HSCT have failed, and the patients eventually rebounded[166]. Recently, however, a second case of HIV-1 remission has been described. The "London patient", which underwent a HSCT with cells homozygous for the CCR5Δ32 allele[167] and up to now, HIV-1 RNA levels in plasma have remained undetectable for more than a year. Although this apparent "success" gives hope of achieving a HIV-1 cure through HSCT, it is necessary to bear in mind that this kind of procedure is complex, dangerous, expensive, and requires a compatible donor with the homozygous CCR5Δ32 allele,

making it an unrealistic approach to achieve a cure on a global scale. Aside from that, another limitation of this strategy may be the viral evasion by shifting to a HIV-1 X4-strain[145].

Gene editing: A different approach to achieve resistance to HIV-1 infection would be the direct disruption of the CCR5 receptor with genome-editing techniques in order to generate autologous CCR5-disrupted T cells and infuse them to HIV-1+ patients. Those techniques may take advantage of genome editing techniques such as zinc fingers nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) or clustered regularly interspaced short palindromic repeats/CRISP-associated protein nuclease-9 (CRISP/Cas9)[168–170]. In clinical trials, infusion of CCR5-disrupted cells exhibited a half-life of 48 weeks and the viremia of all the patients evaluated post-ART interruption decreased after a previous viral rebound, although a serious adverse event was reported for one of the participants[171]. Aside from targeting HIV-1 co-receptors, disruption of sequences from the integrated provirus has also been proposed. Studies targeting viral LTR have been reported, being able to remove proviral DNA from latently infected cells and prevent *de novo* infection[172]. Overall, these studies support the use of gene editing as a new therapeutic strategy to treat HIV-1; however, several issues need to be addressed, including the cost and complexity of the procedure, the cytotoxicity and off-target effects, the viral escape and, in the case of the targeting of integrated provirus, the lack of an efficient vector and human experimental data[173].

Shock and kill: One of the causes for the viral rebound is the spontaneous reactivation of latently infected cells, which are not recognized by the host as infected cells. One of the proposed methods to purge this latent reservoir consists in, first, reactivating those cells with latency-reversing agents (LRAs) (**shock**) and, second, eliminating those reactivated cells (**kill**)[174], either due to the immune system or the virus-induced cytolysis (**Figure 8**). First attempts to induce a latency reversal involved the activation of T cells with interleukin-2 (IL-2)[175], showing a decrease in infectious units per million cells (IUPM) but no change in the levels of viral DNA, and IL-2 in combination with anti-CD3 antibodies[176], where serious side-effects were reported and early antibodies against the anti-CD3 antibodies were generated, thus, limiting the stimulatory effect. Until now,

several latency-reversing compounds have been proposed, mainly acting by releasing sequestered cellular transcription factors or by epigenetic modulation[177]. Current LRAs include bromodomain and extraterminal (BET) inhibitors, DNA methyltransferase (DNMT) inhibitors and protein kinase C (PKC) agonists, among others (reviewed in[178]). However, the most studied mechanism for HIV-1 reactivation has been the chromatin remodelation using HDAC inhibitors like vorinostat, panobinostat and romidepsin. Studies with HDACi have demonstrated that they are able to induce viral RNA production *in vivo*[179–182]. However, other studies denied the compound-dependent reduction of the latent reservoir[183–185], highlighting the need to further study and find better LRAs. In fact, several authors propose that total viral reactivation will be the result of a combination of LRAs, instead of a unique holy grail, as specific LRAs seems to be able to reactivate the provirus depending on its insertion site[178,186,187].

In addition to the LRAs that are mainly directed to the "shock" process, there is also a need to further study the "kill" aspect. The premise for the "shock and kill" strategy was that the reactivated cells would be eliminated by HIV-1-induced cytolysis or by cytotoxic T-cell (CTL) response; however, current LRAs seem to lack the ability to reduce the reservoir by themselves. Different hypothesis have been proposed to explain the inability to clear reactivated cells, including the anti-apoptotic activity of certain viral proteins like Tat, Nef and Vpr at early stages of the viral cycle[188], or even the impairment of the CTL response mediated by HDAC inhibitors panobinostat, vorinostat and romidepsin[189], negatively affecting the elimination of the infected cells[177]. All these observations suggest that the use of LRAs alone may not be sufficient to trigger a response potent enough to eliminate the reactivated cells. Thus, in order to enhance the "kill" process, several approaches have been proposed, including the use of antibodies, chimeric antigen receptor (CAR) engineered T cells, therapeutic vaccination and pro-apoptotic compounds like second mitochondria-derived activator of caspases (SMAC) mimetics, Bcl-2 antagonists, PI3K/Akt inhibitors and RIG-I inducers (reviewed in[177,190]).

The use of compounds to treat HIV-1, in comparison to strategies like gene editing or HSCT, would reduce the complexity and cost of the procedure, making it available to a

wider target population. However, there is still a long way to go until "shock and kill" can be considered a feasible strategy to cure HIV-1. Thus, novel LRAs able to reactivate HIV-1 latency in the absence of side-effects or T cell activation are needed, along with additional compounds or strategies able to eliminate the reactivated cells in a specific way. Furthermore, a deeper understanding on the role that the HIV-1 integration sites have on latency reversal is also essential in order to find the right LRA cocktail that would induce HIV-1 reactivation regardless of any genomic location in where the provirus could have been integrated.

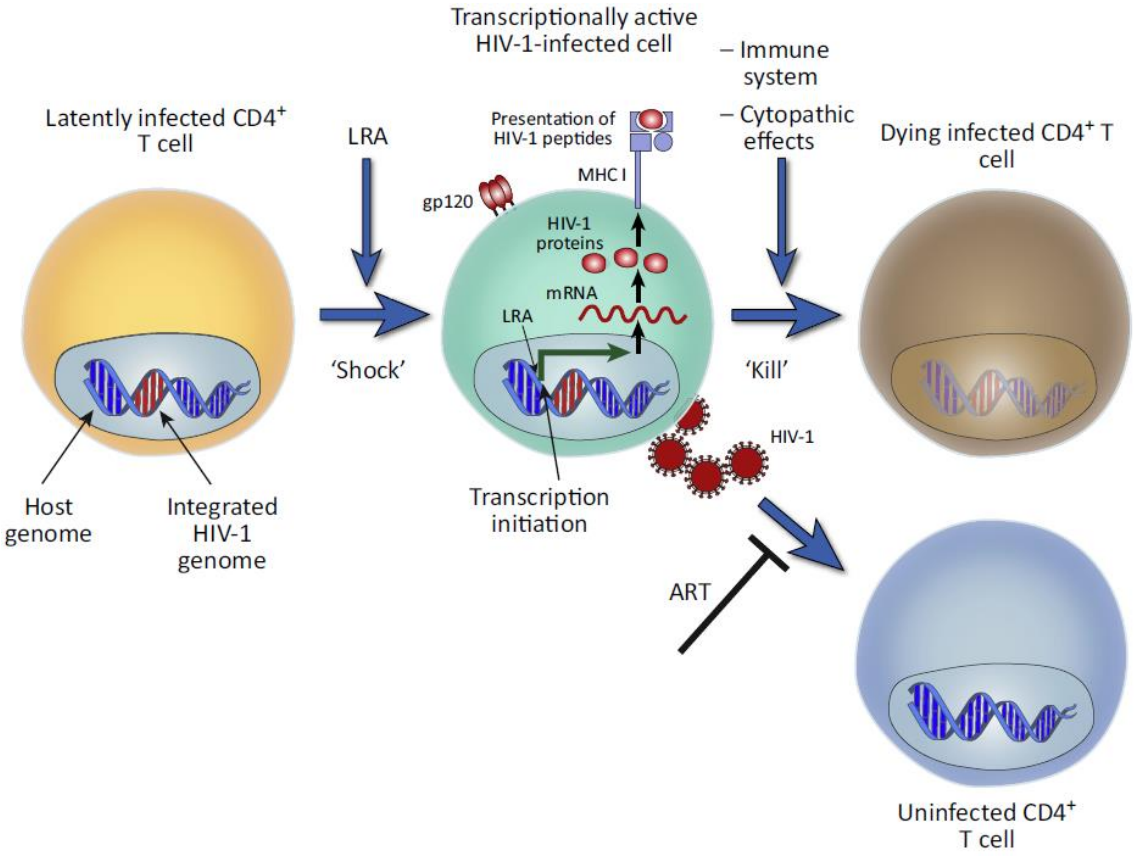

Figure 8. Shock and kill strategy. Schematic representation of the reactivation and elimination of latently infected cells. Latent proviruses become transcriptionally active after the LRA-dependant "shock". After reactivation, the infected cell will produce virions, viral proteins and peptides that will allow the recognition of the immune system, leading to the "kill" of those cells. The presence of ART during the process will inhibit the *de novo* infection of cells by the generated virions[153].

HYPHOTHESIS AND OBJECTIVES

Despite the fact that current combination antiretroviral therapy (ART) effectively suppresses viral replication, HIV-1 remains an incurable viral infection. Moreover, ART discontinuation results in a quick viral rebound due to viral replication from the latent reservoir, which is established very early in the first stages of the infection. Thus, many approaches have been investigated and developed aimed at finding the cure for HIV, either by achieving a complete eradication of HIV-1 reservoirs (sterilizing cure) or a control of the viral replication in the absence of ART (functional cure).

Although promising, current "shock and kill" strategies based on commonly employed LRAs have not succeed in providing a definite answer for HIV cure. To be successful, it might be necessary to: (i) reactivate proviral expression in all infected T cells, targeting a wide distribution of integrational genomic loci, (ii) express minimal side-effects to other cell types, (iii) stimulate cell death mechanisms specific to those infected cells and (iv) the latent reservoir within the resting CD4+ cells should ideally be reactivated without complete T cell activation to avoid toxicity.

Based on the need of novel agents and strategies to achieve an efficient clearance of the latent reservoir, and taking into account the characteristics that the ideal therapy should meet, we hypothesize that agents developed as modulators of innate immune response or designed to modulate the cell cycle progression might represent an alternative to current inducers of HIV-1 reactivation, alone or in combination with already described LRAs.

Hence, the **principal aim** of the present thesis is the evaluation and characterization of novel agents triggering latency reactivation and death of infected cells with the final objective of proposing novel strategies to eliminate the latent HIV-1.

The **specific objectives** of the thesis are:

1. To evaluate the role of innate immune-based strategies in HIV-1 latency reversal and elimination of the viral reservoir
   1.1. To study the capacity of the retinoic acid derivative acitretin to reactivate HIV-1 latency and to induce apoptosis of the infected cells by stimulating the RIG-I pathway

2. To explore the latency reactivation properties of small molecules affecting cell cycle progression
   2.1. To determine the potential of aurora kinase inhibitors as a novel class of LRAs
   2.2. To assess the effect of midostaurin, a multiple kinase inhibitor, in acute and latent HIV-1 infection

RESULTS

CHAPTER 1

EVALUATION OF THE INNATE IMMUNE MODULATOR ACITRETIN AS A STRATEGY TO CLEAR THE HIV RESERVOIR

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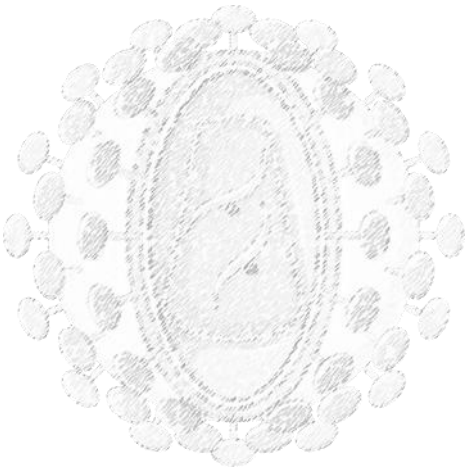

1.1 ABSTRACT

The persistence of HIV despite suppressive antiretroviral therapy is a major roadblock to HIV eradication. Current strategies focused on inducing the expression of latent HIV fail to clear the persistent reservoir, prompting the development of new approaches for killing HIV-positive cells. Recently, acitretin was proposed as a pharmacological enhancer of the innate cellular defense network that led to virus reactivation and preferential death of infected cells. We evaluated the capacity of acitretin to reactivate and/or to facilitate immune-mediated clearance of HIV-positive cells. Acitretin did not induce HIV reactivation in latently infected cell lines (J-Lat and ACH-2). We could observe only modest induction of HIV reactivation by acitretin in latently green fluorescent protein-HIV-infected Jurkat cells, comparable to suboptimal concentrations of vorinostat, a known latency reversing agent (LRA). Acitretin induction was insignificant, however, compared to optimal concentrations of LRAs. Acitretin failed to reactivate HIV in a model of latently infected primary CD4+ T cells but induced retinoic acid-inducible gene I (RIG-I) and mitochondrial antiviral signaling (MAVS) expression in infected and uninfected cells, confirming the role of acitretin as an innate immune modulator. However, this effect was not associated with selective killing of HIV-positive cells. In conclusion, acitretin-mediated stimulation of the RIG-I pathway for HIV reactivation is modest and thus may not meaningfully affect the HIV reservoir. Stimulation of the RIG-I-dependent interferon (IFN) cascade by acitretin may not significantly affect the selective destruction of latently infected HIV-positive cells.

1.2 INTRODUCTION

The use of antiretroviral therapy (ART) has significantly transformed HIV-1 infection from a terminal illness to a chronic manageable disease[101]. Despite intensive investigation, no strategy so far has resulted in sustained control of HIV in the absence of ART, and HIV persists through multiple mechanisms[191]. Thus, the eradication of HIV-1 will require novel approaches to purge the reservoir of latently infected cells from a patient[192,193]. The quest for long-term control of HIV-1 in the absence of ART has led to numerous therapeutic approaches aimed at increasing host-mediated control of HIV

and clearance of latent virus reservoirs[194–196] while maintaining the beneficial effects of immune reconstitution.

HIV infection and recognition by infected cells trigger a signaling cascade that leads to increased activity of interferon (IFN) regulatory factors (IRFs) and production of IFNs and inflammatory cytokines[197]. The innate immune response may also be responsible, in part, for a virus-induced cell death response, either through caspase-1-mediated programmed cell death triggered by abortive viral infection[198] or IFN-induced apoptosis[199,200]. Like others, we have shown that established HIV-1 infection of monocyte-derived cells induces upregulation of the pattern recognition receptors melanoma differentiation-associated protein 5 (MDA-5) and retinoic acid-inducible gene I (RIG-I), production of IFN- , and transcription of IFN-stimulated genes (ISGs)[201,202]. Additionally, HIV-1 infection may limit the deoxynucleotide pool through downregulation of the ribonucleotide reductase subunit R2 (RNR2) and reactivation of the virus restriction factor SAMHD1[77,203] together with increased cell death[201], mediated in part by the HIV-1 Vpr[204,205].

Pharmacological stimulation of the RIG-I pathway has been proposed as an alternative mechanism to kill cells in the latent HIV reservoir, following viral reactivation. Enhancement of RIG-I signaling ex vivo was shown to increase HIV transcription and to induce preferential apoptosis of HIV-infected cells[66], recapitulating the effect observed with chronic infection of macrophages[201]. Li et al.[66] showed that the retinoic acid derivative acitretin enhanced RIG-I signaling ex vivo, increased HIV transcription, and induced preferential apoptosis of HIV-infected cells. Acitretin is an oral retinoid that may be used in the treatment of severe resistant psoriasis in HIV-positive individuals[206], suggesting that treatments to revert HIV latency and potentially to eliminate the virus reservoir are already available and thus require further careful examination.

Here, we have explored the effects of acitretin in HIV infection, hoping to confirm and to expand the observations made by Li et al.[66]. We found that acitretin-mediated stimulation of the RIG-I pathway over HIV reactivation was modest and lacked selective destruction of HIV-positive cells; therefore, it may not meaningfully affect the HIV reservoir.

1.3 MATERIALS AND METHODS

1.3.1 Viruses and cells

The HIV-1 viral strain NL4–3 was obtained from the MRC Centre for AIDS Reagents (London, UK). The NL4-3 strain was grown in the lymphoid MT-4 cell line. The envelope-deficient HIV-1NL4-3 clone (HIG) encoding internal ribosome entry site (IRES)-GFP (NL4-3-GFP) was pseudotyped with vesicular stomatitis virus G protein (VSV-G) by cotransfection of HEK293T cells using polyethylenimine (Polysciences), as described previously[207,208]. Viral stocks were titrated for use in MT-4 cells.

Peripheral blood mononuclear cells (PBMCs) from buffy coats of healthy donors were obtained by Ficoll-Paque density gradient centrifugation and used for fresh purification of CD4+ T lymphocytes, naïve CD4+ T lymphocytes, or monocytes by negative selection (StemCell Technologies). The purity of the populations was confirmed by flow cytometry. Buffy coats were purchased anonymously from the Catalan Banc de Sang i Teixits. The buffy coats received were totally anonymous and untraceable, and the only information given was whether or not they had been tested for disease. CD4+ T lymphocytes were kept in complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco, Life Technologies). Monocytes were cultured in complete culture medium (RPMI 1640 medium supplemented with 10% heat inactivated FBS [Gibco] and penicillin-streptomycin [Gibco]) and differentiated to monocyte-derived macrophages for 4 days in the presence of macrophage colony-stimulating factor (M-CSF) (Peprotech). Macrophages were infected with a VSV-G-pseudotyped NL4-3-GFP virus, and viral replication was measured 48 h later by quantification of GFP expression by flow cytometry.

CD4+ T cells were activated with anti-CD3 and anti-CD28 (at 1µg/ml each; StemCell Technologies) for 3 days or left untreated with interleukin 2 (IL-2) (16 U/ml; Roche). Cells were acutely infected with a VSV-G-pseudotyped NL4-3-GFP virus by spinoculation before the addition of the corresponding drugs and incubation for 48 h.

Human cell line ACH-2[209], Jurkat (J-Lat) clone 9.2 and clone 8.4[210], and CD4+ TZM-bl[211] cells were obtained from the AIDS Reagent Program, National Institutes of Health (Bethesda, MD). All cell lines were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Gibco) and antibiotics (100U/ml penicillin and 100µg/ml streptomycin; Life Technologies) and were maintained at 37°C in a5% CO2 incubator. TZM-bl cells were infected with the NL4-3 virus, and drugs were added at the time of infection. Viral replication was measured 48 h later by quantification of luciferase production in a luminometer.

1.3.2 Generation of latently infected cells

Latently infected Jurkat cells (J-Hig) were generated by following a modification of the protocol described by Li et al.[66]. Briefly, cells were generated after acute infection of CD4+ Jurkat cells with HIV-1 HIG and were maintained in culture for 10 days to allow the attrition of productively infected cells.

Latently infected primary CD4+ T cells were generated according to the cytokine-polarized primary T cell model of latency[212,213], with few modifications. Briefly, naive CD4+ T cells were activated with anti-CD3 and anti-CD28 antibodies (1µg/ml each; BD, Madrid, Spain) and supplemented with transforming growth factor β1 (TGF-β1) (10 µg/ml; Peprotech), anti-IL-12 (2 µg/ml), and anti-IL-4 (1 µg/ml; Peprotech). Medium supplemented with recombinant IL-2 (rIL-2) (30 IU/ml; Roche) was replaced every 3 days. CD4+ T cells were infected with VSV-NL43-GFP by spinoculation (1,200 x g for 90 min at 37°C) after 7 days of activation, and latently infected/GFP-negative cells were sorted 3 days later using a FACSAria II flow cytometer (BD Biosciences).

1.3.3 Compounds

Acitretin was purchased from Selleckchem, vorinostat was purchased from Prochifar srl (Italy), and panobinostat was purchased from LC Laboratories. The antiretroviral agent 3’-azido-3’-deoxythymidine (AZT) (zidovudine) was obtained from the NIH AIDS Research and Reference Reagent Program. The P300 inhibitor curcumin was purchased from Sigma-Aldrich. All compounds were reconstituted in dimethyl sulfoxide (DMSO)

and stored at -20°C until use. Control (untreated) cell cultures contained a DMSO concentration equivalent to that of drug-treated cultures.

1.3.4 HIV reactivation in vitro in latently infected cells

HIV reactivation was measured as described previously[212]. Briefly, J-Lat cells, which harbor a HIV provirus containing the GFP open reading frame (ORF) instead of nef and a frameshift mutation in env[210], or J-Hig cells were incubated for 24 h with different concentrations of acitretin or the LRAs panobinostat and vorinostat, which were used as controls for HIV-1 reactivation. Reactivation of HIV was monitored as the percentage of living GFP-positive cells, according to forward and side laser light scatter flow cytometry analysis in a FACS LSRII flow cytometer (BD Biosciences). The data were analyzed using FlowJo software.

Similarly, ACH-2 cells, a T cell latency model with one integrated proviral copy, were cultured for 48h in the presence or absence of a LRA; reactivation was assessed by measurement of the production of HIV CAp24 antigen using the Genscreen HIV-1 Ag enzyme-linked immunosorbent assay (ELISA) (Bio-Rad), according to the manufacturer's instructions, or by detection of viral mRNA by quantitative PCR, as described below. Forty-eight-hour incubations were used to minimize cytotoxic effects commonly observed with known LRAs (data not shown)[212,214].

Sorted, latently infected, GFP-negative, naive CD4+ T cells were incubated for 12 h with panobinostat, vorinostat, or acitretin. Treatment with anti-CD3 and anti-CD28 was used as the reactivation control. Subsequently, cells were washed with phosphate-buffered saline (PBS) and kept for 3 days at 37°C in 5% CO2 in fresh medium containing rIL-2, and reactivation was then measured by flow cytometry (GFP-positive cells).

1.3.5 Quantitative PCR

To assess HIV-1 reactivation in ACH-2 cells, total RNA was isolated using the QIAamp viral RNA minikit (Qiagen, Hilden, Germany), as recommended by the manufacturer, and was retrotranscribed to cDNA by using PrimeScript RT Master Mix (TaKaRa Bio USA, Inc.). Quantification of HIV-1 reactivation was determined by using a two-step quantitative PCR assay described previously[212], with few modifications. Briefly, samples were run in

triplicate on cDNA using TaqMan Universal Master Mix II (Applied Biosystems) in a 7500 real-time PCR system (Applied Biosystems). We used the following set of primers and probe for conserved regions of the 5' long terminal repeat (LTR) of HIV-1 mRNA: forward primer, 5'-GACGCAGGACTCGGCTTG-3'; reverse primer, 5'-ACTGACGCTCTCGCACCC-3'; probe,5'-fluorescein amidite (FAM)-TTTGGCGTACTCACCAGTCGCCG-6-carboxytetramethylrhodamine (TAMRA)-3'. Cycling conditions were as follows: 50°C for 2 min followed by 95°C for 10 min for polymerase activation, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. A standard curve of 106 to 102 copies of the 5' LTR of HIV-1 was prepared using ACH-2 DNA, which was run in parallel with samples in order to quantify absolute viral RNA copy numbers in cell supernatants.

1.3.6 Immunoblotting

Treated cells were rinsed in ice-cold PBS, and extracts were prepared in lysis buffer (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, 10 mM sodium β-glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 270 mM sucrose, and 1% Triton X-100) supplemented with protease inhibitor cocktail (Roche) and 1 mM phenylmethylsulfonyl fluoride. Lysates were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Thermo FisherScientific). The following antibodies were used for immunoblotting: horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG secondary antibodies (1:5,000; Pierce), anti-human Hsp90 (1:1,000, product no. 610418; BD Biosciences), anti-cleaved PARP (1:1,000 or 1:10,000, depending on the cell type used, product no. ab32046; abcam), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1,000,product no. ab9485; abcam), anti-MDA-5 (1:500, product no. 5321; Cell Signaling Technology), and anti-phospho-STAT1 (product no. 9177), anti-RIG-I (product no. 3743), anti-phospho-IRF3 (product no. 4947), anti-IRF3 (product no. 11904), and anti-MAVS (product no. 3993) (all at 1:1,000; Cell Signaling Technology).

1.3.7 Flow cytometry

For evaluation of cell death, cells were stained for 30 min in PBS with the LIVE/DEAD Fixable Near-IR dead cell stain kit (Invitrogen, Thermo Fisher Scientific), according to the manufacturer's instructions. Cells were washed and fixed in 1% formaldehyde before

the analysis. Selective clearance of HIV-positive cells by acitretin was measured as expression of annexin V, which stains apoptotic cells. Cells were suspended with the antigen-presenting cell (APC) annexin V antibody (BD Pharmingen) 30 min before cytometric analysis. Antibodies were diluted 1/20 in 1X annexin V binding buffer (BD Pharmingen). Flow cytometry was performed in a FACS LSRII flow cytometer (BD Biosciences). The data were analyzed using FlowJo software (BD Biosciences). Viability determinations were performed in triplicate, and data were calculated from three independent experiments, as done before[203].

1.3.8 Statistical analyses

Data are presented as means ± standard deviations (SDs). All P values were calculated using Student's t test with GraphPad PRISM software (GraphPad Software, San Diego, CA, USA). A P value of 0.05 was considered to be statistically significant.

1.4 RESULTS

1.4.1 Acitretin efficacy as a HIV-1 latency-reversing agent

To evaluate the efficacy of acitretin as a latency-reversing agent (LRA), we compared its effect to that of the known LRAs panobinostat and vorinostat in two commonly used models of HIV reactivation. We were unable to detect HIV reactivation in cells treated with acitretin (up to 25µM) for two different J-Lat clones (clones 8.4 and 9.2), under conditions in which clear, dose-dependent effects were observed for panobinostat or vorinostat (**Figure 9A and 9B**). The combination of acitretin with the LRA panobinostat or the LRA vorinostat did not show any significant difference in HIV reactivation, compared to the LRA alone (**see Suppl. Figure 1A in the Supplemental material**). Similarly, curcumin, a p300 inhibitor used to counteract the effect of acitretin[66], did not have any relevant effect in the presence of acitretin (**Suppl. Figure 1B and Suppl. Figure 1C**). Moreover, acitretin did not induce significant HIV reactivation in ACH-2 cells, as indicated by both p24 and viral mRNA copies in the cell supernatant (**Figure 9C and 9D**).

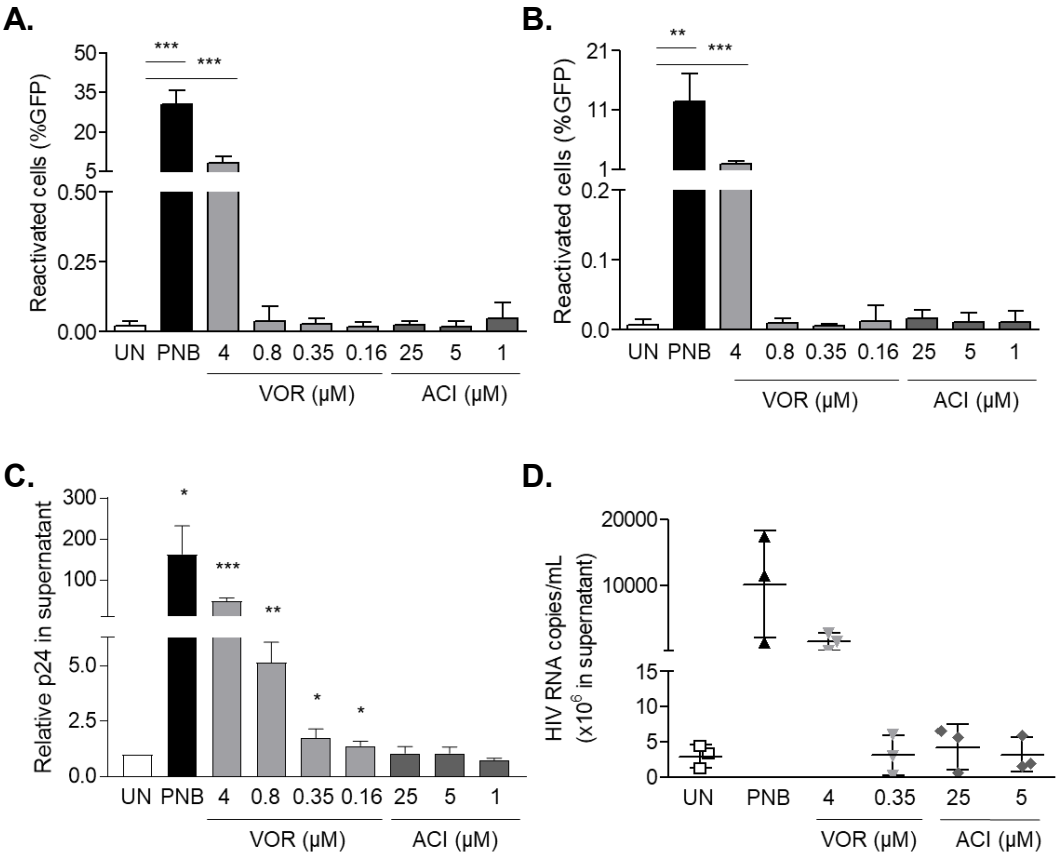

Figure 9. Acitretin does not induce HIV reactivation in J-Lat and ACH-2 cells. (A, B) HIV reactivation induced by the retinoic acid derivative acitretin (ACI) (1 to 25 µM) in J-Lat clone 8.4 (A) and clone 9.2 (B) cells. The HDAC inhibitors panobinostat (PNB) (0.16 µM) and vorinostat (VOR) (0.16 to 4 µM) were used as controls. Reactivation was determined by the quantification of GFP-positive cells after culturing of J-Lat cells with HDAC inhibitors and acitretin for 24 h. (C, D) HIV reactivation induced by acitretin and HDAC inhibitors in ACH-2 cells. Reactivation was determined after 48 h of incubation by quantification of Cap24 (C) and HIV RNA copy number (D) in the supernatant. Values represent means ± SDs of at least three independent experiments performed in triplicate. UN, untreated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

J-Lat clones and ACH-2 cells may differ in the number of integrated HIV copies and their integration sites in the cell genome and thus may have different susceptibilities to LRAs. To exclude a cell-dependent lack of potency, latently HIV-infected cells were generated in-house (J-Hig), and HIV reactivation in the presence of a LRA and acitretin was tested. In this model, acitretin was able to induce HIV reactivation. However, the effect of acitretin on J-Hig cells was modest, compared to those of optimal panobinostat or vorinostat concentrations (**Figure 10A and Suppl. Figure 2**). In addition, acitretin was not able to induce HIV reactivation in latently infected primary CD4+ T lymphocytes

generated in vitro (**Figure 10B**). Conversely, acitretin induced the expression of integrin α4and β7 in activated CD4+ T cells, a common marker of retinoid-induced T cell activation (**Figure 10C**), indicating that acitretin was indeed active at the concentrations used. Taken together, these results indicate that acitretin is, at most, a modest or weak inducer of HIV reactivation.

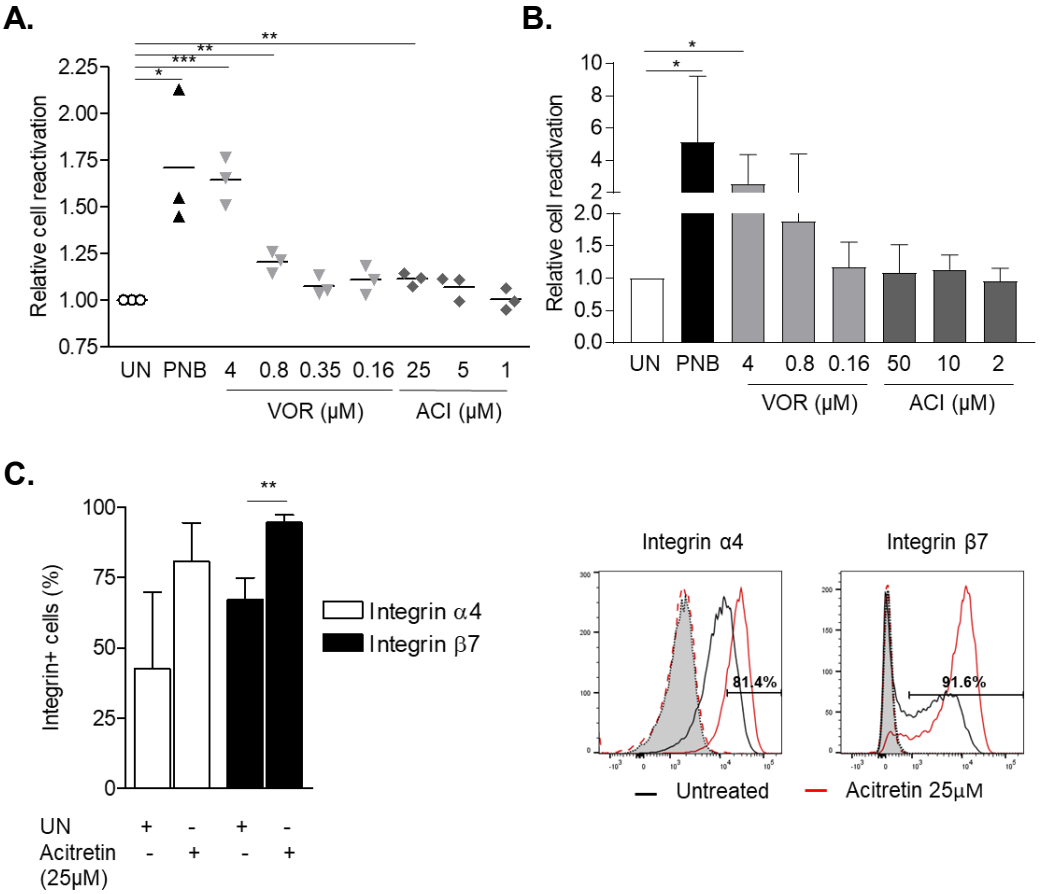

Figure 10. Acitretin effect as a HIV reactivator in latently infected Jurkat cells and primary naïve CD4+ T lymphocytes. (A) HIV reactivation induced by acitretin (ACI) (1 to 25 μM) in latently HIV-infected Jurkat (J-Hig) cells. Panobinostat (PNB) (0.16 μM) and vorinostat (VOR) (0.16 to 4 μM) were used as controls. After 24 h, reactivation was quantified as GFP-positive cells in the non-apoptotic population, measured with an anti-annexin V antibody. (B) HIV reactivation induced by acitretin and the HDAC inhibitors in latently infected primary CD4+ T cells. Naive CD4+ T cells were activated for 7 days, followed by VSV-NL43-GFP infection. After 3 days, GFP-negative cells were sorted and incubated with acitretin for 12 h. Panobinostat (0.16 μM) and vorinostat (0.16 to 4 μM) were used as controls. (C) Acitretin activity as a retinoic acid derivative in activated PBMCs. Integrin α4 and β7 expression induced by acitretin was assessed in PBMCs activated with IL-2, anti-CD3, and anti-CD28 for 7 days, followed by 72 h of incubation with IL-2 in the presence or absence of acitretin (25 μM). Integrin overexpression was measured by flow cytometry. Values represent means ± SDs of at least three independent experiments performed in triplicate. UN, untreated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

1.4.2 Acitretin did not selectively clear HIV-infected cells

Pharmacological HIV reactivation is considered a means to trigger the death of HIV-positive cells that otherwise would remain latent and unrecognized by the immune system[215,216]. We explored the capacity of acitretin to selectively induce apoptosis in HIV-reactivated cells, as suggested by Li et al.[66]. Induction of HIV reactivation in J-Hig cells was followed in parallel with the evaluation of cell death. Panobinostat and vorinostat induced cell-dependent cytotoxic effects in all cell lines tested, but acitretin did not (**Table 1 and Suppl. Figure 3**).

Table 1. Cytotoxic concentrations of latency reversing agents in the cell lines tested

	CC_{50} (μM)[a]			
Drug	J-Lat clone 8.4	J-Lat clone 9.2	ACH-2	J-Hig
Panobinostat	>4	<0.16	<0.16	>4
Vorinostat	>4	1.16	1.16	>4
Acitretin	>125	>125	>125	>25
Curcumin	39.18	34.38	20.64	

[a]CC50, 50% effective concentration, or the concentration needed to induce 50% cell death for the indicated drug.

In order to evaluate the selective killing of HIV-positive cells, we measured annexin V staining in J-Hig and latently HIV-infected CD4+ T cells after 12 h and 24 h of treatment. Following Li et al.[66], the double-positive (annexin-positive/HIV-positive) fraction was compared to the annexin-positive/HIV-negative fraction (**Figure 11A and 11C**). The majority of dead cells induced by panobinostat, vorinostat, or acitretin belonged to the annexin-positive/HIV-negative fraction (**Figure 11B**). That is, neither panobinostat, vorinostat, nor acitretin selectively killed HIV-positive cells (**Figure 11D**). This result was confirmed in primary CD4+ T cells, since none of the conditions demonstrated more apoptotic green fluorescent protein (GFP)-positive (HIV-positive) cells than the untreated control (**Figure 11E and Suppl. Figure 5**). Acitretin concentrations were up to >10-fold higher than those used by Li et al.[66].

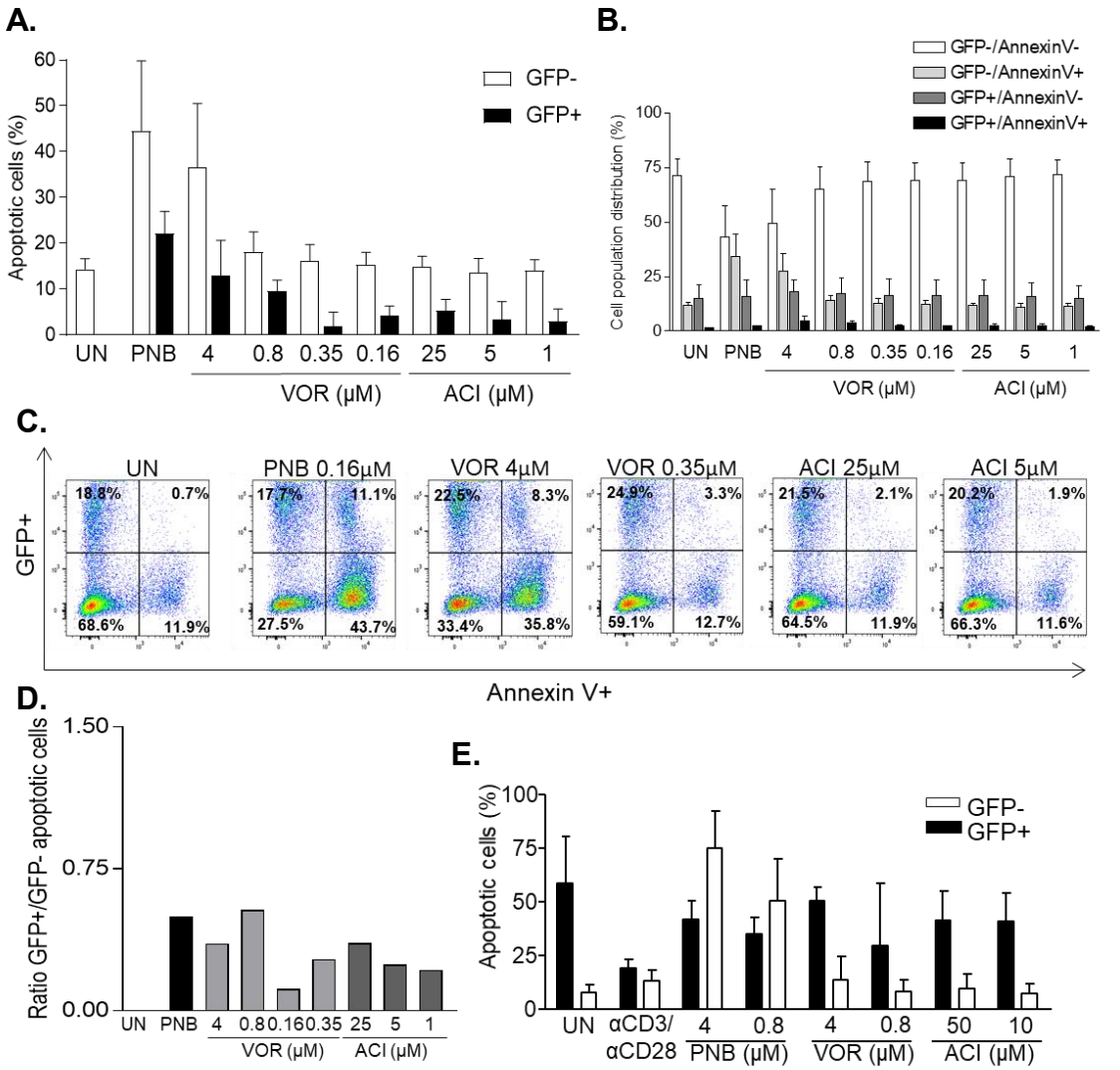

Figure 11. Acitretin does not selectively kill HIV-reactivated cells. (A) Apoptosis percentages in HIV-reactivated (GFP-positive) and not reactivated (GFP-negative) latently infected Jurkat cells (J-Hig). Cells were incubated for 24 h with acitretin (ACI) (1 to 25 μM), panobinostat (PNB) (0.16 μM), or vorinostat (VOR) (0.16 to 4 μM). Apoptosis percentages shown represent fractions of the total GFP-positive/negative subpopulation. Cells were evaluated by flow cytometry with double staining for HIV reactivation (GFP positive) and cell apoptosis (annexin V positive). (B) Cell distribution of apoptotic and/or HIV-reactivated J-Hig cells from the results shown in panel A, without taking into account the fraction of GFP-positive or GFP-negative cells. Assays were evaluated by flow cytometry. (C) Representative cytometry plots from the data in panel A, showing the four subpopulations, i.e., GFP-positive/annexin V-positive, GFP-positive/annexin V-negative, GFP-negative/annexin V-positive, and GFP-negative/annexin V-negative. (D) Ratio of apoptosis values for the HIV-reactivated (GFP-positive) population and the nonreactivated (GFP-negative) population in J-Hig cells. A drug that is selective against HIV-reactivated cells is expected to have a value of >1. I Apoptosis percentages in HIV-reactivated (GFP-positive) and nonreactivated (GFP-negative) latently infected primary CD4+ T cells. Cells were incubated for 12 h with acitretin, panobinostat, or vorinostat. The anti-CD3/anti-CD28 condition was used as a reactivation control. Apoptosis percentages shown represent fractions of the total GFP-positive/negative subpopulation. Values represent means ± SDs of at least three independent experiments performed in triplicate. UN, untreated.

1.4.3 Acitretin is a weak inducer of the RIG-I signaling pathway

To evaluate acitretin capacity to enhance the RIG-I signaling pathway, we observed and quantified RIG-I protein expression and its downstream effectors such as mitochondrial antiviral signaling (MAVS) and IRF3 in several cell lines. Similar to the study by Li et al.[66], TZM-bl cells were treated with acitretin, vorinostat, and panobinostat for 48 h in the presence or absence of HIV-1. Acitretin enhanced RIG-I and MAVS expression in both uninfected and infected cells (**Figure 12A**). Moreover, a slight and not significant increase in the apoptosis marker cleaved poly(ADP-ribose) polymerase (PARP) was observed. Similar results were obtained in ACH-2 cells (**Figure 12B**), uninfected and infected primary monocyte-derived macrophages (**Figure 13A**), and primary resting and activated CD4+ T cells (**Figure 13B**); all of them showed modest increases in MDA-5, RIG-I, MAVS, or IRF-3 expression after acitretin treatment. The modest effects on the RIG-I signaling pathway after culture with acitretin concur with the lack of antiviral effects of acitretin observed in acute infection (**Suppl. Figure 4**). However, LRAs such vorinostat also are able to induce some of the observed changes (**Figure 12 and 13**).

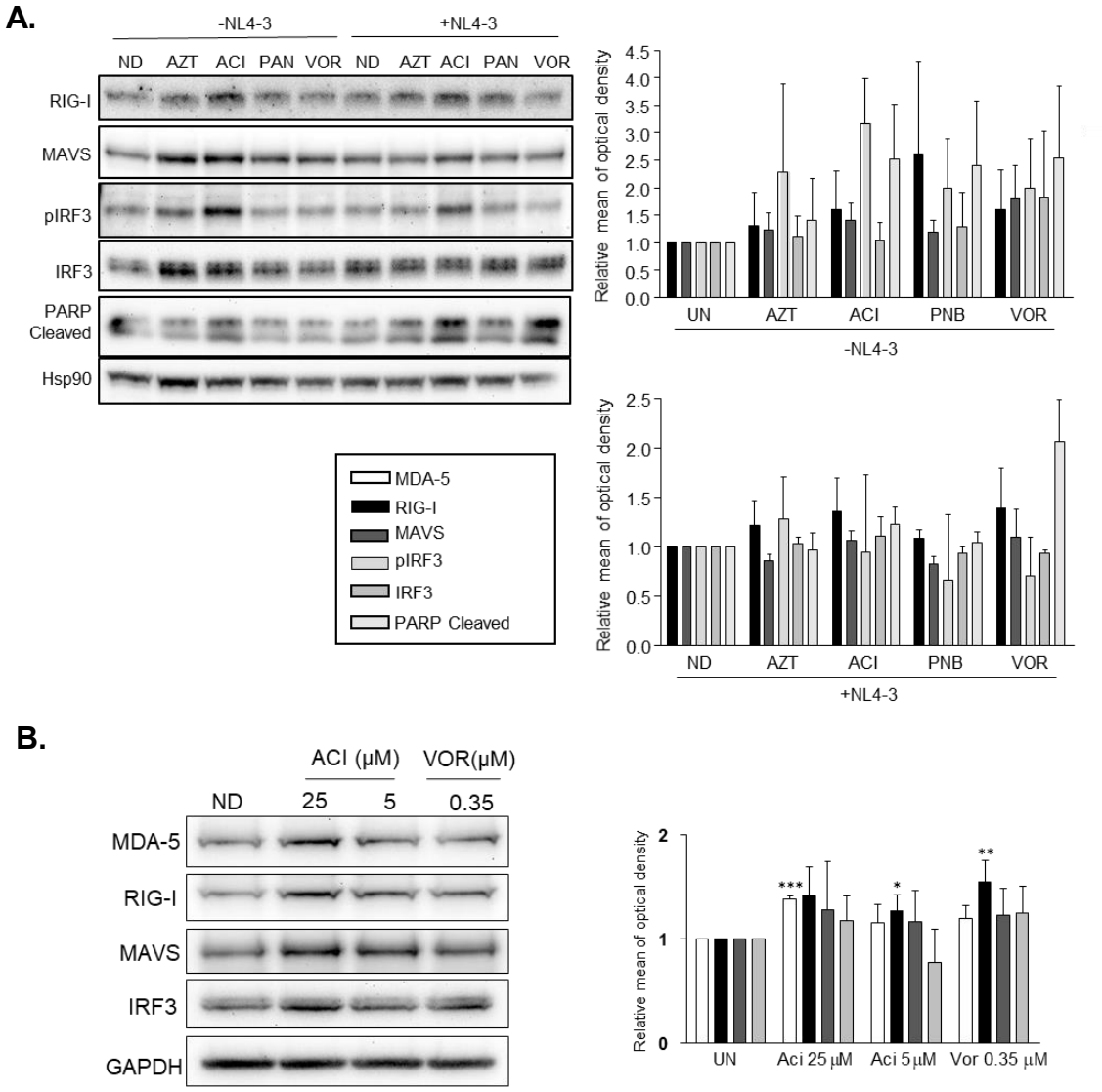

Figure 12. Effects of acitretin versus panobinostat and vorinostat in different cell lines. (A) Representative immunoblot (left) and graphs representing quantification of band density (right) for TZM-bl cells with expression of RIG-I, MAVS, pIRF3, total IRF3, cleaved PARP, and Hsp90 under uninfected or NL4-3-infected conditions, after 48 h of treatment with AZT (1 μg/ml), acitretin (ACI) (1 μM), panobinostat (PAN or PNB) (0.5 μM), or vorinostat (VOR) (0.35 μM). Data were calculated relative to untreated controls in at least three independent experiments. Data for a representative donor are shown. (B) Representative immunoblot (left) and graph representing quantification of band density (right) for ACH-2 cells with protein expression of MDA-5, RIG-I, MAVS, total IRF3, and GAPDH, after 48 h of treatment with acitretin (25 μM or 5 μM) or vorinostat (0.35 μM). Data were calculated relative to untreated controls in at least three independent experiments. A representative experiment is shown. ND, no drug; UN, uninfected. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

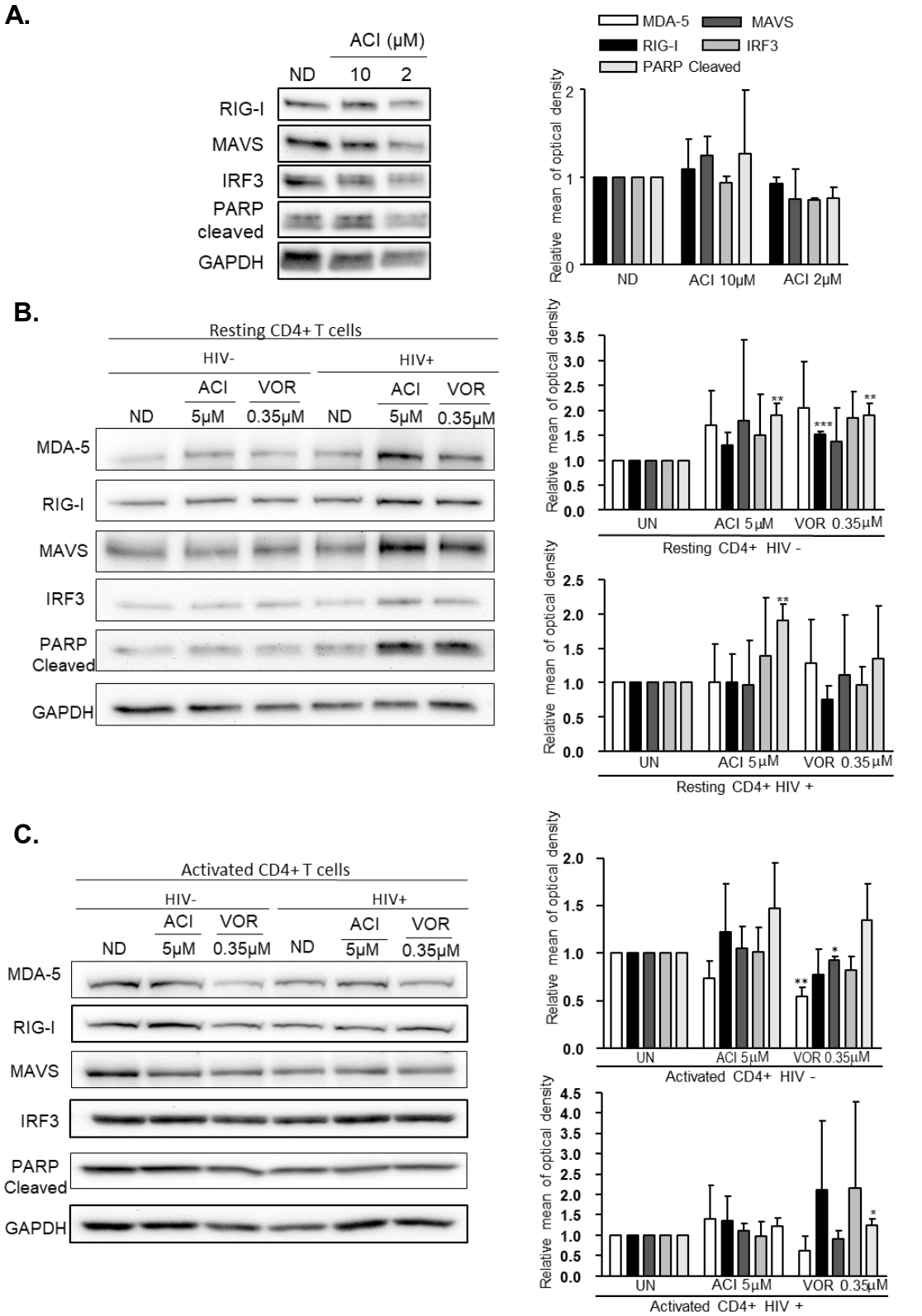

Figure 13. Effects of acitretin versus panobinostat and vorinostat in primary cells. (A) Representative immunoblot (left) and graph representing quantification of band density (right) for monocyte-derived macrophages treated with acitretin (ACI) at 10 μM or 2 μM. Protein expression of RIG-I, MAVS, total IRF3, cleaved PARP, and GAPDH after 24 h of treatment is shown. Data were calculated relative to untreated controls in at least two independent

experiments. Data for a representative donor are shown. (B and C) Representative immunoblots (left) and graphs representing quantification of band density (right) for resting (B) or activated (C) CD4+ T cells with protein expression of MDA-5, RIG-I, MAVS, total IRF3, cleaved PARP, and GAPDH, under uninfected or infected conditions, after 48 h of treatment with acitretin (5 μM) or vorinostat (VOR) (0.35 μM). Data were calculated relative to untreated controls in at least three independent experiments. Data for a representative donor are shown. ND, no drug; UN, uninfected. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

1.5 DISCUSSION

The identification of acitretin as a LRA and selective inducer of HIV-positive cell death[66] offered a fast-lane opportunity to treat and to cure HIV infections; it is an approved drug used to treat autoimmune diseases such as psoriasis, it triggers what appears to be a favorable immune response, and it was shown to culminate in the preferential apoptotic death of reactivated HIV reservoir cells[66]. We strongly challenge these conclusions, as we could not recapitulate the promising results previously shown for acitretin.

Li et al.[66] compared the effect of acitretin at 5μM to that of vorinostat at 0.350μM, that is, a vorinostat concentration 11.3-fold lower than its reported 50% effective concentration (EC50)[214]. Indeed, we found that the latency reversal activity of acitretin at 25μM was commensurate only with suboptimal concentrations of an already relatively weak LRA, vorinostat[217,218], and was negligible in comparison to other, more potent histone deacetylase (HDAC) inhibitors such as panobinostat[214] (**Figure 10A**). Acitretin LRA activity was undetectable in a HIV latency model in primary CD4+ T cells[212] and was only weakly observed in one of three different cell line models used, suggesting that acitretin's effect on HIV reactivation would be insufficient to trigger a desirable effect.

Despite early indications that RNA sensing of HIV-1 infection may be counteracted by protease-mediated sequestration of RIG-I[219], we and others have shown that HIV-1 infection may induce the expression of genes involved in antiviral signaling, including MDA-5 and RIG-I, in primary cell cultures[201,202,220]. RIG-I expression may also be associated with disease progression in HIV-positive individuals[221]. These effects have been associated with IFN-mediated cell death. However, the effect of acitretin in the stimulation of the RIG-I pathway leading to IFN production was again mild in the

laboratory-adapted HeLa-derived TZM-bl cells, in ACH-2 cells or primary macrophages, and in activated and resting CD4+ T cells. Importantly, the effect of acitretin on RIG-I stimulation was neither specific nor selective for HIV-positive cells (Fig. 4), as stimulation of RIG-I, MDA-5, and IRF3 was not differentially observed in infected acitretin treated cells. These results are in line with a mild cytotoxic effect of acitretin in both infected and uninfected cells.

The proposed selective killing of latently infected, HIV-positive cells deserves particular attention, considering the method employed to evaluate its significance by Li et al.[66]. Comparing the percentage of HIV-positive dead cells to that of HIV-negative dead cells in the presence or absence of an apoptosis inducer may indeed provide clues to purging and eliminating unwanted HIV-positive cells. Selective destruction of HIV-positive cells is the goal of a "shock and kill"[174,215] therapeutic strategy. However, we failed to detect a significant number of HIV-positive dead cells, compared to uninfected dead cells, with acitretin at concentrations that effectively affect α4 and β7 integrin expression. Of note, the absolute number of uninfected dead cells was significantly higher than that of infected dead cells, indicating a lack of selectivity for HIV-positive cells. We do not discard the possibility of differences in virus strains or cell culture conditions used that could explain the discrepancies between our results and the results shown by Li et al.[66]. The virus genomes in the two studies were different, and we used a Nef-deleted virus in our cultures. However, loss of the accessory protein Nef is not necessary for HIV replication in tissue culture.

Innate immune protection from HIV-1 infection may be associated with the inability of the virus to surpass cell restriction without negatively affecting the cells' proliferative capacity[222] and to be detected by pattern recognition receptors[201,220], indicating that stimulation of such recognition is justified as a potential therapeutic strategy. However, our results suggest that acitretin-mediated stimulation of the RIG-I pathway for HIV reactivation is modest and thus may not meaningfully affect the HIV reservoir. Effective LRAs may be defined only when they can, by themselves, induce measurable clearance of persistent HIV infections or when they can be appropriately paired with viral clearance strategies that result in latency depletion[223]. Stimulation of the RIG-I-

dependent IFN cascade by acitretin alone may not significantly affect the selective destruction of latently infected, HIV-positive cells.

1.6 ACKNOWLEDGMENTS

We thank the National Institutes of Health (AIDS Research and Reference Reagent Program) for reagents.

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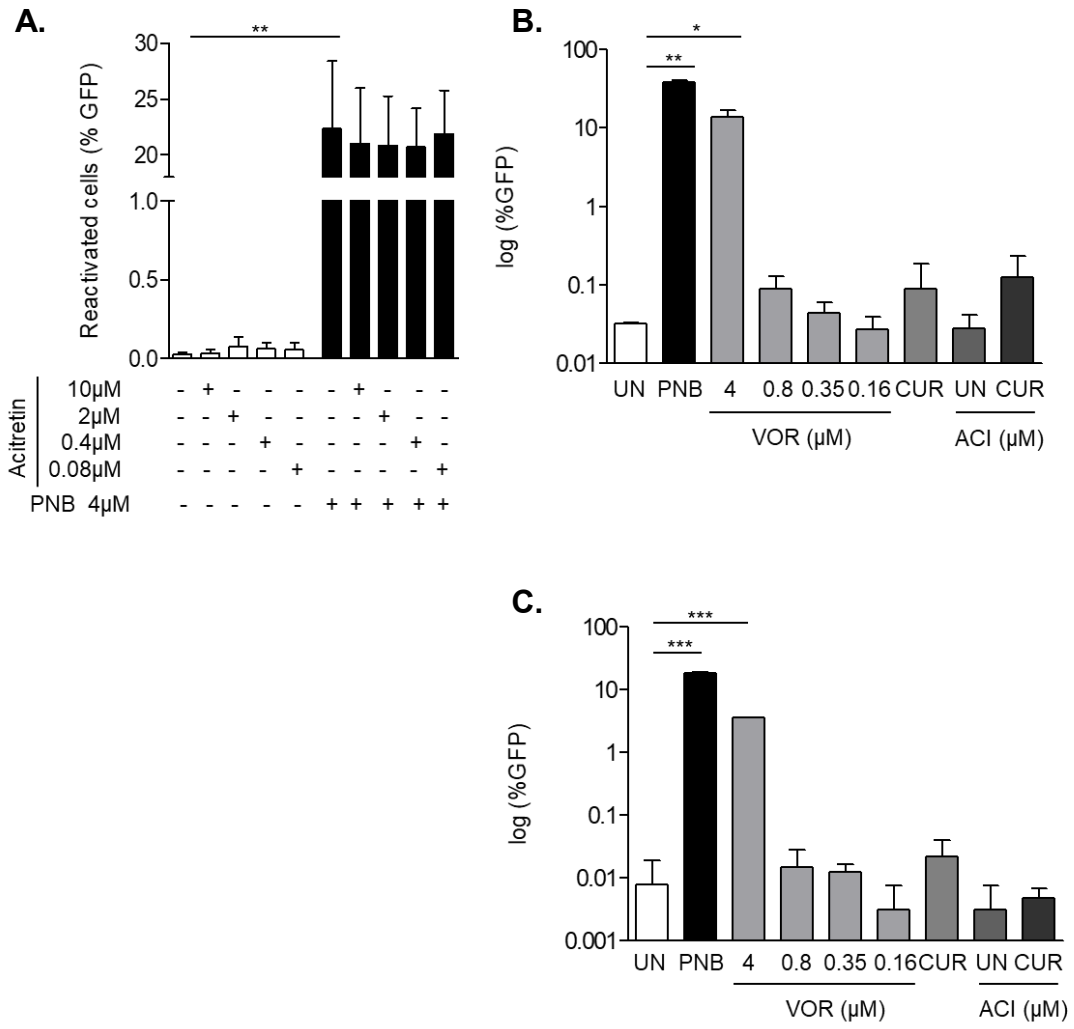
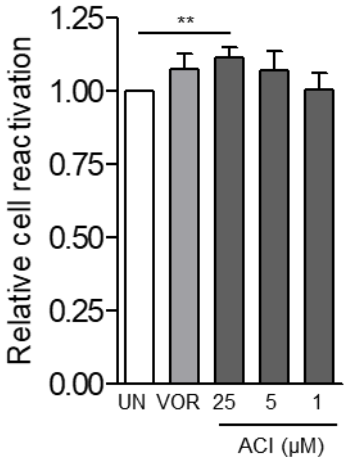
We declare no conflicts of interest.

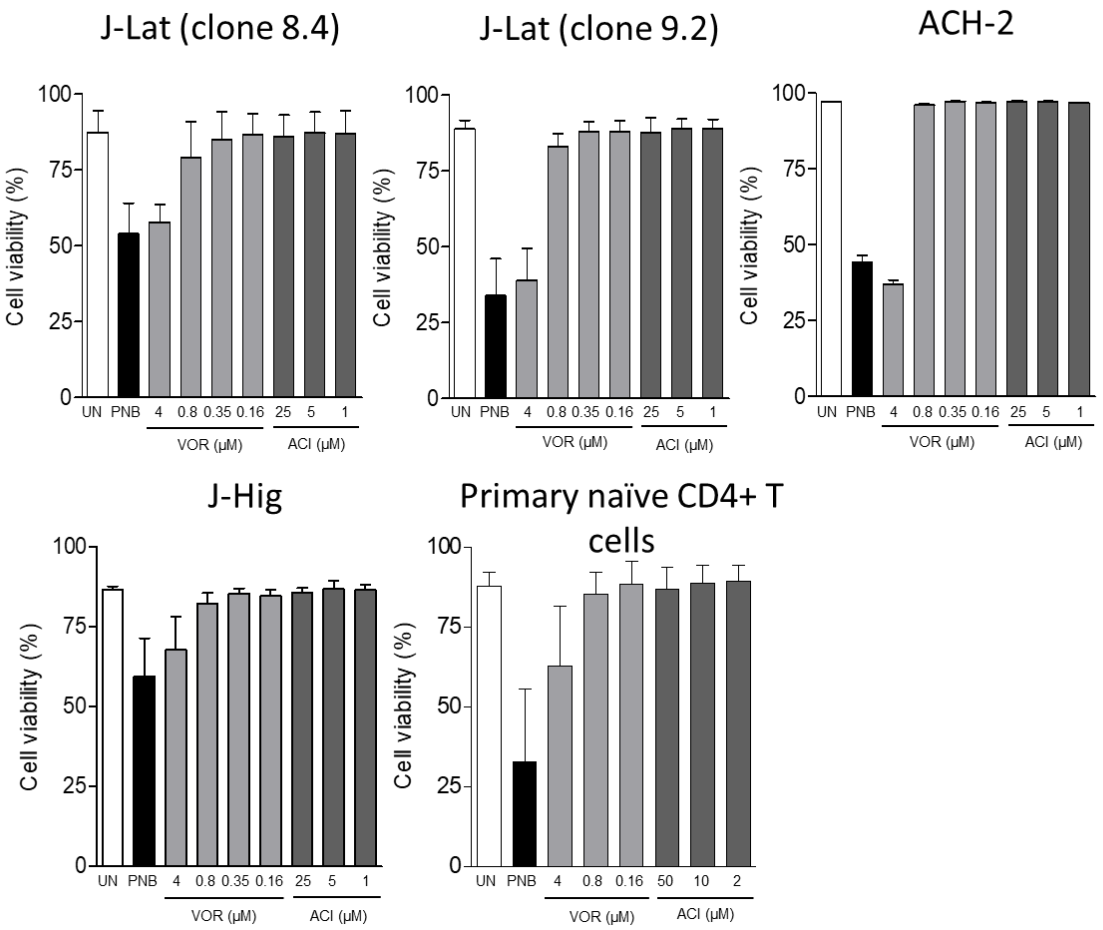
1.7 SUPPLEMENTAL MATERIAL



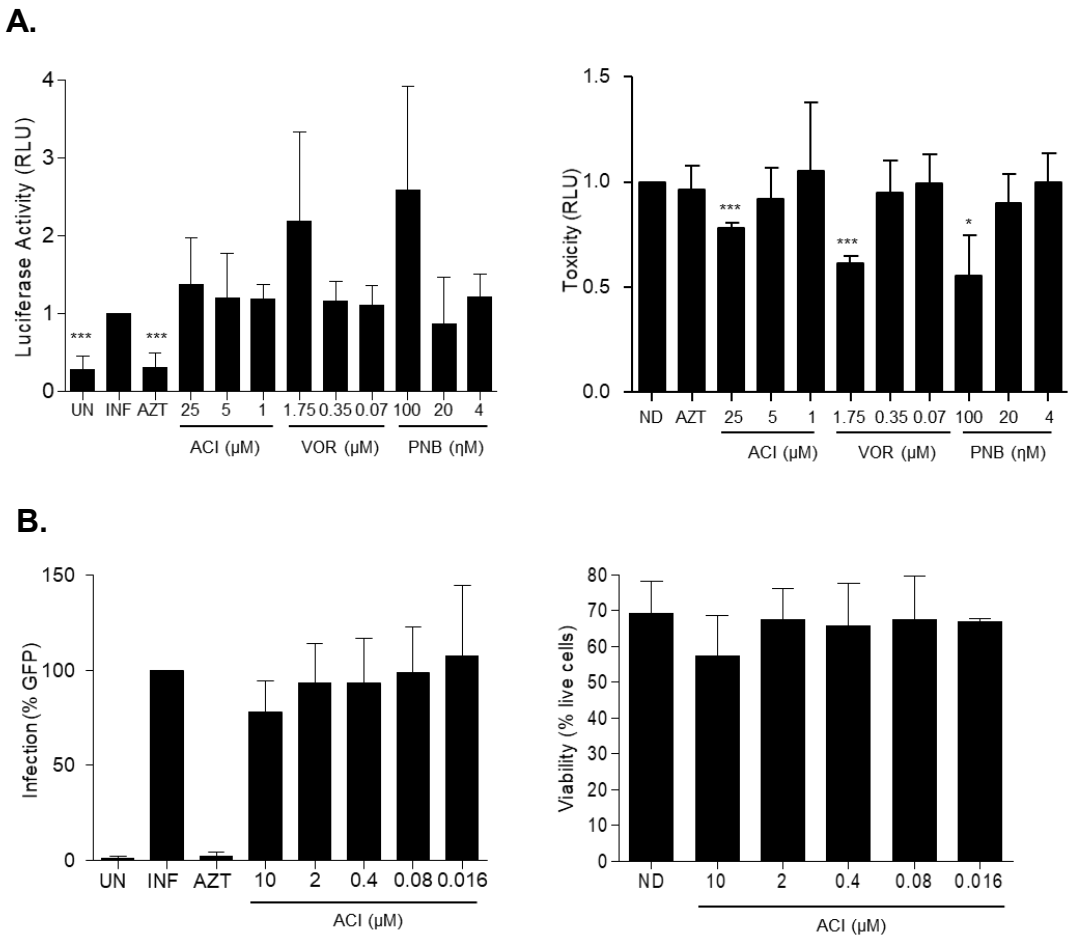
Supplemental figure 1. Acitretin does not affect Panobinostat activity and its effect is not suppressed by Curcumin. (A) HIV reactivation induced by Acitretin (10 – 0.08μM) in the absence or presence of the HDACi Panobinostat (4μM). Reactivation was determined by quantification of GFP+ (%) cells in J-Lat clone 9.2 after 24h of incubation. (B, C) HIV reactivation induced by Acitretin (5μM) in the absence or presence of p300 inhibitor, Curcumin (10μM). HDAC inhibitors Panobinostat and Vorinostat were used as controls. Reactivation was evaluated by quantification of GFP+ (log %) cells in J-Lat clones 8.4 (B) and 9.2 (C) after 24h of incubation. Values represent mean±SD of triplicate samples representative of two (B, C) and three (A) independent experiments. Student's t test was used to compare experimental conditions. UN, untreated; CUR, curcumin. *P < 0.05; **P < 0.01; ***P < 0.001.



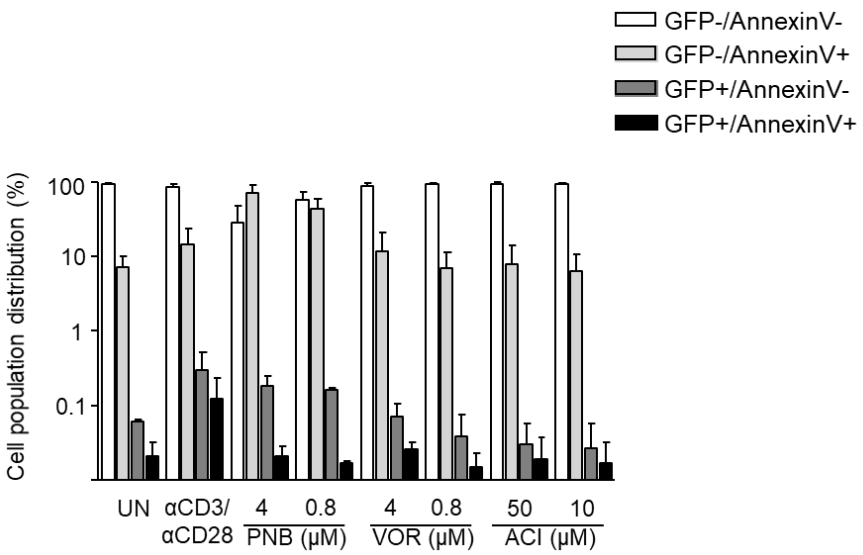
Supplemental figure 2. Comparison of Acitretin effect to a suboptimal concentration of Vorinostat in J-Hig cells. HIV reactivation by Acitretin (25 - 1µM) and HDACi Vorinostat (VOR, 0.35µM) was evaluated by the quantification of GFP+ cells (%) in the non-apoptotic population after culturing Jurkat latently infected cells (J-Hig) for 24h. Values represent mean±S.D. of triplicate samples representative of three independent experiments. Student's t test was used to compare experimental conditions. UN, untreated. **P < 0.01.



Supplemental figure 3. Acitretin does not induce cell toxicity. Cell viability in the presence of Acitretin (25 – 1μM) in J-Lat cells (clones 8.4 and 9.2), ACH-2 cells and J-Hig cells and primary naïve CD4+ T cells, respectively. HDAC inhibitors Panobinostat (PNB, 0.16μM) and Vorinostat (VOR, 4 – 0.16μM) were used as controls. Viability assays were assessed with a death-staining kit (J-Lat, ACH-2 and CD4+ T cells) and using αAnnexin V antibodies (JHig) by flow cytometry. UN, untreated.



Supplemental figure 4. Antiviral and cytotoxic effect of acitretin in TZMbl cells and M-CSF MDMs. (A) Evaluation of HIV susceptibility (left panel) and drug toxicity (right panel) of TZMbl cells 48h post-treatment with acitretin, vorinostat and panobinostat. Infection and toxicity were measured by luciferase reporter assay and was relativized to infected and untreated control, respectively. Data represents mean and SD of at least three independent experiments. (B) Evaluation of HIV susceptibility (left panel) and viability (right panel) of M-CSF MDMs 48h post-treatment with acitretin. Infection using a VSV-pseudotyped, GFP-expressing HIV-1, was measured by GFP content, and viability was estimated with flow cytometer FSC and SSC. Data represent percentage of replication relative to infected cells, and percentage of live cells. Mean ± SD of 3 different experiments in triplicate is shown. ND; No drug, AZT; Zidovudine, ACI; Acitretin, PNB; Panobinostat, VOR; Vorinostat, UN; uninfected, INF; infected. * $p<0.05$; ** $p<0.005$; *** $p<0.0005$.



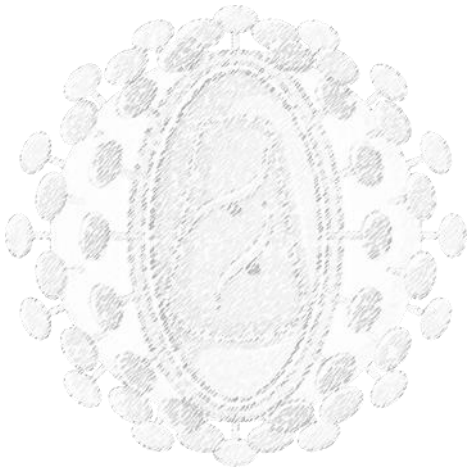
Supplemental figure 5. Acitretin does not selectively kill HIV-reactivated cells. Cell distribution of apoptotic and/or HIV reactivated latently infected primary CD4+ T cell evaluated by flow cytometry showing the four subpopulations: GFP+/AnnexinV+, GFP+/AnnexinV-, GFP-/AnnexinV+ and GFP-/AnnexinV-. Cells were incubated for 12h with acitretin, panobinostat and vorinostat. Anti-CD3 anti-CD28 condition was used as a reactivation control. Values represent mean ± SD of at least three independent experiments performed in triplicate. UN, untreated.

CHAPTER 2

AURORA KINASE-DEPENDENT REACTIVATION OF HIV-1

Edurne Garcia-Vidal, Lucía Gutiérrez, Maria Pujantell, Roger Badia, Teresa Puig, Cristina Ramírez, Bonaventura Clotet, Eva Riveira-Muñoz, Ester Ballana, and José A. Esté

Data not published.
This study is related to the patent with the PCT application number PCT/ES2019/070596.



2.1 ABSTRACT

HIV-1 reactivation from latency in the presence of antiretroviral therapy has been proposed as a strategy to purge the viral reservoir. Unfortunately, all attempts brought to the clinic to reactivate HIV-1 and destroy the infected cells have failed owe to the limited capacity or potency of virus latency-reversing agents (LRA) or the lack of an effective mechanism to induce the elimination of infected cells. Here, we screened an anti-cancer compound library to identify agents that may selectively reactivate and kill HIV-1+ cells. 62 compounds were identified in a non-clonal HIV-1 latency model in Jurkat cells, including histone deacetylase inhibitors, aurora kinase (AURK) inhibitors, agents targeting IκB/Ikk pathway, JAK inhibitors and heat shock protein inhibitors as the classes with the most number of agents identified. The prototype AURKB inhibitor, barasertib, reactivated HIV-1 from stable cell lines and primary memory CD4+ T cells, as other AURKi did too, and enhanced the activity of HDAC inhibitors vorinostat (SAHA) and panobinostat. However, unlike the later, barasertib and other AURKi induced cell cycle arrest in a polyploid state in immortalized cells, suggesting a distinct mode of action. Antiretroviral agents did not affect the latency-reversing activity of the AURK inhibitors; however, an additive effect was observed when AURKi and antiretroviral agents were used in combination in HIV-1 replication assays, as the inhibition of the aurora kinases by itself was able to inhibit HIV-1 acute infection too. Aurora kinase A and B play crucial roles in cellular division, being AURKA associated with the centrosome and required for a normal spindle positioning whereas AURKB takes part in the chromosomal passenger complex (CPC), which controls chromatid segregation and contributes to centromeric cohesion. Our results suggest that inhibition of the centromere function by AURKi triggers the reactivation of specific HIV-1 provirus that may be located in low-transcription sites such as the centromeres.

2.2 INTRODUCTION

Despite significant efforts in identifying strategies to reactive (shock) HIV-1 from latently infected cells and induce the elimination (kill) of infected cells, the so called, shock and kill strategy has failed. The lack of adequate models of latent infection and sufficient

understanding of the mechanisms driving latency and reactivation have hindered the, much needed, eradication of infection from the organism of HIV+ individuals.

The HIV-1 reservoir is generally defined as an infected cell population that allows the persistence of replication-competent HIV-1 in patients on optimal treatment regimens on a timescale of years. To date, the latent reservoir in resting CD4+ T cells is the major reservoir shown to fit this definition[224,225]. Resting CD4+ T cells with integrated HIV-1 DNA are actually derived from activated CD4+ T cells that became infected and then reverted back to a resting memory state[134].

A number of latency-reversing agents (LRA) have been identified as the means to shock HIV-1, with some of them reaching clinical evaluation[178,226]. Unfortunately, all attempts to reduce the viral reservoir have failed. LRAs studied in clinical trials may not have effectively cleared latently infected cells for several reasons. These include limited potency, toxicity, insufficient delivery to lymphoid tissue, post-transcriptional blocks limiting viral protein expression, competing mechanisms of HIV persistence(5), the size of the HIV-1 reservoir[227] and, importantly, variability in HIV-1 integration sites that may be differentially affected by distinct LRA[186,228]. We and other have often observed synergistic effects of combination of two or more LRA with distinct mechanisms of action[229–231].

Two individuals, one in Berlin[164] and one in London[167], are the only two cases of HIV-1 long-term remission following hematopoietic stem-cell transplantation (HSCT) using CCR5Δ32/Δ32 cells. In both cases, patients received conditioning regimens prior to HSCT, consisting of exclusively chemotherapy agents with known activity against lymphoma (London patient) or total body irradiation in conjunction with cyclophosphamide (Berlin patient), suggesting that chemotherapeutic treatment may have contributed to the loss of HIV-1 infected cells[167].

Here, we have screened an anti-cancer compound library to identify agents that may selectively reactivate and kill HIV-1+ cells and affect the viability of infected cells. We have identified aurora kinase (AURK) inhibitors, and particularly barasertib, a reversible ATP-competitive inhibitor of aurora kinase B, as latency-reversing agents in stable and primary cells models of latent HIV-1 infection.

2.3 MATERIALS AND METHODS

2.3.1 Cells

Peripheral blood mononuclear cells (PBMCs) from buffy coats of healthy donors were obtained by Ficoll-Paque density gradient centrifugation and used for fresh purification of CD4+ T lymphocytes, naïve CD4+ T lymphocytes or monocytes by negative selection (StemCell Technologies). Purity of the populations was confirmed by flow cytometry. Buffy coats were purchased anonymously from the Catalan Banc de Sang i Teixits (http://www.bancsang.net/en/index.html). The buffy coats received were totally anonymous and untraceable and the only information given was whether or not they have been tested for disease. CD4+ T lymphocytes were kept in complete culture medium (RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco, Life Technologies)) and rIL-2 (6.5 IU/mL, Roche). Monocytes were cultured in complete culture medium and differentiated to monocyte-derived macrophages (MDM) for 4 days in the presence of monocyte-colony stimulating factor (M-CSF, Peprotech) at 100 ng/mL.

The human cell lines ACH-2[209], Jurkat and Jurkat (J-Lat)[210] cells were obtained from AIDS Reagent Program, National Institutes of Health (Germantown, MD). Cells were grown in complete culture medium, as the primary cells, and maintained at 37ºC in a 5% CO2 incubator.

2.3.2 Viruses and virus infections

HIV-1 viral strain NL4–3, expressing green fluorescent protein (GFP), was obtained from the MRC Centre for AIDS Reagents (London, UK). The envelope-deficient HIV-1 NL4-3 clone (HIG) encoding internal ribosome entry site (IRES)-green fluorescent protein (GFP) (NL4-3-GFP) was pseudotyped with vesicular stomatitis virus G protein (VSV-G) by cotransfection of HEK293T cells using polyethylenimine (Polysciences) as previously described[73,232]. For the production of viral-like particles carrying Vpx (VLP-Vpx), HEK293T cells were cotransfected with pSIV3+ and a VSV-G–expressing plasmid[233]. Three days after transfection, supernatants were harvested, filtered, and stored at -80°C. The protocol for production of HIV-1 NL4-3 virus modified to carry Vpx (NL4-3*Vpx) was

performed as described previously[79]. In some cases, viral stocks were concentrated using a Lenti-X concentrator (Clontech).

Macrophages were infected with HIG in the presence or not of VLP-Vpx and viral replication was measured 48h later by quantification of GFP+ expression by flow cytometry. Similarly, CD4+ T lymphocytes were infected with HIG and viral replication was measured 48h later, as with the macrophages.

2.3.3 Generation of latently infected cells

Latently infected cells (J-Hig) were generated following a modified protocol described before[66,230]. Briefly, cells were generated after acute infection of CD4+ Jurkat cells with HIG and maintained in culture for 10 days to allow for the attrition of productively infected cells.

Latently infected primary CD4+ T cells were generated according to the cytokine-polarized primary T cells model of latency[212,234] with few modifications. Briefly, naïve CD4+ T cells were activated with αCD3/αCD28 (ImmunoCult™ Human CD3/CD28 T Cell Activator, 25µL/mL, StemCell) and supplemented with TGFβ1 (10 µg/mL, Peprotech), αIL-12 (2 µg/mL) and αIL-4 (1 µg/mL, Peprotech). Medium supplemented with rIL-2 was replaced every 3 days. After 7 days of activation, CD4+ T cells were infected with NL4-3*Vpx by spinoculation (1200xg, 1h 30 min at 37 ºC). Three days later, GFP negative cells containing both latently infected and uninfected cells were sorted using a FACSAria II flow cytometer (BD Biosciences).

2.3.4 Compounds

The anti-cancer compound library and the individual aurora kinases inhibitors barasertib, MK-5108, alisertib, hesperadin, SNS-314 mesylate and tozasertib were purchased from Selleckchem (Houston, TX). The histone deacetylase (HDAC) inhibitors vorinostat (VOR) and panobinostat (PNB) were purchased from Prochifar srl (Italy) and LC Laboratories, respectively. Antiretroviral agent 3-azido-3-deoxythymidine (zidovudine; AZT) was obtained from the NIH AIDS Research and Reference Reagent Program. The integrase inhibitor raltegravir (RAL) was received from Merck Sharp and

Dohme (MSD, Spain). All compounds were resuspended in DMSO and stored at -20º C until use.

2.3.5 HIV-1 reactivation in vitro in latently infected cells

HIV-1 reactivation was measured as described before[230,234]. Briefly, clonal J-Lat or non-clonal J-Hig cells were incubated for 24h with subtoxic concentrations of the AURKi. LRAs panobinostat and/or vorinostat were used as controls for HIV-1 reactivation. Reactivation of HIV-1 was monitored as the percentage of living GFP positive cells. Drug screening for latency reversal agents was performed in J-Hig cells. To assess HIV-1 reactivation, cells were incubated with the drugs at 5µM for 24h before measuring GFP expression in live cells. Panobinostat at 0.16µM was used as a positive control for HIV reactivation and as a mean to sort the drugs into the categories "hit", "pseudo-hit" and "out-of-range". Drugs that induced a relative HIV reactivation equal or higher than that of panobinostat minus 2 standard deviations (-2SD) were classified as "hit" compounds; those with relative reactivation values ranging from less than that of panobinostat-2SD to 1.4-fold were classified as "pseudo-hit" compounds; and those with a relative reactivation value lesser than 1.4-fold were classified as "out-of-range" compounds.

For the HIV-1 reactivation assay in sorted latently infected/GFP negative naïve CD4+ T cells, cells were incubated for 24h with subtoxic concentrations of barasertib and MK-5108. Treatment with αCD3/αCD28 was used as an HIV-1 reactivation control. Previously, cells were washed with PBS after the sorting and kept in fresh media containing rIL-2 overnight at 37 ºC and 5 % CO2. Reactivation was measured as the percentage of GFP positive cells by flow cytometry.

For the p24 in supernatant quantification, ACH-2 cells, a clonal T cell HIV-1 latency model, were cultured for 48h in the presence or absence of vorinostat and the AURKi; reactivation was assessed by measurement of the production of HIV CAp24 antigen using the Genscreen HIV-1 Ag enzyme-linked immunosorbent assay (ELISA) (Bio-Rad), according to the manufacturer's instructions.

2.3.6 Flow cytometry

For evaluation of cell death, cells were stained for 30 minutes with LIVE/DEADTM Fixable Near-IR Dead Cell Stain Kit (Invitrogen, Thermo Fischer Scientific) in PBS according to manufacturer's instructions. Alternatively, viable cells were identified according to forward and side laser light scatter flow cytometry analysis. Cells were washed and fixed in 1% formaldehyde before the analysis.

For cell cycle analysis, primary cells were treated with 7-aminoactinomycin D (7AAD; Sigma-Aldrich) and pyronin Y (Sigma-Aldrich) as described previously[208]. Alternatively, cell cycle evaluation in J-Hig cells was performed following the 2-step cell cycle assay in a NucleoCounter® NC-3000™ (ChemoMetec) according to manufacturer's instructions.

Flow cytometry assays were performed in a FACS LSR II or a FACSCanto II flow cytometer (BD Biosciences). The data was analyzed using the FlowJo software (BD Biosciences). The cell separation of the latently infected primary naïve CD4+ T cells was performed by the Flow Cytometry facility at the Germans Trias i Pujol Research Institute, using a FACSAria II cell separator (BD Biosciences).

2.3.7 Statistical and mathematical analyses

Data are presented as mean ± standard deviation (SD). All p-values were calculated using a t-Student's test calculated with the GraphPad PRISM software (GraphPad Software, San Diego, CA). A p-value of 0.05 was considered to be statistically significant.

2.4 RESULTS

2.4.1 Aurora kinase inhibitors are identified as hits in a drug screening for latency-reversing agents

In order to identify novel compounds with HIV-1 latency-reversing activity, 426 anti-tumoral drugs for multiple cancers were tested in the non-clonal HIV-1 latency model J-Hig cells. After 24h of incubation at 5 µM, 62 compounds showed latency-reversing activity and were then classified as hits (16 compounds) or pseudo-hits (46 compounds) depending on their latency-reversing potency (**Figure 14A, Table 2 and Suppl. Table 1**).

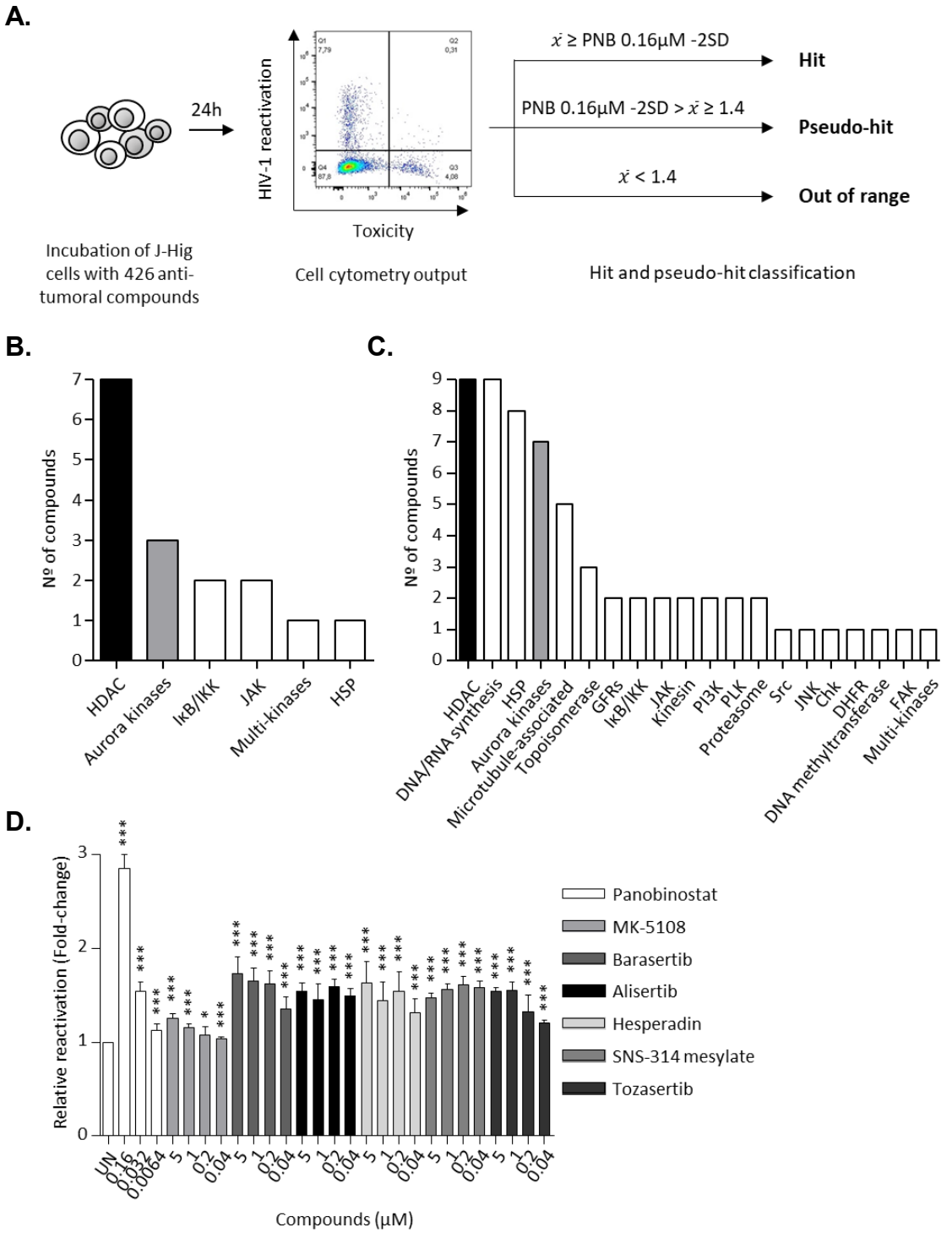


Figure 14. Aurora kinase inhibitors display HIV-1 latency-reversing activity in a drug screening. (A) Flowchart used for drug screening and hit classification. J-Hig cells were incubated with drugs at 5µM for 24h. Relative HIV-1 reactivation of each condition, measured as percentage of GFP+ cells and normalized to untreated condition, was compared to that of the control panobinostat (PNB) at 0.16µM. Tested drugs were sorted into "hit", "pseudo-hit" and "out of range" categories based on that. (B) Classification of the hit compounds by their target. After 24h incubation, compounds able to achieve a relative reactivation equal or higher to that of PNB 0.16µM were classified as hit compounds. (C) Classification of the hit and pseudo-hit compounds altogether by their target. After 24h incubation, compounds able to achieve a relative

reactivation equal or higher than 1.4 were classified as either hit or pseudo-hit compounds. (D) HIV-1 reactivation reported by 6 aurora kinase inhibitors (AURKi) at decreasing concentrations. Six AURKi from the drug screening, including both hits and pseudo-hits, plus an additional AURKB inhibitor, were incubated in J-Hig cells for 24h and their reactivation was assessed by percentage of GFP expression by flow cytometry. The drug screening was assessed in three independent experiments, while (D) values represent mean ± SD of at least three independent experiments performed in triplicate. Statistical significance in (D) was assessed by comparing the conditions with latently infected untreated control (UN). UN; untreated, PNB; panobinostat. *P < 0.05; ***P < 0.001.

The drugs tested were then classified according to their targets, as reported by the manufacturer, revealing that the most common target among the hit compounds was the histone deacetylase (HDACs) family, a well-known LRA target, with seven compounds. Considering both hit and pseudo-hit compounds together, HDAC inhibitors were still the most common class (9 compounds) along with a mixture of compounds targeting the RNA/DNA synthesis pathway (9 compounds). Additionally, the Aurora kinase (AURK) family was the second most common target among the hits (3 compounds) and the fourth when including the pseudo-hits (7 compounds) (**Figure 14B and 14C**).

Retesting of AURK inhibitors (AURKi) used in the screening (barasertib, tozasertib, alisertib, SNS-314 mesylate and MK-5108), along with an additional AURKi (hesperadin), confirmed their activity and potency as LRAs, some of them active at concentrations as low as 0.04 μM (**Figure 14D**).

Table 2. Reactivation, viability and inhibitory selectivity of the aurora kinase inhibitors included in the LRA screening

Compound	Relative reactivation		Viability (%)		IC50 (nM)*		
	Mean	SD	Mean	SD	AURKA	AURK B	AUKRC
Hits							
AMG-900	1.89	0.30	92.70	1.91	5	4	1
Barasertib (AZD1152-HQPA)	1.80	0.06	93.29	3.21	-	0.37	-
Tozasertib (VX-680, MK-0457)	1.7	0.03	94.04	3.16	0.6 (Ki app)	18 (Ki app)	4.6 (Ki app)
Pseudo-hits							
Alisertib (MLN8237)	1.62	0.02	93.89	1.75	1.20	-	-
SNS-314 Mesylate	1.55	0.25	92.22	3.89	9	31	3
MK-5108	1.51	0.11	93.50	3.81	0.06	-	-
ENMD-2076	1.44	0.22	95.13	0.46	14	350	-
Out-of-range							
Danusertib (PHA-739358)	1.29	0.03	93.9	1.43	13	79	61
AT9283	1	0.08	91.3	4.19	3	3	-
JNJ-7706621	0.9	0.14	94.52	0.67	11	15	-
CYC116	1.17	0.24	95.69	1	8 (Ki)	9 (Ki)	-
BI-847325	n.d.**	n.d.	n.d.	n.d.	25	3 (X. laevis)	15

*Inhibitory selectivity reported by the manufacturer.

**No data. The compound could not be analyzed due to interferences with the flow cytometer lasers.

2.4.2 The AURKi barasertib enhances the latency-reversing activity of the HDACi vorinostat and panobinostat

To test whether AURKi could have an impact in the effect of other LRAs, a clonal (J-Lat clone 9.2) and a non-clonal (J-Hig) model for HIV-1 latency were used. When the most specific AURKi hit compound, barasertib, was used in the clonal J-Lat cells, the

compound was not able to induce HIV-1 reactivation by itself. However, when barasertib was used in combination with the HDACi vorinostat or panobinostat, the effect observed was slightly higher than that achieved by the HDACi alone, although non-significant (**Figure 15A**). This could be better observed in J-Hig cells, where both barasertib and the HDACi had activity by themselves that resulted in a significant additive effect when used in combination (**Figure 15B**).

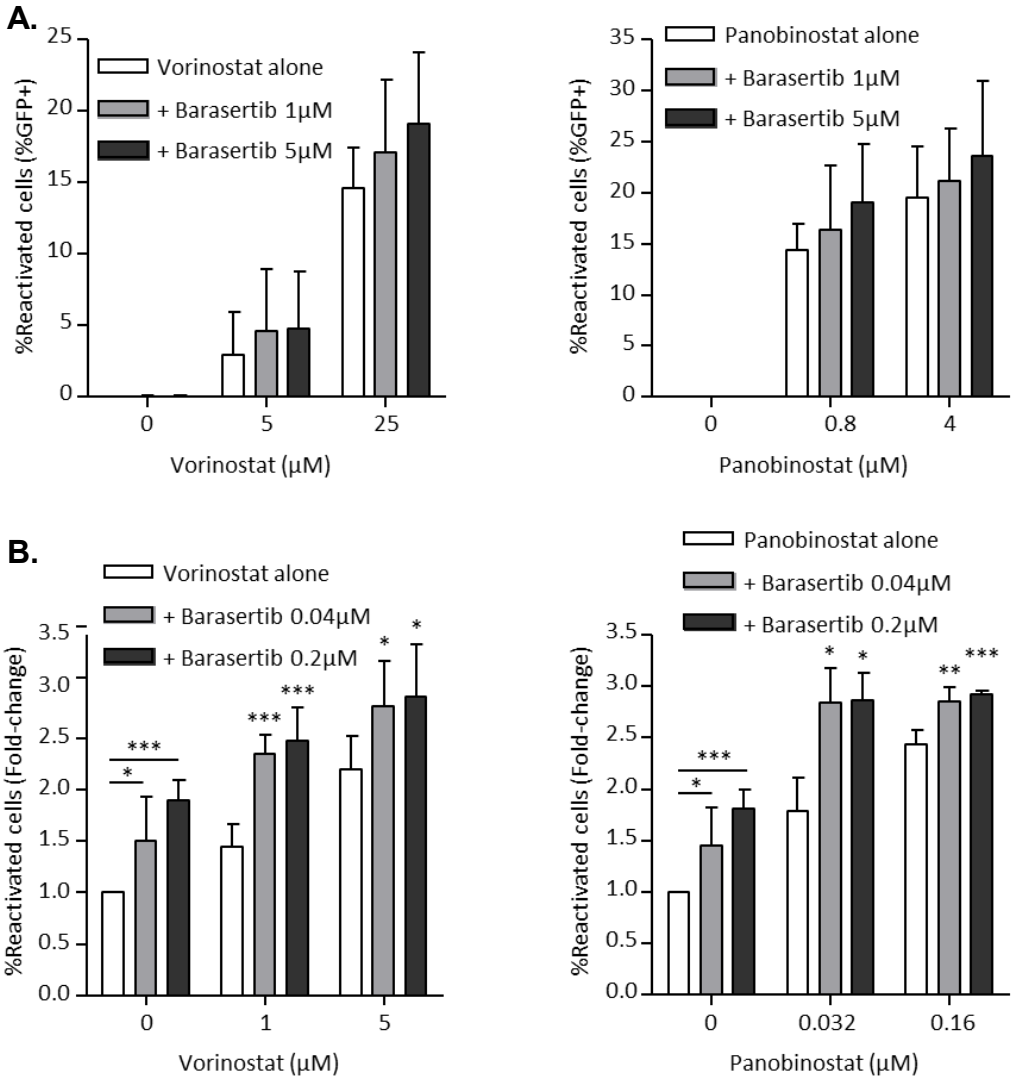


Figure 15. AURKB inhibition by barasertib increases the latency-reversing activity of the HDACi vorinostat and panobinostat. (A) HIV-1 reactivation in the clonal latency model J-Lat clone 9.2 cells in the presence of the HDACi vorinostat and panobinostat (left and right panel, respectively) in combination with the AURKi barasertib. Compounds were incubated together for 24h and HIV-1 reactivation was assessed by GFP expression in a flow cytometer. (B) HIV-1 reactivation of the non-clonal latency model J-Hig cells in the presence of vorinostat and panobinostat (left and right panel, respectively) in the presence of barasertib. Incubations and reactivation was performed and detected as in (A). Values represent mean±SD of at least three independent

experiments. Statistical significance was assessed by comparing each combination with the "only HDACi" condition at the same HDACi concentration. *p < 0.05; **p < 0.01; ***p < 0.001.

2.4.3 AURKi reactivated HIV-1 in a latency model of primary CD4+ T cells

To assess the latency-reversing activity of AURKi in latently infected primary cells, we used a model of memory CD4+ T cells from healthy donors, infected with a GFP-expressing virus. After sorting the cells by their GFP expression, the negative cells that included both non-infected and latently infected cells were incubated with the AURKi barasertib and MK-5108, the two most specific AURKi for AURKB and AURKA, respectively. Both compounds induced a dose-dependent reactivation of the latently infected cells comparable to that of cells stimulated with anti-CD3/anti-CD28 (**Figure 16A**). The reactivation activity of vorinostat and panobinostat was concomitant to their cytotoxic effect. Conversely, drug-induced toxicity was not observed with AURKi or the anti-CD3/anti-CD28 control at the concentrations required to reactivate HIV-1 (**Figure 16B**).

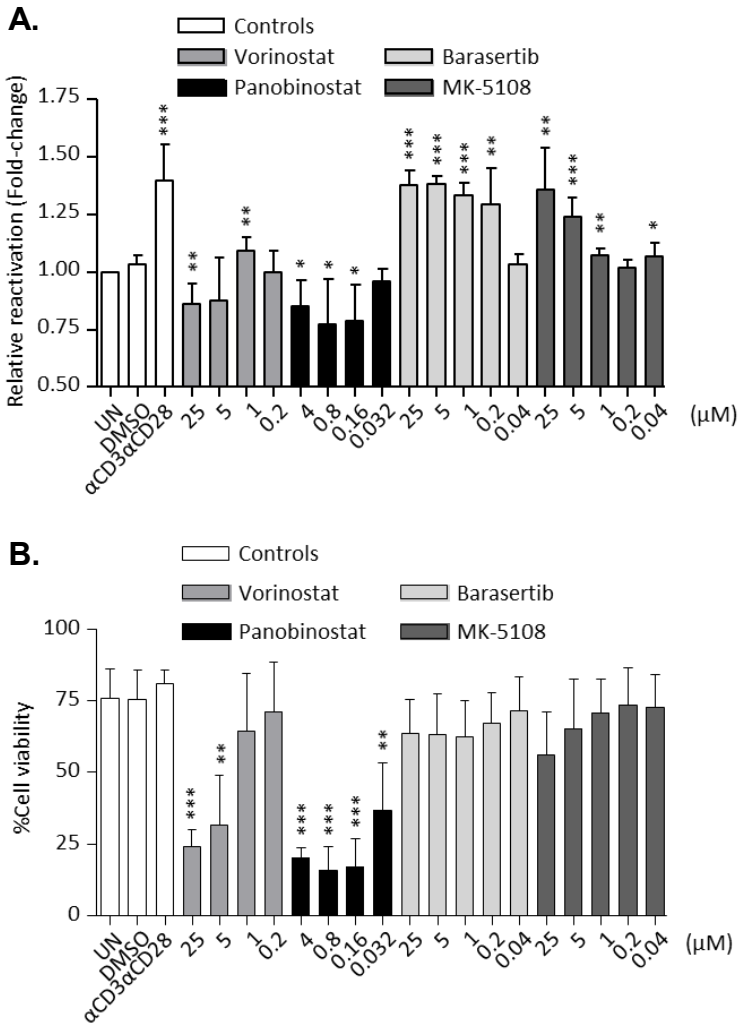


Figure 16. Aurora kinase inhibition reverts HIV-1 latency in a primary memory CD4+ T cell model. (A) HIV-1 reactivation induced by the AURKi barasertib and MK-5108 in latently infected memory CD4+ T cells from healthy donors. Cells were incubated for 24h with the AURKi compounds, in the presence of IL-2, and HIV-1 reactivation was assessed as the percentage of GFP by flow cytometry. HDAC inhibitors vorinostat and panobinostat were used as positive controls, as well as antibodies αCD3αCD28. (B) Cell viability in the conditions tested in (A). Viability was assessed as the percentage of negative cells stained by a cell death dye. A control just with DMSO at the same concentration than in the compounds tested was used to rule out unspecific toxicity. Values represent mean±SD of at least three independent experiments performed in triplicate. Statistical significance was assessed by comparing the treated conditions with the untreated control (UN). UN; untreated, DMSO; dimethyl sulfoxide. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

2.4.4 AURK inhibitors inhibit HIV-1 acute infection

In addition to the activity of AURKi as LRAs, the aurora kinase inhibitors were also tested in acute HIV-1 infection in CD4+ T and monocyte-derived macrophages (MDM) isolated

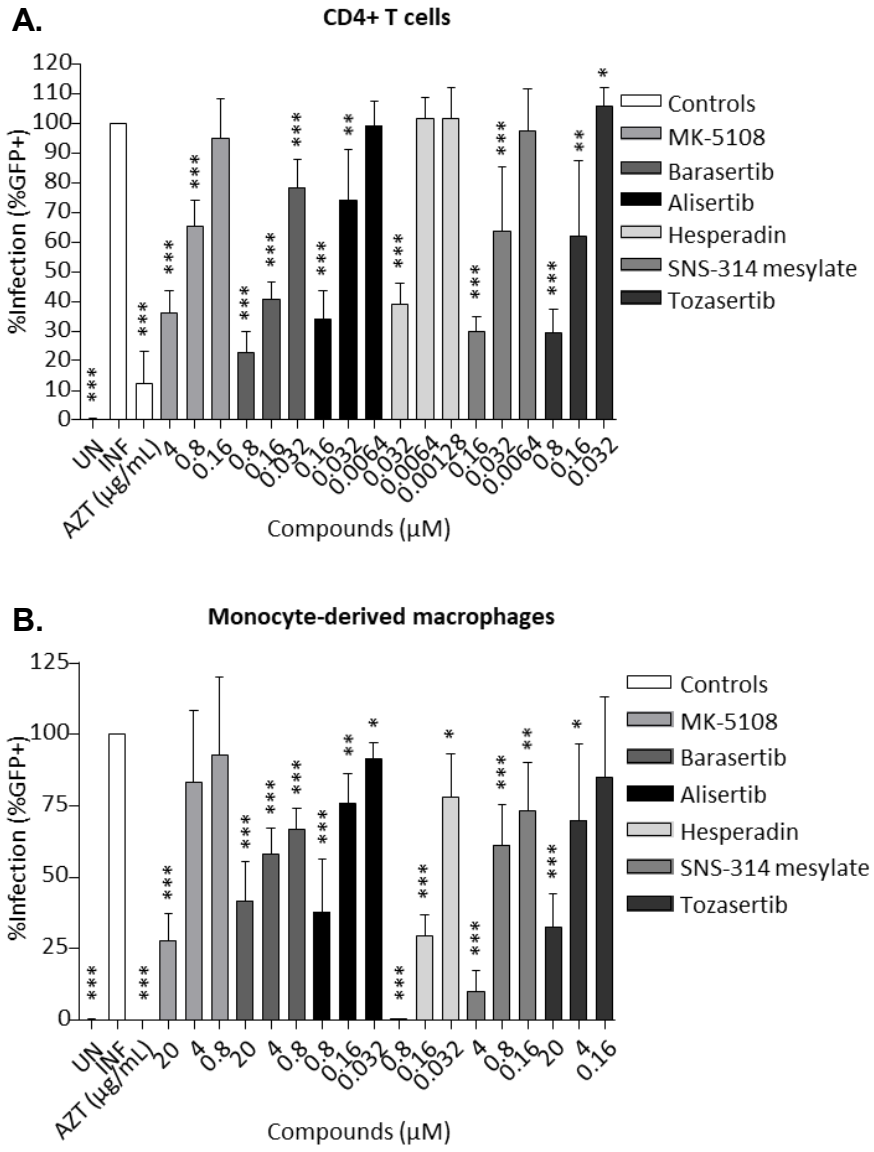


Figure 17. Inhibition of aurora kinases blocks HIV-1 replication in primary cells. (A) HIV-1 replication in primary CD4+ T cells isolated from PBMCs from healthy donors and activated with IL-2 and PHA for 72h. Cells were incubated with the compounds 24h prior infection, then they were infected with a GFP-expressing virus for over 48h. The whole experiment was performed in the presence of IL-2. HIV-1 infection was assessed as the percentage of GFP+ cells by flow cytometry. (B) HIV-1 replication in monocyte-derived macrophages stimulated with macrophage colony-stimulating factor (M-CSF) for 96h. Cells were isolated from PBMCs from healthy donors. Compounds and virus incubation, as well as HIV-1 replication, was assessed as in (A). Zidovudine (AZT) was used as a control and was added at the same time as the virus in both assays. Values represent mean±SD of at least three independent donors performed in triplicate. Statistical significance was assessed by comparing the treated conditions with the untreated, infected control (INF). UN; untreated, INF; infected, AZT; zidovudine. *p < 0.05; **p < 0.01; ***p < 0.001.

from healthy donors. The compounds, which were incubated for 24h prior infection, showed antiviral activity in both cell types (**Figure 17A and 17B**). Except for MK-5108 (EC_{50} 1.25 µM), all AURKi were active at sub-µM concentration in CD4+ T cells (**Table 3**). In MDM, EC_{50} values ranged from roughly 10.5 µM to less than 1 µM, being MK-5108 the least potent AURKi tested. Additionally, Vpx-dependent degradation of SAMHD1, did not affect the anti-HIV-1 potency of the compounds except for hesperadin and SNS-314 mesylate (**Figure 18 and Table 3**).

Table 3. EC50 values of que AURK inhibitors in primary CD4+ T cells and monocyte-derived macrophages

	EC_{50}* (µM)			
	CD4+ T cells	**Monocyte-derived macrophages**		
		+ SAMHD1	**- SAMHD1**	**Fold change**
AZT	-	0.004	0.05	13
MK-5108	1.25	10.46	11.92	1
Barasertib	0.11	8.95	18.98	2
Alisertib	0.08	0.48	0.71	1
Hesperadin	0.02	0.08	>0.8	>10
SNS-314 mesylate	0.06	1.13	>4	>4
Tozasertib	0.29	9.43	11.04	1

*EC50, concentration of a compound in which half its maximal response is achieved.

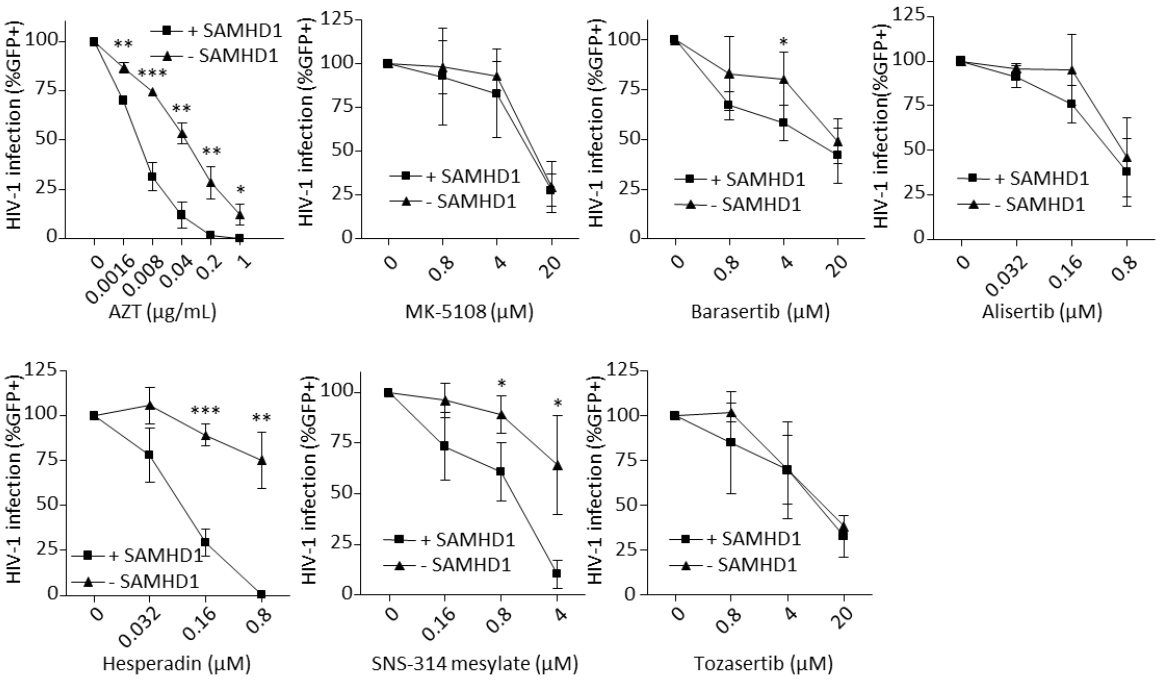


Figure 18. Some AURKi have their antiviral activity modulated by the HIV-1 restriction factor SAMHD1 in monocyte-derived macrophages. After M-CSF treatment of monocytes isolated from PBMCs from healthy donors for 96h, monocyte-derived macrophages were treated with the compounds. 24h post-treatment, cells were incubated with or without Vpx in order to degrade SAMHD1 and then were infected. Infections in the presence (+SAMHD1) or absence (-SAMHD1) of SAMHD1 were assessed as the percentage of GFP+ cells by flow cytometry and were then compared. Zidovudine (AZT) was used as a positive control, known to lose its antiviral activity in the absence of SAMHD1. Values represent mean±SD of at least three independent donors performed in triplicate. Statistical significance was assessed by comparing the same conditions with and without SAMHD1. AZT; zidovudine. *p < 0.05; **p < 0.01; ***p < 0.001.

2.4.5 The cell cycle is blocked by aurora kinase inhibitors in primary and immortalized cells

To further determinate the mechanism underlying the effect of aurora kinase, we evaluated the cell cycle progression in our experimental models of HIV-1 acute and latent infection. Primary CD4+ T cells and monocyte-derived macrophages were incubated with the test compounds, along with the HIV-1 latency model J-Hig cells, and their amount of DNA was quantified. In both primary cells, the AURKA inhibitor MK-5108 and the AURKB inhibitor barasertib induced a slight decrease in the percentage of cells in the G0-G1 phases and an increase in the cells in the G2-M phases in comparison to the untreated (UN) condition (**Figure 19A and 19B**). Likewise, AURKi prevented cell division in the immortalized and latently infected J-Hig cells. Treatment with AURKi

promoted an accumulation of cells in the G2-M phases, which is also seen as an accumulation of polyploid cells. The control panobinostat, a pan-HDAC inhibitor and known LRA, also increased the percentage of cells in the G2-M phase. However, it had little impact on the polyploid cell population, suggesting distinct mechanisms for the latency-reversing effects of HDACi and AURKi (**Figure 19C**).

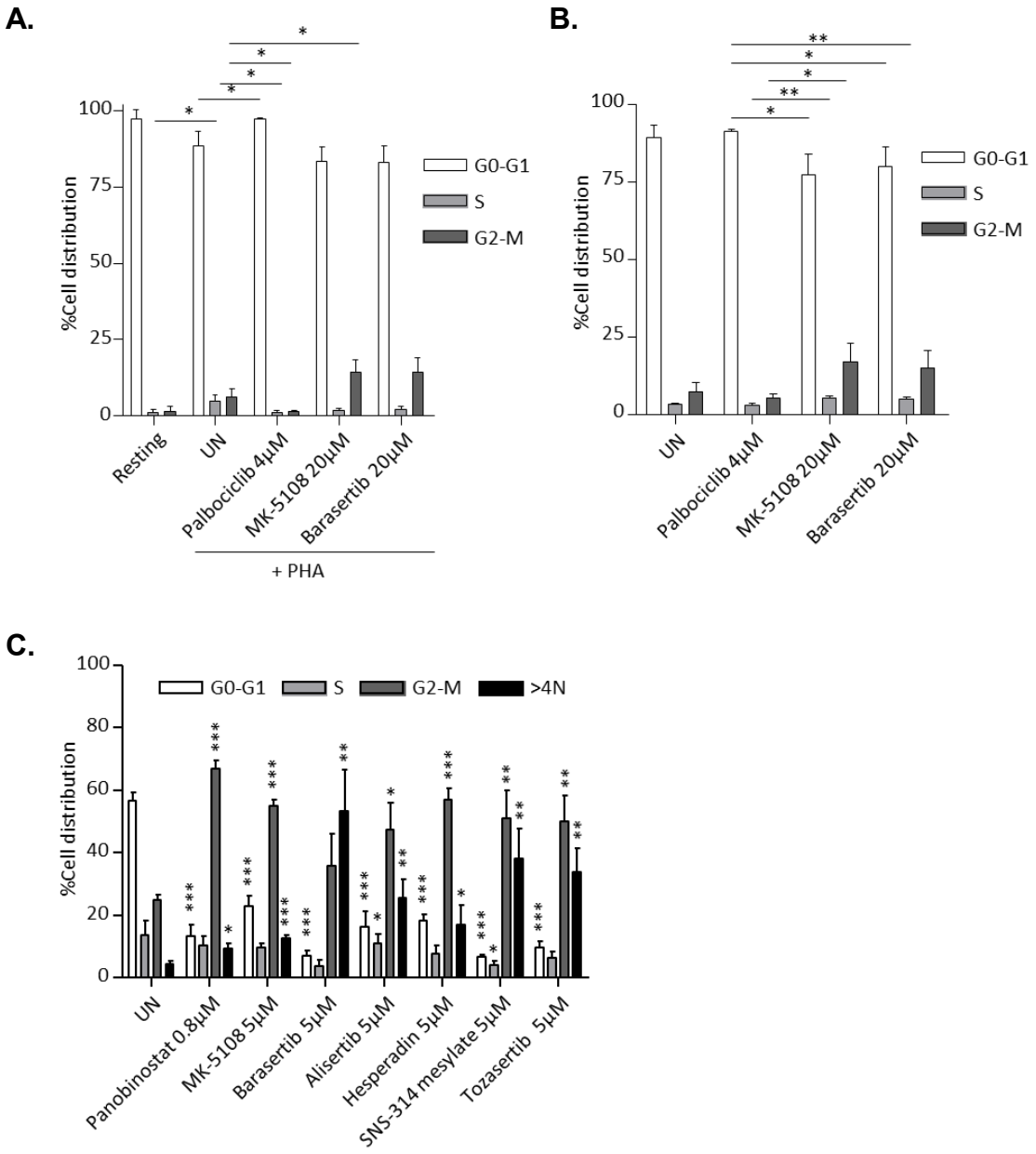


Figure 19. AURKi prevents cell division by accumulation of cells in G2-M phases or in a polyploid state. (A) Cell cycle distribution in primary CD4+ T cells isolated from PBMCs from healthy donors and activated with PHA and IL-2 for 72h. After activation, cells were incubated for 24h with the compounds and classified according their DNA content by flow cytometry (G0-G1, low DNA content; S, intermediate DNA content; G2-M, high DNA content/twice as G0-G1). A resting control without cell activation (no PHA incubation) was added. (B) Cell cycle

distribution in primary monocyte-derived macrophages, isolated from PBMCs from healthy donors and differentiated with M-CSF for 96h. After differentiation, cells were treated and classified as in (A). (C) Cell cycle distribution in the HIV-1 latency model J-Hig cells. Cells were treated and classified as in (A) and (B), however, cells with a DNA content higher than those labelled as in G2-M phases were classified as polyploid cells (>4N). Values represent mean ± SD of three independent donors/experiments in (A) and (C), while four independent donors were used in (B). Statistical significance was assessed by comparing each cell cycle phase to that of the untreated (UN) control. UN; untreated. $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

2.4.6 AURKi and antiretroviral compounds do not negatively interfere with each other when used in combination

To test whether the latency-reversing activity of the AURKi could be blocked by antiretroviral drugs or whether the activity of antiretroviral compounds could be interfered by AURKi, zidovudine (AZT, a reverse transcriptase inhibitor) and raltegravir (Ralt, an integrase inhibitor) were used in combination with the AURKi in models of HIV-1 latency and acute infection.

The latency-reversing activity of AURKi was not affected by AZT or Ralt (**Figure 20A and 20B**). The combination of AURKi with AZT or Ralt showed additive effect in their anti-HIV-1 activity (**Figure 20C and 20D**). These results suggest that AURKi do not interfere with the activity of antiretroviral agents and vice versa.

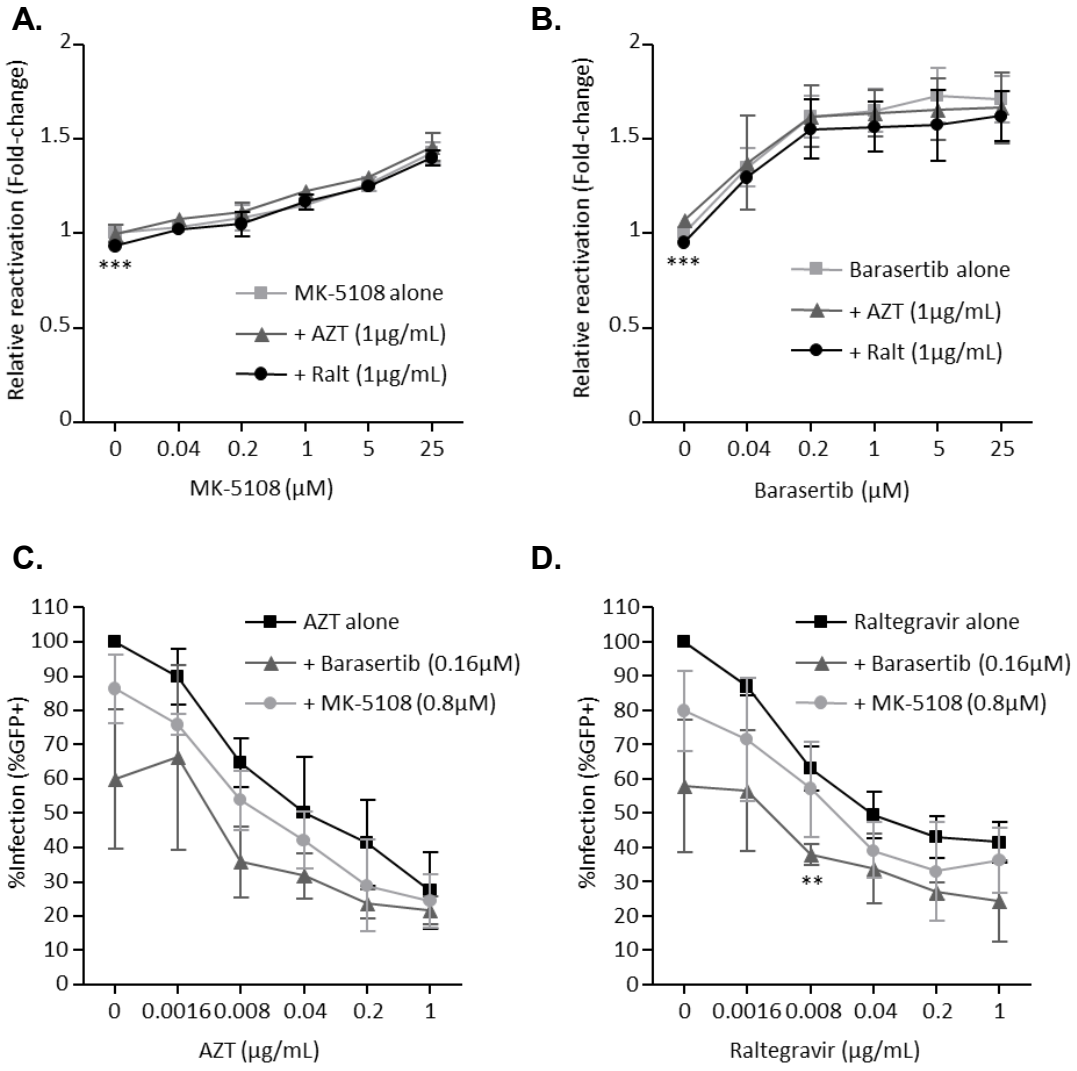


Figure 20. Aurora kinase inhibitors do not block the activity of antiretroviral compounds nor vice versa. (A, B) HIV-1 latency reversal in the J-HIG cells model triggered by the AURKAi MK-5108 (A) and AURKBi barasertib (B) in the presence of the antiretroviral compounds zidovudine (AZT) and raltegravir (Ralt). Cells were incubated for 24h with the compounds, which were added at the same time, and HIV-1 reactivation was assessed as percentage of GFP+ cells by flow cytometry and relativized to that of the untreated (MK-5108/barasertib alone, 0µM) control. (C, D) HIV-1 acute replication in primary CD4+ T cells isolated from PBMCs from healthy donors and activated with IL-2 and PHA for 72h. Cells were treated with the AURKi MK-5108 and barasertib for 24h prior incubation with zidovudine (C) or raltegravir (D) and the virus for 48h. HIV-1 replication was then assessed by flow cytometry as the percentage of GFP+ cells. Values represent mean ± SD of three independent experiments/donors performed in triplicate. Statistical significance was assessed by comparing the same conditions with and without SAMHD1 in (A) or comparing each combination to that of the AURKi alone in (A) and (B) or to that of the antiretroviral alone (C) and (D). AZT; zidovudine, Ralt; raltegravir. **$p < 0.01$; ***$p < 0.001$.

2.5 DISCUSSION

Despite all efforts, HIV-1 latency is still one of the major barriers to an effective cure for HIV-1 infection. Known LRA used in Shock and Kill strategies have failed to effectively reduce the HIV proviral load in HIV+ individuals[178]. Furthermore, the apparent association of LRA activity and the specificity of the HIV-1 integration site(s)[186] as opposed to the multiple sites where HIV-1 may integrate[235] signifies the need to find new LRAs.

Until now, most of LRAs have not been originally proposed for HIV-1 reactivation, including anti-tumoral compounds such as HDACi[236] or the recently FDA-approved compound for acute myeloid leukemia, midostaurin[230]. In this study, we used an anti-tumoral compound library in order to find novel LRAs. Along with known LRAs such as the HDACi, we have found other anti-tumoral drugs able to induce HIV-1 reactivation. In here, we focused on the aurora kinase inhibitors, compounds able to cooperate with HDACi suggesting a distinct mode of action.

The aurora kinases are a family of proteins that have been considered as potential anticancer agents, as these serine/threonine kinases are required for the control of the mitosis and meiosis. Aurora kinase A (AURKA) function is mainly associated to the centrosome maturation and separation and the bipolar spindle assembly, although it can also control the mitotic entry through the indirect activation of Cyclin-B/CDK1[237]. Inhibition of AURKA causes defects in cell cycle progression and in several mitosis-related processes[238,239]. Aurora kinase B (AURKB) phosphorylates histone H3 and Variant centrosome protein A during the early G2 step of the cell cycle, leading to condensation of chromatin[240]. AURKB interacts with essential proteins affecting chromosome segregation[241,242]. Inhibition of AURKB induces grow arrest, leading to G2/M accumulation[243] or even to the formation of 8N polyploid cells[244] by blocking chromosome segregation during mitosis and cytokinesis, which has also been observed in our study (**Figure 19**).

The tumor suppressive effect of Aurora B and HDAC inhibition is due to the induction of cell cycle arrest and/or apoptosis but they act synergistically as tumor suppressors by cooperatively regulating distinct steps of the cell cycle[243,245]. Here, we show that AURK

inhibitors are effective inducers of HIV-1 reactivation able to cooperate with HDAC inhibitors panobinostat or vorinostat, suggesting that the mechanisms associated to tumor suppression are, in fact, also associated to reverting HIV-1 latency.

HIV-1 integrates into host chromatin to transcribe and replicate. HIV-1 preferentially integrates into intronic regions of actively transcribed genes, as well as regions within or nearby Alu elements throughout the human genome[37,246]. Because HIV integration is a stochastic event and the integration site may influence the responsiveness to T cell activation signals or chromatin-modifying agents[235,247,248], cooperativity between AURK inhibitors and other LRA may just be a reflection of their distinct capacity to reactivate proviruses at different integration sites. AURKA inhibitor MK-5108 and AURKB inhibitor barasertib induced a modest reactivating effect in the ACH-2 cells (**Suppl. Figure 6**) with only two major integration sites[249]. Furthermore, in lymphocyte cell lines carrying a single latent provirus like the J-Lat clone 9.2 cells[210], the AURK inhibitors tested were unable to induce HIV-1 reactivation by themselves, even though they were able to do it in non-clonal models such as the J-Hig cells or the latently infected memory CD4+ T cells. Although speculative, our data suggest that AURKi may present certain selectivity, as has been previously suggested for other LRAs[186,235], towards specific HIV-1 integration sites that may have been present in the non-clonal models but not in the J-Lat clone 9.2 cells.

Additionally, AURKi were also able to inhibit HIV-1 replication in CD4+ T cells and macrophages, suggesting a more complex role for the aurora kinases regarding the viral cycle. The observation that Vpx-dependent SAMHD1 degradation did not affect AURKi potency, opposed to that of AZT, suggests that the antiviral mechanisms of both compounds are distinct. Off-targets reported for hesperadin and SNS-314 mesylate, which partially lost their potency depending on SAMHD1 expression, may explain the changes in SAMHD1-dependent anti-HIV-1 activity but reflect the complex interplay between cycle progression and virus replication.

Because AURK inhibitors are able to affect centromere function[239], our results may indicate that viral integration at the centromere or chromosome perinuclear sites with low transcriptional activity may be preferentially affected by this class of agents[250].

In conclusion, we identified the aurora kinase family as a novel target for HIV-1 reactivation. Aurora kinase inhibitors were able to reactivate the HIV-1 reservoir and also to inhibit HIV-1 replication by a SAMHD1-independent pathway. Their ability to cooperate with other LRAs like the HDACi, along with the centromere-related function of the aurora kinases, suggests a different integration site specificity that may involve the centromeres. Thus, making the aurora kinase inhibitors attractive candidates in potential LRAs cocktails aimed at the reactivation of the whole HIV-1 latent reservoir.

2.6 ACKNOWLEDGMENTS

We thank the National Institutes of Health (AIDS Research and Reference Reagent Program) for the reagents and the Flow Cytometry facility of the Institute Germans Trias i Pujol (IGTP) for the cell separation assays.

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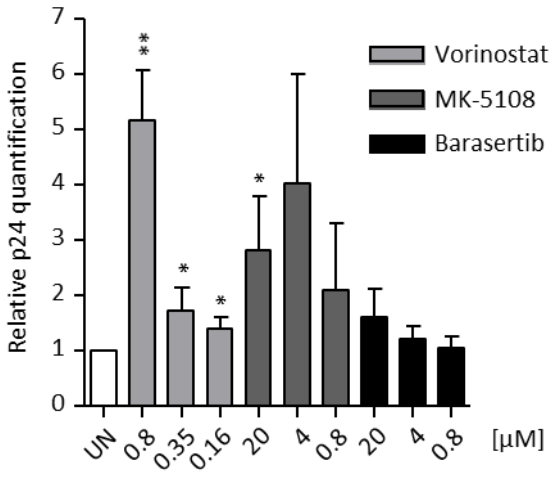
2.7 SUPPLEMENTAL MATERIAL

Supplemental table 1. List of the compounds tested in the LRA screening that classified as hits or pseudo-hits, their reactivation values and their main target

Compound	Mean relative reactivation*	SD	Main target**
Hits			
AMG-900	1.89	0.30	Aurora Kinase
Barasertib (AZD1152-HQPA)	1.80	0.06	Aurora Kinase
Tozasertib (VX-680, MK-0457)	1.70	0.03	Aurora Kinase
Trichostatin A (TSA)	2.06	0.16	HDAC
Abexinostat (PCI-24781)	2.01	0.25	HDAC
Belinostat (PXD101)	1.98	0.20	HDAC
Vorinostat (SAHA, MK0683)	1.97	0.15	HDAC
Quisinostat (JNJ-26481585) 2HCl	1.90	0.19	HDAC
Pracinostat (SB939)	1.82	0.13	HDAC
CUDC-101	1.76	0.32	HDAC
TPCA-1	1.84	0.16	IκB/IKK
BX-795	1.80	0.06	IκB/IKK
AZD1480	1.86	0.13	JAK
Fedratinib (SAR302503, TG101348)	1.80	0.27	JAK
Midostaurin	1.80	0.16	Protein kinase inhibitor
Geldanamycin	1.64	0.25	HSP
Pseudo-hits			
Alisertib (MLN8237)	1.62	0.02	Aurora Kinase
SNS-314 Mesylate	1.55	0.25	Aurora Kinase
MK-5108	1.51	0.11	Aurora Kinase
ENMD-2076	1.44	0.22	Aurora Kinase
Bleomycin Sulfate	1.59	0.23	DNA/RNA Synthesis
Floxuridine	1.58	0.10	DNA/RNA Synthesis
Cladribine	1.58	0.19	DNA/RNA Synthesis
Gemcitabine	1.52	0.10	DNA/RNA Synthesis
Raltitrexed	1.51	0.12	DNA/RNA Synthesis
Nelarabine	1.51	0.11	DNA/RNA Synthesis
Mitomycin C	1.50	0.16	DNA/RNA Synthesis
Chloroambucil	1.43	0.11	DNA/RNA Synthesis
Fludarabine	1.41	0.13	DNA/RNA Synthesis
LY2874455	1.51	0.27	FGFR,VEGFR
Tivozanib (AV-951)	1.44	0.06	PDGFR,VEGFR
4SC-202	1.54	0.21	HDAC
Mocetinostat (MGCD0103)	1.41	0.08	HDAC
CH5138303	1.51	0.01	HSP
Luminespib (AUY-922, NVP-AUY922)	1.50	0.06	HSP

Compound	Mean relative reactivation*	SD	Main target**
Ganetespib (STA-9090)	1.49	0.05	HSP
VER-50589	1.45	0.04	HSP
PU-H71	1.42	0.08	HSP
HSP990 (NVP-HSP990)	1.42	0.14	HSP
Alvespimycin (17-DMAG) HCl	1.41	0.13	HSP
ARQ 621	1.55	0.37	Kinesin
GSK923295	1.48	0.24	Kinesin
Cucurbitacin B	1.63	0.22	Microtubule Associated
Nocodazole	1.54	0.19	Microtubule Associated
Epothilone A	1.50	0.05	Microtubule Associated
Docetaxel	1.46	0.06	Microtubule Associated
Lexibulin (CYT997)	1.44	0.13	Microtubule Associated
HS-173	1.51	0.27	PI3K
Buparlisib (BKM120, NVP-BKM120)	1.42	0.11	PI3K
Ro3280	1.51	0.21	PLK
GSK461364	1.44	0.13	PLK
MLN9708	1.55	0.16	Proteasome
Ixazomib (MLN2238)	1.42	0.12	Proteasome
Teniposide	1.61	0.06	Topoisomerase
Irinotecan HCl Trihydrate	1.61	0.10	Topoisomerase
Etoposide	1.60	0.05	Topoisomerase
AZD7762	1.53	0.11	Chk
Methotrexate	1.44	0.06	DHFR
Azacitidine	1.50	0.33	DNA Methyltransferase
PND-1186 (VS-4718)	1.49	0.14	FAK
JNK Inhibitor IX	1.45	0.12	JNK
KX2-391	1.52	0.19	Src

*****Relative reactivation relativized to the untreated conditions.
**According to the manufacturer.



Supplemental figure 6. Aurora kinase inhibitors are able to mildly reactivate the clonal model for HIV-1 latency, ACH-2 cells. HIV-1 reactivation induced by the AURK inhibitors MK-5108 and barasertib was assessed after 48h of incubation. Cell supernatant was then harvested and HIV CAp24 antigen was quantified by ELISA and relativized to the untreated control (UN). The HDACi vorinostat was used as a positive control. Viability of the treated ACH-2 cells was performed in parallel according to forward and side laser light scatter flow cytometry analysis to ensure that the conditions were subtoxic. Values represent mean ± SD of three independent experiments. Statistical significance was assessed by comparing the conditions with latently infected untreated control (UN). *$p < 0.05$; **$p < 0.01$.

CHAPTER 3

DUAL EFFECT OF THE BROAD SPECTRUM KINASE INHIBITOR MIDOSTAURIN IN ACUTE AND LATENT HIV-1 INFECTION 1

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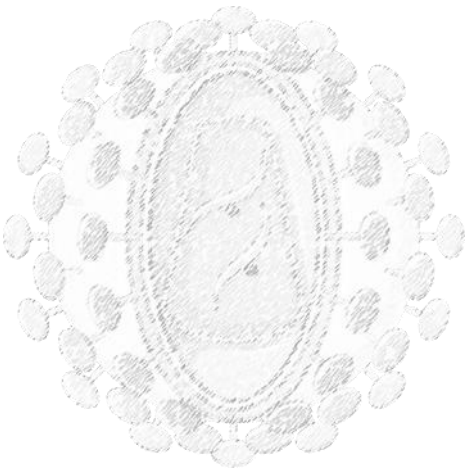
Antiviral Research, **168:18-27. 2019.**



3.1 ABSTRACT

Midostaurin is a multi-kinase inhibitor with antineoplastic activity. We assessed the capacity of midostaurin to affect early and late steps of HIV-1 infection and to reactivate HIV-1 latently infected cells, alone or in combination with histone deacetylase inhibitors (HDACi) known to act as latency-reversing agents (LRA). Acute HIV-1 infection was assessed by flow cytometry in three cell types treated with midostaurin in the presence or absence of SAMHD1. Non-infected cells were treated with midostaurin and harvested for Western blot analysis. Macrophage infections were also measured by quantitative RT-PCR. HIV-1 latency reactivation was assessed in several latency models. Midostaurin induced G2/M arrest and inhibited CDK2, preventing the phosphorylation of SAMHD1 associated to inhibition of its dNTPase activity. In the presence of SAMHD1, midostaurin blocked HIV-1 DNA formation and viral replication. However, following Vpx-mediated SAMHD1 degradation, midostaurin increased viral transcripts and virus replication. In three out of four HIV-1 latency models, including primary CD4+ T cells, midostaurin effectively reversed HIV-1 latency and was synergistic in combination with LRA vorinostat and panobinostat. Our study describes a dual effect for midostaurin in HIV-1 infection, antiviral or proviral depending on SAMHD1 activation, and highlights a role for active SAMHD1 in regulating the activity of potential HIV-1 latency reversal agents.

3.2 INTRODUCTION

Highly active therapy of the human immunodeficiency virus (HIV) has transformed a terminal illness into a manageable disease[101]. However, a cure for HIV has yet to be found. A major hurdle to the eradication of HIV infection is the persistent infection through a latent virus reservoir for which current therapy is ineffective. Purging of latent reservoir through latency reversing agents (LRA), followed by selective destruction of infected cells, the so called shock and kill has been thought as a possible strategy. Despite current LRA showing efficacy in reactivating HIV-1, so far all efforts have failed to reduce the latent virus reservoir in infected individuals[190,251]. There is a critical need for compounds that not only potently reactivate latently infected cells, but also lead to

the death of these reactivated cells[190] while preventing further rounds of infection of bystander cells.

Recent studies have shown that agents targeting protein kinase (PK) C are highly potent in inducing latent HIV-1 expression from the viral reservoirs[252–254] and protecting primary CD4+ T cells from HIV-1 infection through down-modulation of their HIV coreceptor expression[190,255–257]. There are multiple molecular mechanisms that contribute to the establishment of HIV latency, such as the down-regulation of transcription factors required for transcription like nuclear factor κB (NF-κB)[258,259] or the chromatin remodeling by histone deacetylases (HDAC), whose inactivation by HDAC inhibitors, or other types of agents[260], boost HIV-1 reactivation[261]. Therefore, a combination of compounds targeting different mechanisms may have synergistic effects in activating latent HIV-1 expression.

Midostaurin (PKC142) is a tyrosine kinase inhibitor, including the FMS-like tyrosine kinase 3 (FLT3), and was approved in 2017 by the FDA for the treatment of acute myeloid leukemia (AML) with mutations on FLT3[262,263]. However, midostaurin can also inhibit a broad spectrum of serine/threonine kinases, including cyclin-dependent kinases (CDK 1 and 2)[264]. Inhibition of CDK modulates the activation of the HIV-1 restriction factor SAMHD1[77], controlling the dNTP pool required for HIV-1 reverse transcription[203,265,266]. CDK inhibitors have been revealed as potent antiviral agents through the activation of SAMHD1-dependent degradation of dNTPs[266,267]. Inhibition or down-regulation of SAMHD1 allows for effective virus replication in non-proliferating and resting CD4+ T cells[78,79,82]. Additionally, SAMHD1 has been shown to modulate the reactivation of HIV-1 latency in CD4+ T cells[268]. Thus, we hypothesized that midostaurin may effectively exert diverse effect on HIV-1 replication and reactivation from latency.

Here, we show the dual effect of midostaurin in acute and latentHIV-1 infection. These findings provide evidence for the development of a new anti-HIV strategy, aimed at simultaneously reactivating HIV-1and preventing further rounds of infection through the selective modulation of SAMHD1 function.

3.3 MATERIALS AND METHODS

3.3.1 Cells

Peripheral blood mononuclear cells (PBMC) from buffy coats of healthy donors were obtained by Ficoll-Paque density gradient centrifugation and used for fresh purification of CD4+ T lymphocytes, naïve CD4+ T lymphocytes or monocytes by negative selection (StemCell Technologies). Purity of the populations was confirmed by flow cytometry. Buffy coats were purchased anonymously from the Catalan Banc de Sang i Teixits http://www.bancsang.net/en/index.html). The buffy coats received were totally anonymous and untraceable and the only information given was whether or not they have been tested for disease. CD4+ T lymphocytes were kept in complete culture medium (RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 100 U/ml penicillin, 100 µg/mL streptomycin (Gibco, Life Technologies)) and rIL-2 (6.5 IU/mL, Roche). Monocytes were cultured in complete culture medium and differentiated to monocyte-derived macrophages (MDM) for 4 days in the presence of monocyte-colony stimulating factor (M-CSF, Peprotech) at 100 ng/mL. The human cell lines ACH-2[209], Jurkat (J-Lat)[210] clone 9.2, THP-1, HEK293T and MT-4 cells were obtained from AIDS Reagent Program, National Institutes of Health (Germantown, MD). All cell lines were grown in complete culture medium, as the primary cells, and maintained at 37 °C in a 5% CO2 incubator.

3.3.2 Viruses and virus infections

R5-tropic HIV-1 strain BaL was grown in stimulated PBMC and titrated for use in MDMs. The HIV-1 viral strain NL4–3, expressing green fluorescent protein (GFP), was obtained from the MRC Centre for AIDS Reagents (London, UK). The envelope-deficient HIV-1 NL4-3 clone (HIG) encoding internal ribosome entry site (IRES)-green fluorescent protein (GFP) (NL4-3-GFP) was pseudotyped with vesicular stomatitis virus G protein (VSV-G) by cotransfection of HEK293T cells using polyethylenimine (Polysciences) as previously described[208,269]. For the production of viral-like particles carrying Vpx (VLP-Vpx), HEK293T cells were cotransfected with pSIV3+ (Mangeot et al., 2000) and a VSV-G–expressing plasmid. Three days after transfection, supernatants were harvested, filtered, and stored at −80 °C. The protocol for production of HIV-1 NL4-3 virus modified

to carry Vpx (NL4-3*Vpx) was performed as described previously[79]. In some cases, viral stocks were concentrated using a Lenti-X concentrator (Clontech).

MDM were infected with HIG in the presence or not of VLP-Vpx and viral replication was measured 48 h later by quantification of GFP+ expression by flow cytometry. Alternatively, macrophages were infected with HIV-1 BaL in the presence or not of VLP-Vpx. There is a lag time during which completion of HIV-1 reverse transcription and/or integration is not detectable in MDMs[270,271]. Thus, viral replication was measured 18 h later for total viral DNA, 42 h after infection for integrated viral DNA and 66 h after infection for viral transcripts. Viral replication was quantified by two-step quantitative real-time PCR (qPCR).

THP-1 (SAMHD1+) and MT-4 (SAMHD1-) cells were infected with HIG and transduced with VLP-Vpx. Viral replication was measured 48 h later for the MT-4 and 72 h later for the THP-1 cells. Viral replication was quantified as percentage of GFP expression by flow cytometry.

3.3.3 Generation of latently infected cells

Latently infected cells (J-Hig) were generated following a modified protocol[66]. Briefly, cells were generated after acute infection of CD4+ Jurkat cells with HIG and maintained in culture for 10 days to allow for the attrition of productively infected cells.

Latently infected primary CD4+ T cells were generated according to the cytokine-polarized primary T cells model of latency[212,234] with few modifications. Briefly, naïve CD4+ T cells were activated with αCD3/αCD28 antibodies (1 µg/mL each; BD, Madrid, Spain) and supplemented with TGFβ1 (10 µg/mL, Peprotech), αIL-12 (2 µg/mL) and αIL-4 (1 µg/mL, Peprotech). Medium supplemented with rIL-2 (6.5 IU/mL, Roche) was replaced every 3 days. After 7 days of activation, CD4+ T cells were infected with NL4-3*Vpx by spinoculation (1200 x g, 1 h 30 min at 37 °C). Three days later, GFP negative cells containing both latently infected and uninfected cells were sorted using a FACSAria II flow cytometer (BD Biosciences).

3.3.4 Compounds

Midostaurin was purchased from Sigma-Aldrich, vorinostat (VOR) was purchased from Prochifar srl (Italy) and panobinostat (PNB) was purchased from LC Laboratories. Antiretroviral agent 3-azido-3-deoxythymidine (zidovudine; AZT) was obtained from the NIH AIDS Research and Reference Reagent Program. The integrase inhibitor raltegravir (RAL) was received from Merck Sharp and Dome (MSD, Spain). All compounds were resuspended in DMSO and stored at −20 °C until use.

3.3.5 Immunoblot

Treated cells were rinsed, lysed, subjected to SDS-PAGE and transferred to a PVDF membrane as previously described[234]. The following antibodies were used for immunoblotting: anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:5000; Pierce); anti-human Hsp90 (1:1000; 610418, BD Biosciences); anti-SAMHD1 (1:2500; ab67820; Abcam); anti-GAPDH (1:2500; ab9485; Abcam); anti-phospho-pRB (Ser807/811; 9308); antipRB (9309); anti–phospho-CDK2 (Thr160; 2561), anti-CDK2 (2546); anti-phospho-SAMHD1 (Thr592; 15038) and anti-p21 (2947) (all 1:1000; Cell Signaling Technologies).

3.3.6 Flow cytometry

For evaluation of cell death, cells were stained for 30 min with LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (Invitrogen, Thermo Fischer Scientific) in PBS according to manufacturer's instructions. Alternatively, viable cells were identified according to forward and side laser light scatter flow cytometry analysis as described[201]. Cells were washed and fixed in 1% formaldehyde before the analysis.

For cell cycle analysis, cells were treated with 7-aminoactinomycin D (7AAD; Sigma-Aldrich) and pyronin Y (Sigma-Aldrich) as described previously[201,208]. Flow cytometry assays were performed in a FACS LSR II or a FACSCanto II flow cytometer (BD Biosciences). The data was analyzed using the FlowJo software (BD Biosciences).

The cell separation of the latently infected primary naive CD4+ T cells was performed by the Flow Cytometry facility at the Germans Trias i Pujol Research Institute, using a FACSAria II cell separator (BD Biosciences).

3.3.7 Quantification of total viral DNA, integrated provirus and viral transcripts

MDM were infected with HIV-1 BaL and infections were stopped after 18 h to measure total viral DNA or at 42 h to measure viral integration by two-step quantitative Real-Time PCR as previously described[272]. LTR amplification for total and integrated DNA was performed using the following primers and probe: forward 5'-GACGCAGGACTCGGCTTG-3', reverse 5'-ACTGACGCTCTCGCACCC-3', and probe FAM 5'-TTTGGCGTACTCACCAG-3' TAMRA. A pre-amplification Alu-LTR was performed for the integrated DNA with the following primers: forward 5'-GCCTCCCAAAGTGCTGGGATTACAG-3' and reverse 5'-TTGCCCATACTATATGTTTTAA-3. The reverse transcriptase (RT) inhibitor zidovudine (1 µg/mL) and the integrase inhibitor raltegravir (1 µg/mL) were used as negative controls for the total DNA and integrated DNA, respectively.

HIV-1 transcripts were quantified after the reverse transcription using the following primers and probe: forward 5'-GGATCTGTCTCTGTCTCTCTCTCCACC-3', reverse 5'-ACAGTCAGACTCATCAAGTTTCTCTATCAAAGCA-3' and the dual-labeled fluorescent probe FAM 5'-TTCCTTCGGGCCTGTCGGGTCCC-3' TAMRA. All infections were normalized to an untreated control in the presence or absence of VLP-Vpx. All samples were run in duplicate on a 7500 Real-Time PCR System (Applied Biosystems) Real-Time PCR instrument. Cycling conditions were as follows: 50 °C for 2 min followed by 95 °C for 10 min for polymerase activation, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min.

3.3.8 HIV-1 reactivation in vitro in latently infected cells

HIV-1 reactivation was measured as described before[212,234]. Briefly, clonal J-Lat or J-Hig cells were incubated for 24 h with subtoxic concentrations of midostaurin (**Suppl. Table 2**). LRAs panobinostat and vorinostat were used as controls for HIV-1 reactivation. Reactivation of HIV-1 was monitored as the percentage of living GFP positive cells according to forward and side laser light scatter flow cytometry analysis in a FACS LSRII flow cytometer (BD Biosciences). The data were analyzed using the FlowJo software. Similarly, ACH-2 cells, a T cell latent model with one integrated proviral copy, were cultured for 48 h in the presence or absence of LRA and reactivation was measured by

the production of HIV CAp24 antigen using Genscreen HIV-1 Ag ELISA (BioRad) according to manufacturer's instructions.

Sorted latently infected/GFP negative naive CD4+ T cells were incubated for 24 h with subtoxic concentrations of midostaurin (**Suppl. Table 2**). Treatment with anti-CD3 and anti-CD28 was used as reactivation control. Previously, cells were washed with PBS after the sorting and kept in fresh media containing rIL-2 overnight at 37 °C and 5% CO2. Reactivation was measured as the percentage of GFP positive cells by flow cytometry.

3.3.9 Statistical and mathematical analysis

Combination index (CI) was calculated by introducing the reactivation percentages in the CompuSyn software (ComboSyn Inc., Paramus, NJ, USA). Synergistic effect was considered when CI values were below 1. Data are presented as mean ± standard deviation (SD). All p-values were calculated using a t-Student's test calculated with the GraphPad PRISM software (GraphPad Software, San Diego, CA, USA). A p-value of 0.05 was considered to be statistically significant.

3.4 RESULTS

3.4.1 Midostaurin-mediated modulation of cell cycle prevents phosphorylation and inhibition of SAMHD1 in primary macrophages

To confirm the effect of midostaurin in cycle progression[264,273,274], MDM were evaluated for DNA and RNA content as a measure of the distinct cell phases: G0, G1 and G2-M. In comparison to the untreated control (6.8%), a higher percentage of MDM treated with midostaurin (12.5%) were stopped in the G2-M phase (**Figure 21A**), as observed by the increase DNA and RNA content. In parallel, protein from 24 h-treated macrophages was harvested for Western-blot analysis. Midostaurin significantly impaired CDK2 activation, as measured by specifically detecting phosphorylation of CDK2 (pCDK2) or by the disappearance of the corresponding band with an anti-CDK2 antibody (**Figure 21B**). Additionally, midostaurin decreased the expression of p21, a natural CDK2 inhibitor, known to affect SAMHD1 function[275,276]. These changes were accompanied by an

expected inhibition of the phosphorylated forms of pRB and SAMHD1 (**Figure 21C**), both targets of CDK2.

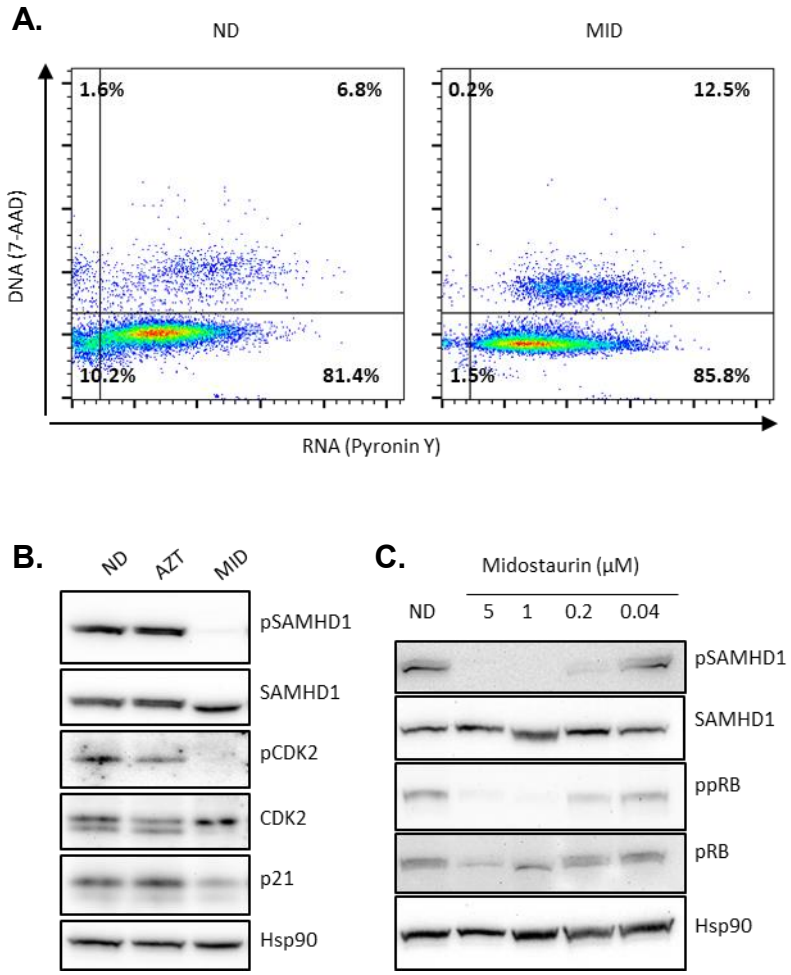


Figure 21. Effect of midostaurin on cell cycle progression. (A) Cell cycle of treated primary monocyte-derived macrophages (MDM) untreated or treated for 24 h with midostaurin at 5 µM was characterized in a flow cytometer by staining RNA with pyronin Y and DNA with 7-aminoactinomycin D (7AAD). Cells in G0 phase are the ones with low amount of RNA and DNA (lower-left quadrant). In G1 phase the RNA is increased (lower -right quadrant), while in G2-M phase both RNA and DNA are increased (upper-right quadrant). Numbers in each quadrant represent the percentage of the population located in that phase. (B) Expression of cell cycle proteins from 24 h MDM untreated or treated with zidovudine (AZT; 0.2 µM) or midostaurin (MID; 5 µM) was assessed by immunoblot. p21, phospho-CDK2 (pCDK2, active form), CDK2, SAMHD1 and phospho-SAMHD1 (pSAMHD1, inactive form) expression levels were analyzed. Hsp90 was used as a loading control. (C) MDM untreated or treated with midostaurin at different concentrations (5–0.04 µM) for 24 h were harvested for protein analysis by immunoblot. pRB, phospho-pRB (ppRB), SAMHD1 and pSAMHD1 expression levels were analyzed. Hsp90 was used as a loading control. A representative experiment is shown. ND; No drug, AZT; zidovudine, MID; midostaurin

3.4.2 Midostaurin effect in acute HIV-1 infection is dependent on SAMHD1 expression

We have previously shown that pharmacological inhibition of CDK prevents SAMHD1 phosphorylation and blocks HIV-1 replication[73,266]. In primary MDM, midostaurin showed anti-HIV-1 activity (50% effective concentration, EC50: 0.416 µM) at subtoxic concentrations (**Suppl. Table 2**) that was abrogated following Vpx-dependent degradation of SAMHD1 (**Figure 22A and 22B**), similarly to the CDK4/6 inhibitor palbociclib[266]. However, a significant (p=0.04 at 1 µM) stimulatory effect on HIV-1 replication was revealed with midostaurin in the absence of SAMHD1, not observed with palbociclib (**Figure 22C**).

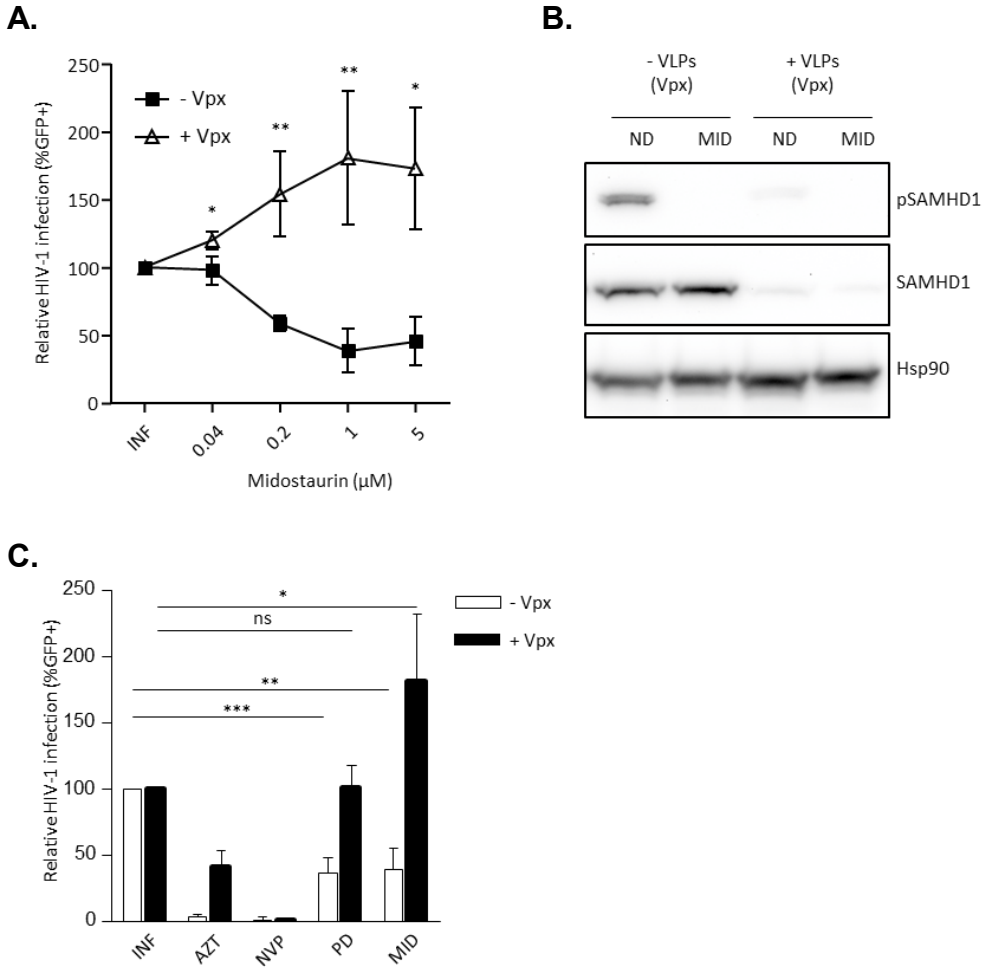


Figure 22. SAMHD1 expression modulates the effect of midostaurin in acute HIV-1 infection in monocyte-derived macrophages. (A) HIV-1 infection in the presence of midostaurin (5–0.008 µM) in untreated MDM (- Vpx) or Vpx-induced degradation (+Vpx) of SAMHD1 MDM. (B) SAMHD1 and pSAMHD1 expression levels in MDM untreated (ND) or treated with midostaurin (MID; 5 µM) in the presence or not of viral-like particles carrying Vpx (+/− Vpx). Hsp90 was used

as a loading control. (C) Effect of zidovudine (AZT; 0.2 µM), nevirapine (NVP; 5 µM), palbociclib (PD; 5 µM) and midostaurin (MID; 1 µM) in the presence (- Vpx) or absence (+Vpx) of SAMHD1. Infection was quantified as percentage of GFP + cells by cell cytometry at 48 h post infection and normalized to the infected non-treated (INF) condition. A representative experiment is shown in (B). Values represent mean ± SD of three independent donors performed in duplicate in (A) and (C). Statistical significance was assessed by comparing the same conditions with and without SAMHD1 in (A) or comparing the treated conditions with the infected non-treated control (INF) in (C). ND; No drug, MID; midostaurin, INF; infected, AZT; zidovudine, NVP; nevirapine, PD; palbociclib. ns (non-significant) $p > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

The effect of midostaurin was also tested in transformed monocytoid THP-1 cells and proliferating lymphoid MT-4 cells, which showed differential basal levels of SAMHD1 (**Figure 23A**). As observed in macrophages, the stimulatory effect in virus replication of midostaurin in the SAMHD1-expressing THP-1 cells was only revealed following SAMHD1 degradation ($p=0.001$ at 5 µM, comparing +/− Vpx conditions) (**Figure 23B**). As for the MT-4 cells with an already negligible SAMHD1 expression, midostaurin alone was enough to increase viral replication ($p=0.03$ at 1 µM and $p=0.0003$ at 5 µM, compared to untreated conditions) (**Figure 23C**).

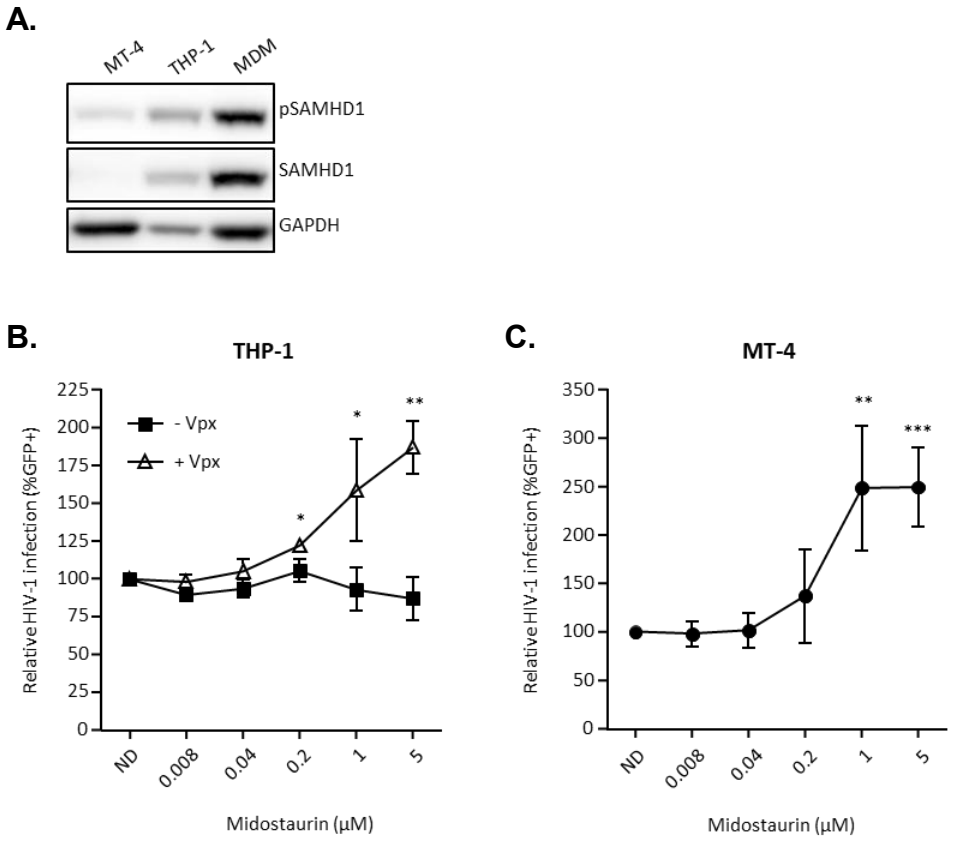


Figure 23. Midostaurin enhances HIV-1 infection in the absence of SAMHD1. (A) Protein levels of SAMHD1 and phospho-SAMHD1 in the leukemic cell lines MT-4 and THP-1 and in primary monocyte-derived macrophages (MDM). GAPDH was used as a loading control. (B) HIV-1 infection in THP-1 with (-Vpx) or without (+Vpx) SAMHD1 and (C) MT-4 in the presence of midostaurin (5–0.008 μM). Infection was quantified as percentage of GFP + cells by cell cytometry 72 h post infection (THP-1) or 48 h post infection (MT-4) and normalized to the infected no-drug (ND) condition. A representative experiment is shown in (A). Values represent mean ± SD of at least three independent experiments performed in duplicate in (B and C). Statistical significance in (B) was assessed by comparing the same conditions with and without SAMHD1 in the THP-1 cells or in (C) by comparing the treated conditions with the no drug control (ND) in the MT-4 cells. ND; No drug. *P < 0.05; **P < 0.01; ***P < 0.001.

3.4.3 Midostaurin affects different steps of the HIV-1 replication cycle

To understand the role of midostaurin on HIV-1 replication, we evaluated its effect at different steps of HIV-1 replication in the presence or absence of SAMHD1. MDM were treated with VLPs carrying Vpx, incubated with midostaurin (1 and 0.2 μM), infected with the fully replicative R5-tropic HIV-1 strain BaL and collected at different times post-

infection. Midostaurin blocked HIV-1 total DNA formation, resembling the effect of the reverse transcriptase inhibitor AZT (**Figure 24A**), integrated DNA (**Figure 24B**) and viral RNA transcripts were decreased in the presence of SAMHD1. However, these effects were lost in SAMHD1-depleted (transduced with VLP-Vpx) cells and an increased number of transcripts (p=0.044 at1 µM) was detected in VLP-Vpx-treated cells (**Figure 24C**). Taken together these results indicate that midostaurin blocks the early formation of viral DNA, prior to integration, in a SAMHD1-dependent manner and has a proviral effect at the later time of transcription of new viral RNA (**Figure 24D**).

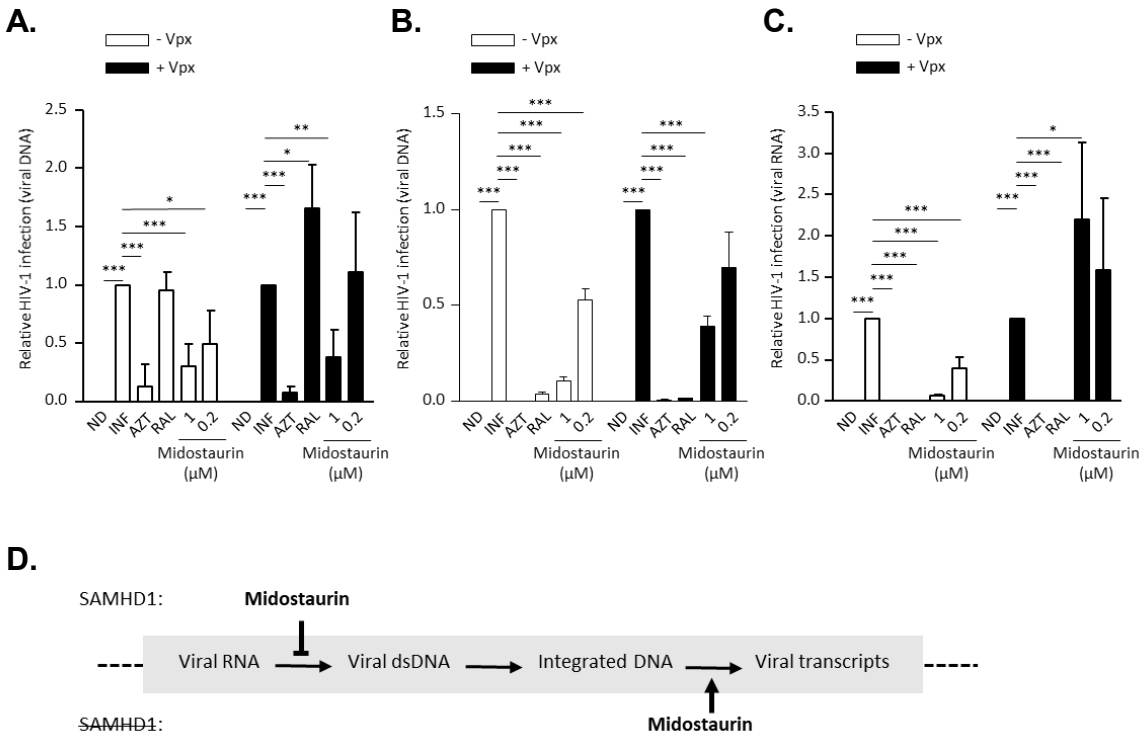


Figure 24. Effect of midostaurin on HIV-1 infection at different stages of the viral cycle. (A) Total viral DNA, (B) integrated viral DNA and (C) transcribed viral RNA in primary monocyte-derived macrophages (MDM) infected with HIV-1 BaL. Infection was quantified by quantitative RealTime-PCR after DNA (A, B) or RNA (C) extraction at different times: 18 h post-infection for the total viral DNA, 42 h post-infection for the integrated viral DNA and 66 h post-infection for the transcribed viral RNA. Infections were performed in the presence (- Vpx) or Vpx-induced degradation (+Vpx) of SAMHD1. Zidovudine (AZT, 1 µg/mL) was used as a control for assessing HIV-1 reverse transcription step. Raltegravir (RAL, 1 µg/mL) was used as a control for assessing HIV-1 integration step. (D) Schematic representation of the dual-effect of midostaurin in the viral cycle of HIV-1 infected MDM with (SAMHD1) or without SAMHD1. Values represent mean ± SD of at least three independent donors performed in duplicate in (A), (B) and (C). Statistical significance in (A), (B) and (C) was assessed by comparing the conditions with the infected non-treated control (INF). ND; No drug, INF; infected, AZT; zidovudine, RAL; raltegravir. *P < 0.05; **P < 0.01; ***P < 0.001.

3.4.4 Midostaurin reverses HIV-1 latency and enhances HDACi-mediated latency reversal

The enhancing effect of midostaurin in acute viral transcription suggests that midostaurin may help revert HIV-1 latency. HIV-1 reactivation was observed in the non-clonal model J-Hig when cells were incubated with midostaurin (p=0.0099) (**Figure 25A**). Midostaurin-mediated HIV-1 reactivation was also observed in the clonal model ACH-2 ($p < 0.0001$), although the effect was less potent compared to that of panobinostat ($p < 0.0001$) (**Figure 25B**). In the clonal model J-Lat clone 9.2, midostaurin did not show any effect at 5 µM, in contrast to the reactivation observed with the HDAC inhibitor (HDACi) panobinostat at 0.16 µM, a known HIV-1 LRA (**Figure 25C**). However a synergic increase in

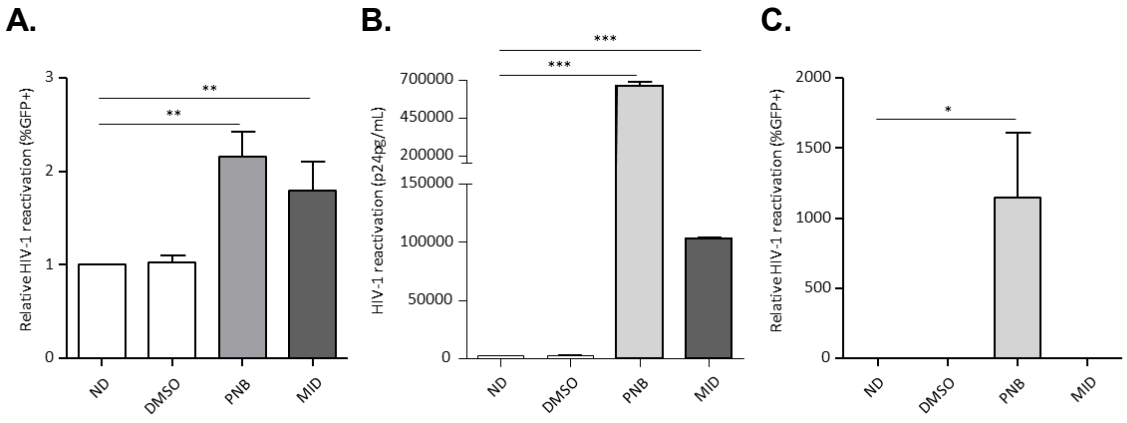


Figure 25. Midostaurin reactivates HIV-1 latent virus in ACH-2 cells and in a nonclonal Jurkat-derived latent model, but not in J-Lat clone 9.2 cells. (A) HIV-1 reactivation in the latently infected Jurkat cells, J-Hig. HIV-1 reactivation was quantified as the percentage of GFP + cells by flow cytometry 24 h after cell treatment with midostaurin (MID; 5 µM). Panobinostat (PNB; 0.16 µM) was used as a positive control. Dimethyl sulfoxide (DMSO) was used at the same concentration present in the midostaurin condition to exclude nonspecific reactivation effects. (B) HIV-1 reactivation in the HIV-1 latent model ACH-2. Cells were treated as in (A) for 48 h and the supernatant was collected. Reactivation was measured as CAp24 quantification in the supernatant by an ELISA. (C) HIV1 reactivation in the HIV-1 latent cell model J-Lat clone 9.2. Experiment was performed as in (A). Values represent mean ± SD of three independent experiments performed in triplicate. Data was normalized to the ND condition in (A) and (C). Statistical significance in (A), (B) and (C) was assessed by comparing the treated conditions with the no drug control (ND). ND; No drug, DMSO; dimethyl sulfoxide, PNB; panobinostat, MID; midostaurin. *P < 0.05; **P < 0.01; ***P < 0.001.

reactivation was observed when midostaurin was combined with either vorinostat or panobinostat (**Figure 26B and Suppl. Figure 7B and Table 4**). In J-Hig cells, midostaurin enhanced the reactivation observed with vorinostat or panobinostat (up to 2.4-fold compared to the HDACi alone) (**Figure 26A and Suppl. Figure 7A**).

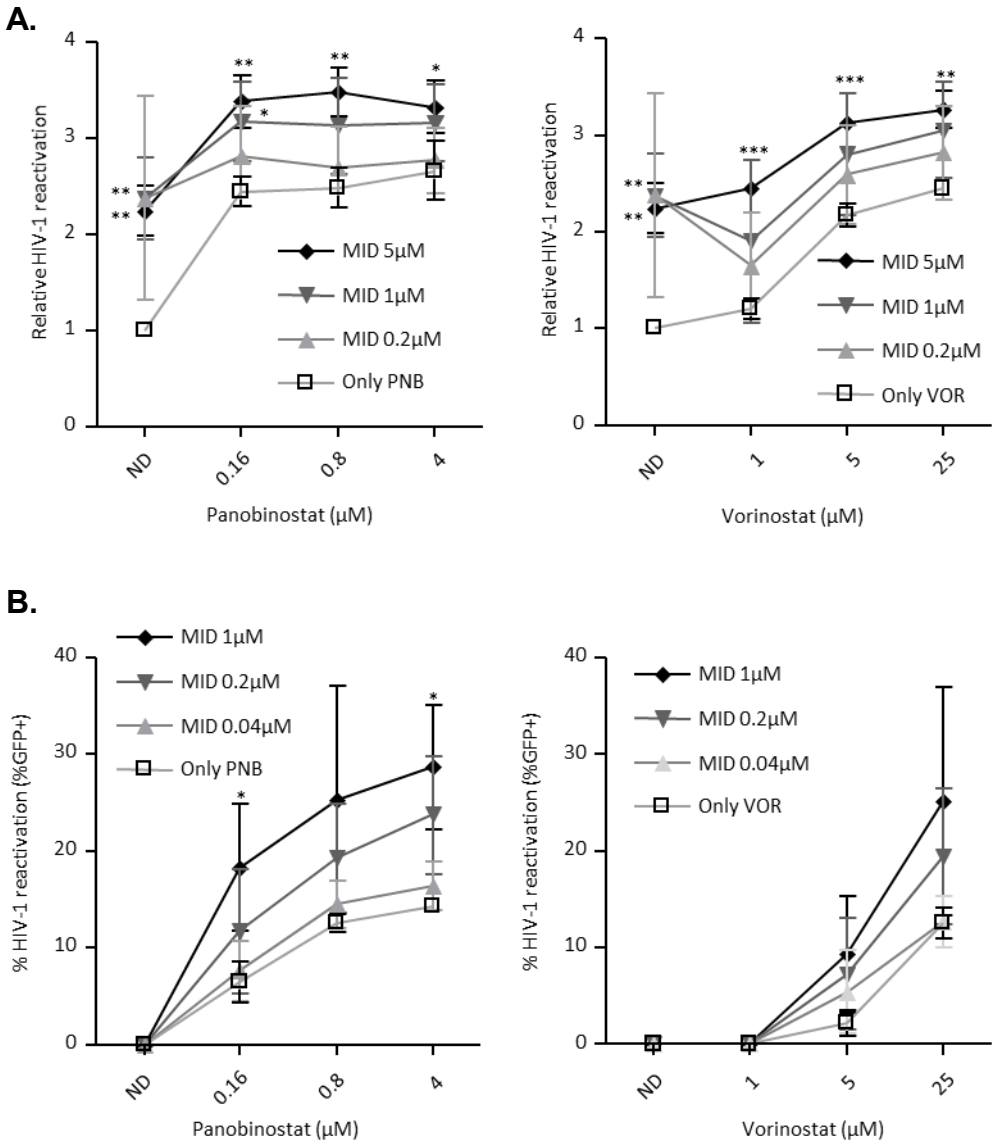


Figure 26. HIV-1 reactivation by panobinostat and vorinostat is enhanced by midostaurin. (A) HIV-1 reactivation in J-Hig cells treated for 24 h with increasing concentrations of midostaurin (5–0.2 µM) and the HDACi panobinostat (PNB; 4–0.16 µM) (left panel) or vorinostat (VOR; 25-1 µM) (right panel). Reactivation was measured as the percentage of GFP + cells by flow cytometry. (B) HIV-1 reactivation in J-Lat clone 9.2 cells. The experiments and the reactivation quantification were performed as in (A). Values represent mean ± SD of three independent experiments. Data was normalized to the ND condition in (A). Statistical significance in (A), (B), (C) and (D) was assessed by comparing the midostaurintreated conditions with the conditions treated just with the HDACi (Only PNB or Only VOR). ND; No drug, PNB; panobinostat, VOR; vorinostat. **P < 0.01; ***P < 0.001.

Table 4. Combinatory index (CI) of midostaurin and HDAC inhibitors in J-Lat clone 9.2 cells.

		Midostaurin					
		CI (1 µM)		CI (0.2 µM)		CI (0.04 µM)	
Vorinostat	25 µM	0.545	Synergy	0.669	Synergy	0.916	Additive
	5 µM	0.226	Synergy	0.268	Synergy	0.327	Synergy
Panobinostat	4 µM	0.011	Synergy	0.039	Synergy	0.366	Synergy
	0.8 µM	0.005	Synergy	0.028	Synergy	0.148	Synergy
	0.16 µM	0.008	Synergy	0.098	Synergy	0.957	Additive

CI values were obtained from the CompuSyn software. Classifications used were: CI > 1 antagonism, CI = 1 additive, CI < 1 synergism. Data represent the mean of at least two independent evaluations.

The activity of midostaurin to reactivate HIV-1 latency was also assessed in a latent model of primary naive CD4+ T cells. A significant increase in HIV-1 reactivation was observed for midostaurin (p=0.03) (**Figure 27**) in the absence of the acute toxicity observed with known LRA (**Suppl. Figure 8**).

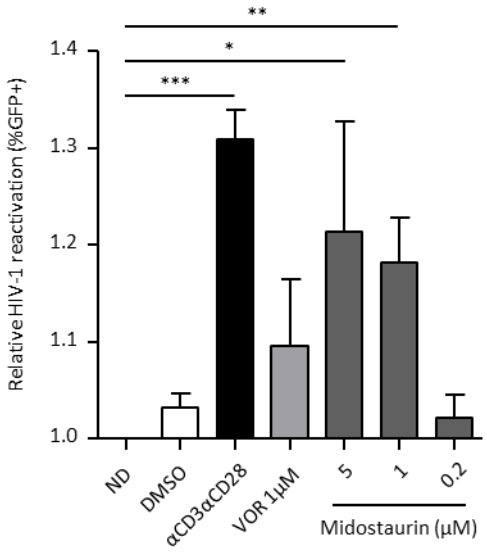


Figure 27. Midostaurin reactivates latent HIV-1 in a model of primary CD4+ T cells. Latently infected naïve CD4+ T cells isolated from peripheral blood mononuclear cells (PBMCs) from healthy donors were incubated for 24 h with different concentrations of midostaurin (5-0.2 µM). HIV-1 reactivation was quantified as percentage of GFP + cells by flow cytometry. The HDACi vorinostat (VOR; 1 µM) was used as a positive control for HIV-1 reactivation. Antibodies

αCD3αCD28 were used as a control of cell activation and expansion and HIV-1 reactivation. Dimethyl sulfoxide (DMSO) was used at the same concentration present in the condition with higher DMSO content (VOR 25 µM, data not shown) to exclude nonspecific HIV-1 reactivation. Values represent mean ± SD of three independent donors performed in triplicate. Data was normalized to the ND condition. Statistical significance was assessed by comparing the treated conditions with the no drug control. ND; No drug, DMSO; dimethyl sulfoxide, αCD3αCD28; antibodies αCD3αCD28, VOR; vorinostat. *P < 0.05; **P < 0.01; ***P < 0.001.

3.5 DISCUSSION

There is no effective cure for HIV-1 infection yet. Identification of molecules able to induce HIV-1 reactivation and inhibit further infection may help developing new strategies for HIV-1 eradication. In this study, we used several cellular models to assess the effect of the multi-kinase inhibitor midostaurin in both acute and latent HIV-1 infection. Using a flow cytometry assay we identified a dual effect of midostaurin in HIV-1 acute infection: inhibition of virus DNA formation following reverse transcription and a later proviral effect at increasing viral transcripts (**Figure 28**). Midostaurin had already surfaced, in a screen of over 1500 small molecules and kinase inhibitors, as an enhancer of viral transcription through a mechanism involving NF-κB signaling[277]. Here, we extend this finding to other models of HIV-1 latency, including latently-infected primary resting CD4+ T cells, and provide evidence of the role of the virus restriction factor SAMHD1 in the activity of midostaurin.

SAMHD1 is a major restriction factor of HIV-1 infection in restingCD4+ T cells[79]. Overcoming this restriction by degrading SAMHD1 through expression of HIV-2 Vpx and/or RNA interference has been used in attempts to identify correlates of HIV-1 latency and/or reactivation[224,278]. Moreover, overexpression of wild-type SAMHD1 was shown to suppress HIV-1 LTR-driven gene expression at a transcriptional level[268], indicating a possible role of SAMHD1 function in regulation viral and cellular transcription. In turn, degradation of SAMHD1, increases HIV-1 replication[78,82] and negatively affects the antiviral potency of nucleoside analogues commonly used in the treatment of HIV-1[232,279]. Pharmacological activation of SAMHD1[77] increased expression

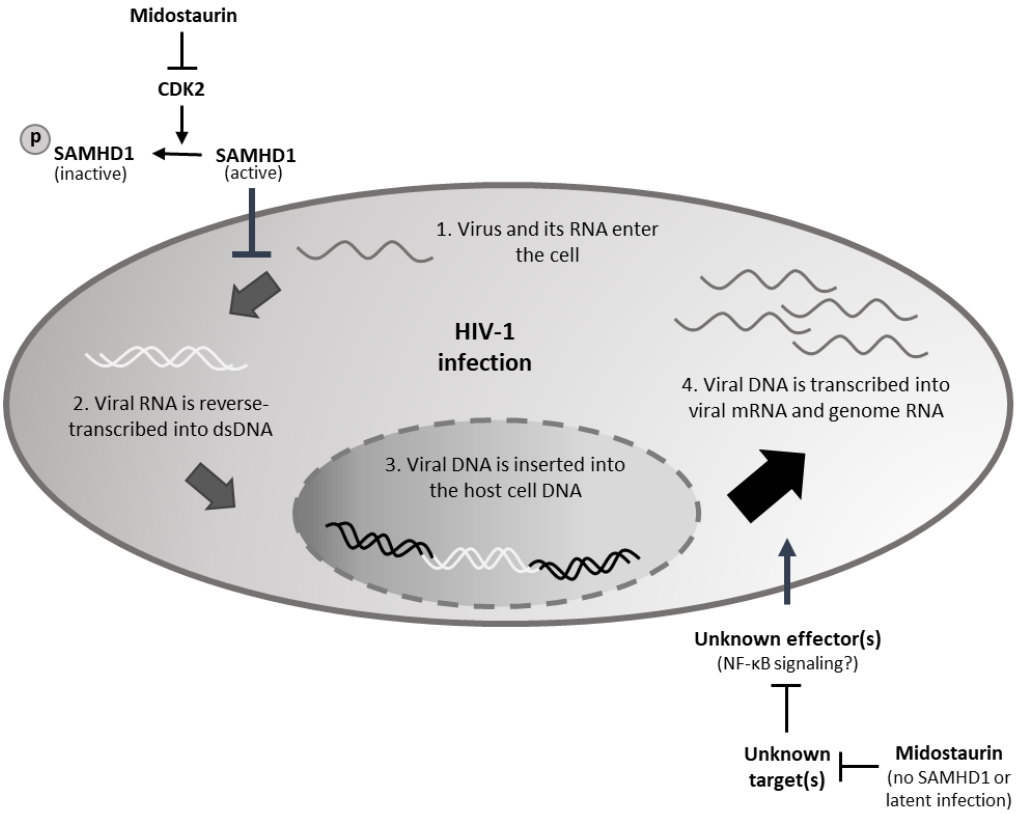


Figure 28. Proposed model for the effect of midostaurin in HIV-1 infection. In the presence of the HIV-1 restriction factor SAMHD1, midostaurin prevents its phosphorylation and inactivation by inactivating CDK2. Thus, leading to a SAMHD1-restricted pool of dNTPs that prevents HIV-1 reverse transcription. Aside from the effect on SAMHD1, the multi-kinase inhibitor midostaurin is also able to inhibit other proteins. The inhibition of one or more of those targets leads to a direct or indirect increase in HIV-1 transcription observed in both HIV-1 acute and latent infection. However, this effect is only noticeable when SAMHD1 is not performing its function due to inactivation, degradation or in latent infection, where the virus is already integrated in the host genome. As proposed in previous studies, midostaurin-mediated increase in HIV-1 transcripts could be the response to an increase in the NF-κB signaling.

of host factors that activate it such as p21[275,276,280,281] and higher SAMHD1 expression[222,282] appears also to affect HIV-1infection.

Here, we show that midostaurin blocked CDK2 activation, a kinase known to phosphorylate SAMHD1[275,276], providing evidence of the mode of action of the anti-HIV-1 activity of midostaurin, i.e. activation of SAMHD1 and restriction of HIV-1 through inhibition of HIV DNA formation. This inhibition was completely lost, and even reversed to a proviral effect, upon SAMHD1 degradation, indicating that the antiviral effect of midostaurin depends on SAMHD1 function. Alternative mechanisms of action, although

not formally excluded, were not apparent in our cell culture conditions. Our analysis showed that midostaurin also increased HIV-1 transcription and reversed HIV-1 latency in a non-clonal model of virus latency. This increase in HIV-1 transcription may be due to a NF-κB-dependent mechanism, as suggested by other authors before[277]. However, we did not observe any reactivation in the J-Lat cell clone 9.2. An explanation for this apparently contradictory result may lie in the insertion site of the virus in those cells. LRA can differentially target and reactivate HIV depending on the viral insertion site[186], indicating that the specific integration site in J-Lat clone 9.2 cells may not be accessible to the activity of midostaurin, and partly explaining the combined effect of midostaurin with the LRAs panobinostat and vorinostat. Effective reactivation of latent HIV-1 may come from combinations of different but possibly overlapping LRAs able to target HIV-1 provirus integrated at different genomic locations. Future studies involving the sequencing of the midostaurin-reactivated provirus and their insertion sites may prove helpful to corroborate our assumptions on the differential specificity of midostaurin on HIV-1 insertion sites.

Thus, agents conveyed of a SAMHD1-dependent antiviral activity that at the same time promote virus reactivation from latently infected cells could provide an alternative to effectively purge the virus reservoir. Curiously, histone deacetylase inhibitors (HDACi) such as vorinostat, have been shown to induce a SAMHD1-dependent block to HIV-1[283], revealing an intricate mechanism associated to cell cycle control and eventual cell death that could be exploited for the selective killing of latently infected cells.

Resting CD4+ T cells with an active (unphosphorylated) form of SAMHD1 are the major reservoir driving HIV-1 persistence. Nevertheless, acute infection of monocyte-derived macrophages and their susceptibility to antiviral drugs as well as to subtle variation in SAMHD1 function provide an excellent model for the evaluation of new strategies to purge and eliminate the HIV-1 reservoir that can be later translated to T-cell models of virus persistence.

In conclusion, we identified and characterized the effect of midostaurin in both acute and latent HIV-1 infection. This compound showed dual but opposing effects by inhibiting HIV-1 reverse transcription in acute HIV-1 infection and enhancing viral

transcription in SAMHD1-depleted cells or in latently infected cells, reversing HIV-1 latency and enhancing the potency of known HDAC inhibitors. Our results suggest that the use of agents with similar properties to that of midostaurin could be useful to develop new anti-HIV-1 strategies, by reactivating the HIV-1 latent reservoir while preventing subsequent rounds of infection.

3.6 ACKNOWLEDGMENTS

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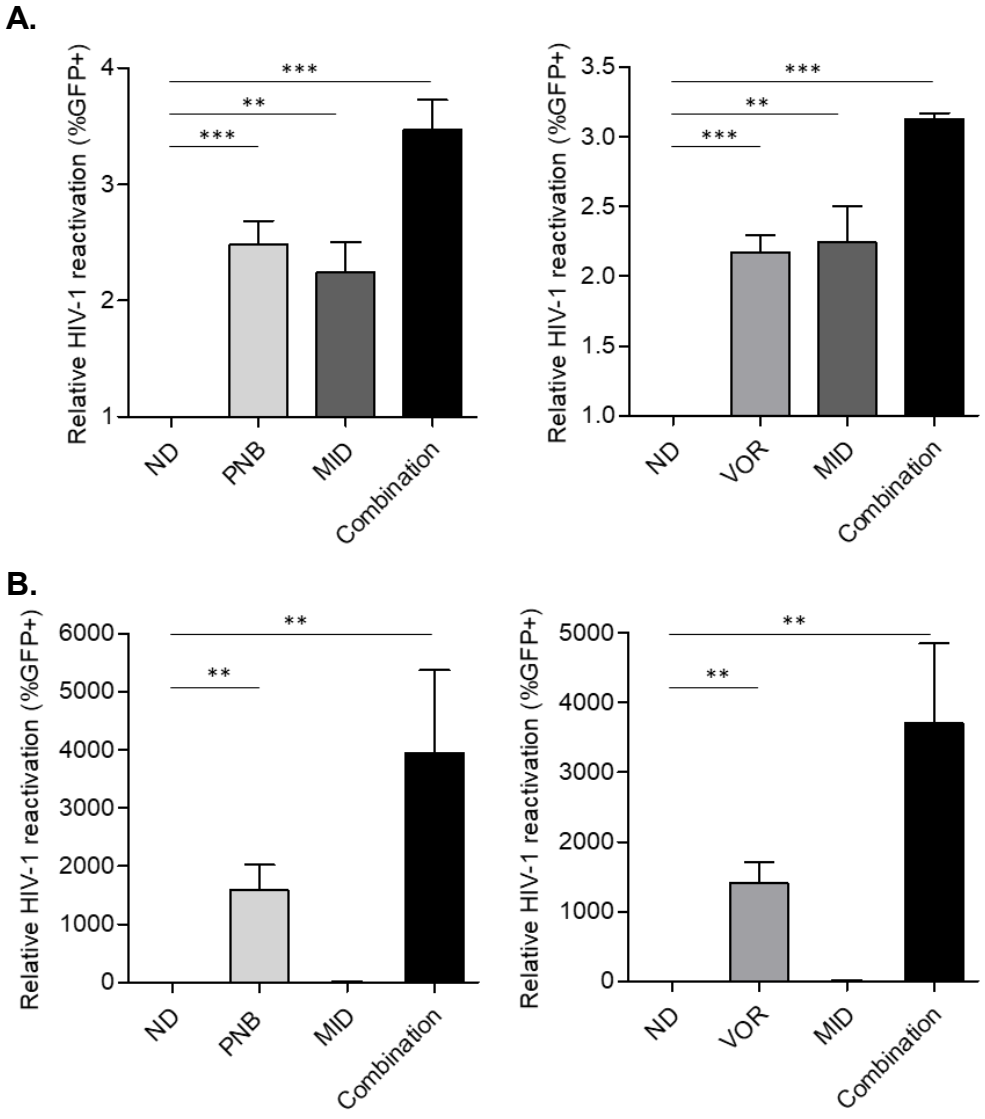
3.7 SUPPLEMENTAL MATERIAL

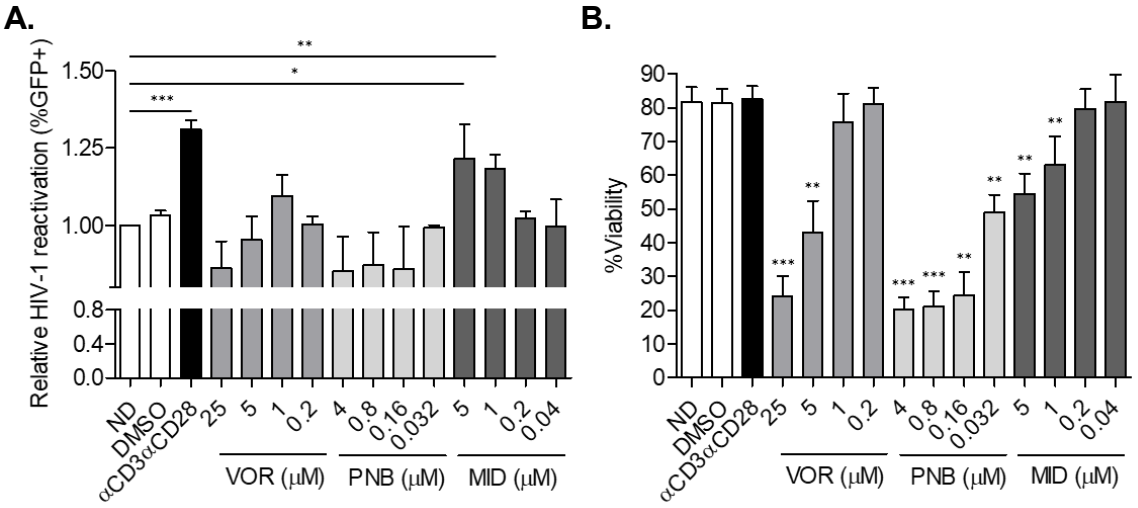
Supplemental table 2. Cytotoxicity of HIV-1 latency reversing agents in different cell types

	Midostaurin	Panobinostat	Vorinostat
	CC_{50} (μM)	CC_{50} (μM)	CC_{50} (μM)
Macrophages	>5	-	-
THP-1	>5	-	-
MT-4	>5	-	-
ACH-2	>5	<0.16	-
J-Lat clon 9.2	>5	<0.16	10.7
J-Hig	>5	>20	>25
Jurkat	>5	>20	>25
Naïve CD4+ T	>5	<0.16	6.0

CC50: 50% Cytotoxicity concentration (CC50) or the concentration required to induce 50% cell death as measured by the LIVE/DEADTM Fixable Near-IR Dead Cell Stain Kit (Invitrogen, Thermo Fischer Scientific) according to manufacturer's instructions. Data represent the mean of two independent experiments.



Supplemental figure 7. Midostaurin increases HIV-1 reactivation induced by LRAs in J-Hig and J-Lat clone 9.2 cells. (A) J-Hig cells were reactivated with midostaurin (MID; 5µM) and the HDAC inhibitors (HDACi) panobinostat (PNB; 0.8µM) (left panel) or vorinostat (VOR; 5µM) (right panel). Combination of the HDACi and midostaurin is also shown in each panel (Combination). Cells were treated for 24h and HIV-1 reactivation was determined by the percentage of GFP+ cells by cell cytometry. (B) HIV-1 reactivation in J-Lat clone 9.2 cells incubated with midostaurin (MID; 1µM) and the HDACi panobinostat (PNB; 4µM) (left panel) or vorinostat (VOR; 25µM) (right panel). The quantification of the HIV-1 reactivation was performed as in (A). Values represent mean±SD of three independent experiments. Data was normalized to the ND condition. Statistical significance in (A) and (B) was assessed by comparing the treated conditions with the no drug control (ND). ND; No drug, PNB; panobinostat, VOR; vorinostat, MID; midostaurin. **P < 0.01; ***P < 0.001.



Supplemental figure 8. The HIV-1 reactivation induced by the latency-reversing agents vorinostat and panobinostat is masked by their toxicity. (A) Latently infected naïve CD4+ T cells were incubated for 24h with different concentrations of midostaurin (5-0.04μM). HIV-1 reactivation was quantified as percentage of GFP+ cells by flow cytometry. The HDACi vorinostat (VOR; 25-0.2μM) and panobinostat (PNB; 4-0.032μM) were used as positive controls for HIV-1 reactivation. Antibodies αCD3αCD28 were used as a control of cell activation and expansion and HIV-1 reactivation. Dimethyl sulfoxide (DMSO) was used at the same concentration present in the condition with higher DMSO content (VOR 25μM) to exclude nonspecific HIV-1 reactivation. (B) Viability of the latently infected naïve CD4+ T cells after 24h of incubation with the compounds from (A). Viability assay was performed in parallel to (A) as the percentage of negative cells for a dead cell stain kit by flow cytometry. Values represent mean±SD of three different donors performed in triplicate. Data was normalized to the ND condition in (A). Statistical significance in (A) and (B) was assessed by comparing the treated conditions with the no drug control (ND). ND; No drug, DMSO; dimethyl sulfoxide, αCD3αCD28; antibodies αCD3αCD28, VOR; vorinostat, PNB; panobinostat, MID; midostaurin. *P < 0.05; **P < 0.01; ***P < 0.001.

DISCUSSION AND PERSPECTIVES

In the past decades, HIV-1 infection has evolved from a terminal illness to a manageable chronic disease thanks to the introduction of ART[101]. However, HIV-1 is still an incurable infection with only two reported cases of apparent HIV-1 remission. The HIV-1 latent reservoir represents one of the main barriers for the HIV-1 cure[109–111], therefore, several strategies have been proposed directed at its eradication, one of them being the "shock and kill" strategy[174].

Compared to the other sterilizing strategies like stem cell transplantation or genome editing, "shock and kill" has the advantage of being less complex, more affordable and, consequently, having a wider target population range, as it does not require a suitable donor or the *ex vivo* engineering of autologous cells. Furthermore, infusion of cells with a disrupted CCR5 coreceptor, either by using a CCR5Δ32 donor or using gene editing procedures, would not prevent the infection by X4-using strains, favoring its selection and therefore rendering the strategy ineffective for those infected individuals harboring X4 HIV-1 strains. In comparison, the ideal "shock and kill" strategy would consist in oral doses of one or more LRAs, ideally in the form of a tablet, which represent a much easier and cheaper procedure, as the only personalized treatment needed would be the choice of the adequate LRA or LRA combination.

More than twenty years have passed since the first reports on HIV-1 latency reactivation[284], and, to date, several agents with different molecular targets have been proposed for HIV-1 reactivation, including histone and non-histone chromatin modulators, NF-κB stimulators, TLR agonists and extracellular receptor ligands, among others (reviewed in[178]). However, complete reactivation and the subsequent eradication of the HIV-1 latent reservoir has not been achieved despite all efforts devoted to identify agents able to fulfill the requirements of the prototype LRA: efficient reactivation of latent virus and selective killing of infected cells. Although some LRA have reached clinical trials, none of them were able to impact the reservoir[181–183,285]. Moreover, a recent study reported that current LRAs lack selectivity and are not able to reactivate more than 5% of the latently infected cells[286], prompting the need to find better but also more specific compounds. On the other hand, the clearance of reactivated cells also needs further review, given the inability of current LRAs to effectively reduce the latent reservoir. Several alternatives have been assayed, including pairing LRAs with other

strategies to enhance the killing of the infected cells, like the use of antibodies, CAR engineered T cells, therapeutic vaccination and pro-apoptotic compounds[177,190], albeit the best option would be the identification of novel LRAs with the ability to decrease the reservoir by themselves. To this end, we evaluated the use of modulators of the innate immune response and agents able to affect cell cycle progression in HIV-1 latently infected cells, as both latency reversal agents and putative reservoir eradication strategies.

Innate immune responses are key steps in the control of any infection and, in the case of HIV-1, partially control virus replication during natural infection. Thus, the boosting of host immunity has been proposed as a way to achieve the purge of HIV-1 reservoirs. The study of modulators of innate immune pathways as agents able to clear the HIV-1 reservoir experienced a breakthrough with the description of acitretin[66], a retinoic acid derivative approved for the treatment of psoriasis, that has recently been proposed to induce HIV-1 reactivation and selective killing of the infected cells[66,234]. Retinoic acid, and thus acitretin as well, upregulates the activity of the histone acetyltransferase p300[287,288], which in turn regulates transcription through acetylation of the histones in the nucleosomes, providing a molecular mechanism explaining the ability of acitretin as a LRA. Moreover, retinoic acid induces the activity of the PRR RIG-I[289], boosting an innate immune response and inducing the killing of infected cells[201,202,220]. Therefore, acitretin could be a valuable asset for the shock and kill strategy, being able to both promote the "shock" in a p300-dependent manner and enhance the "kill" by boosting the innate immunity through the RIG-I pathway. However, we found that the effect of acitretin on HIV-1 reactivation was negligible in the vast majority of the models tested, albeit activation of RIG-I pathway was detected and a mild induction of viral reactivation was observed in a non-clonal T cell model of HIV latency. Furthermore, acitretin was not able to induce the selective killing of infected cells, which collides once more with the results previously reported[66]. Overall, the use of retinoic acid derivatives, and specifically the already FDA-approved acitretin, seem to be an attractive approach, given their proposed roles on both transcription and innate immunity. However, the discrepancies reported on the potential of acitretin to either induce HIV-1 reactivation or selective cell death

suggest that further studies are needed in order to prove their value for the “shock and kill” strategy.

Indeed, evaluation of the “shock and kill” strategy *in vitro* has important considerations and unsolved technical challenges which impede the translation of *in vitro* approaches to *in vivo* therapies, and also influence the reproducibility of results. In the case of acitretin, discrepancies may be the result of differences in the cell model and methodology used to assess HIV-1 reactivation. Although the quantification of viral proteins is thought to represent the more accurate readout of efficient viral reactivation, viral reactivation is often assessed by quantification of HIV-1 nucleic acids, a method that does not discriminate between infectious and defective viruses, or by viral outgrowth assays, which require large numbers of cells and long-term cultures. Focusing on acitretin as a potential LRA, Li et al. evaluated viral RNA transcription levels whereas in our study we directly quantified the percentage of reactivated cells with a fluorescent reporter. Higher levels of cellular HIV-1 RNA may not correlate with cells undergoing HIV-1 reactivation and virion production[286,290] and, although a reported gene is not a viral protein, the detection of a GFP reporter might represent a step closer to "real" viral production, validating our approach.

The type of cellular latency model used can also affect the results obtained. The use of latently infected T cell lines (usually expanded from a single cell clone as the widely used J-Lat and ACH-2 cell models) and primary cells has contributed enormously to the study of HIV-1 latency and reactivation. However, as it has already been suggested[248], differences among the diverse HIV-1 latency models may be the reason why potential LRAs do not always show the same latency-reversing capacity, which means that the results obtained in a specific cell model cannot be applied to other models or to infected patient cells, tested *ex vivo*. Indeed, this is the case for acitretin, where HIV-1 reactivation could only be detected in the J-Hig cells but not the J-Lat cells, whereas Li et al. was able to report HIV-1 reactivation in several models, including ACH-2 and CEM-T4 cell lines, a model of primary CD4+ T cells from healthy donors and CD4+ T cells from aviremic HIV-1+ patients.

HIV-1 latency establishment depends on different factors, one of them being the site and orientation of the integrated provirus, which differs significantly between clonal cell models, where the number and site of integrated provirus is fixed and equal in all cells, and non-clonal cell models, where the number and genomic location of integrated HIV-1 is different in each cell. Thus, integration site heterogeneity in those cells could have been another key point to explain the differences observed between the previous acitretin work[66] and ours, as the HIV-1 reactivation observed in their models was not observed in the clonal models for HIV-1 latency that we used. This observation may also imply that each LRA could exhibit specificity towards certain HIV-1 integration sites which, in the case of acitretin, are not present in the J-Lat clones used but they are in the non-clonal HIV-1 latently infected jurkat cells, the J-Hig cells.

In support of this theory, Chen et al.[186] mapped the HIV-1 insertion sites by coupling whole genome sequencing and RNA transcriptomics, being able to identify the chromosomic location of reactivated proviruses. As a result, Chen et al. concluded that each LRA was able to reactivate different latent HIV-1 provirus depending on integration site, supporting the idea that, in order to reactivate the whole latent reservoir, a cocktail of several LRA should be used. The findings in Chen et al. also raises concerns on the models used to study HIV-1 latency since, as we observed with acitretin, the use of clonal models could overlook the reversal capacity of potential compounds and negatively affect the results. In this regard, the use of non-clonal HIV-1 latency models like the J-Hig cells, in which each cell can harbour one or more proviruses integrated in different locations, seems to be a more sensitive assay and, therefore, it would be recommended for screening studies instead of clonal models.

Regarding the boosting effect of the innate immunity and the selective killing of latently infected cells proposed for acitretin, the cell model used also seemed to influence the discrepancies observed. In our latently infected jurkat model, J-Hig cells, acitretin was not able to specifically target and promote the elimination of the infected cells, in contrast with Li et al. results. This is also suggested by the acitretin-mediated mild activation of the RIG-I pathway observed by western-blot in any of the cells we used, in comparison to that of Li et al. Regardless of that, it should also be taken into

consideration that the methodology used to assess the apoptosis, which was the same in both studies, has its own flaws, as the cytoplasmic GFP reported could leak out the cell in response to the permeabilization of the plasmatic membrane in late-apoptotic cells, also referred as secondary necrosis[291,292]. Thus, this approach could lead to an underestimation of the real population of GFP+ dead cells, making it unsuitable for apoptosis assays.

In the search for activators of latent HIV-1, anti-cancer compounds have also been proposed as candidate therapies targeting the latent reservoir, including HDAC inhibitors, PKC agonists, DNMT inhibitors and BET inhibitors (reviewed in[293]). This suggests that, even though HIV-1 infection and oncogenic processes are completely different diseases, there are indeed similarities between them. In fact, the presence of rare cells able to promote the viral rebound or cancer relapse is characteristic of both processes[293]. Moreover, epigenetic gene silencing, which has already been reported for cancer[294], is one of the contributing factors for HIV-1 latency establishment. Consequently, epigenetic modifying compounds that were originally developed as anti-cancer agents are now being tested as LRAs, and pro-apoptotic compounds are being evaluated to eliminate infected cells[293]. Thus, we performed a screening of a library of more than 400 anticancer compounds including more than 100 distinct molecular targets, all being structurally diverse, cell permeable, medicinally active and previously used in tumor research. In order to identify as many potential LRAs as possible, the screening was performed in a non-clonal HIV-1 latency model. As a result, we identified a series of compounds with latency reactivating capacity that include the well-known HDAC inhibitors, but also Janus kinases (JAKs), IκB kinases (IKKs) and heat shock proteins (HSPs), whose activity as LRA has not been reported before, and the compound midostaurin, a multi-kinase inhibitor. In fact, in clear contrast to our data, the use of JAK inhibitors has been reported to block IL-15-induced viral reactivation[295] and so has been for Hsp90 inhibitors in TPA-reactivated cells[296], due to the ability of Hsp90 to control NF-κB pathway. In addition, since NK-κB stimulators are a well-known class of LRAs, it is surprising that compounds able to inhibit that pathway, like the HSP inhibitors or even some IKK inhibitors[297,298], could also induce HIV-1 reactivation, as we have shown. Taking

all this into account, it is clear that pathways modulating HIV-1 latency are complex, being indicative of the complexity and high heterogeneity of the viral reservoir.

However, the most interesting finding of the screening was the identification of Aurora kinase inhibitors (AURKi) as latency reactivation agents. The Aurora kinases are a family of serine/threonine kinases with, to date, 3 identified members in mammalian cells: AURKA, AURKB and AURKC. AURK kinases are known to play crucial roles in cell cycle, with AURKA being associated to centrosome maturation and mitotic spindle assembly and AURKB and AURKC to the binding of the chromosomes to the kinetochore and chromosomic segregation, and therefore, their therapeutic inhibition has been considered as a potential anti-cancer treatment (reviewed in[299]). In our screening, 7 distinct AURKi showed consistent latency reactivation capacity. Importantly, barasertib, the best candidate, was highly selective for AURKB in comparison to other AURK members[300], and showed low to none activity for other kinases including a panel of more than 50 other serine-threonine and tyrosine kinases[244].

Similar to acitretin, AURKi have also been associated to contradictory results as to their role in HIV-1 latency. The AURKi danusertib was suggested to block HIV-1 latency reversal[260] instead of promoting transcription. However, as discussed above, the distinct cell models used may account for the differences observed. Indeed, and contrary to the danusertib study that used only a clonal model of HIV-1 latency, we consistently show that 7 distinct AURKi are able to induce HIV-1 reactivation in models of non-clonal latently infected cells and also primary cells, supporting the role of AURKi as latency-reversing agents. Furthermore, the observed inability of AURKi to induce HIV-1 reactivation in the clonal J-Lat cells suggests that, as proposed for acitretin, AURKi could also have specificity towards certain HIV-1 insertion sites.

The mechanism by which AURKi are able to influence HIV-1 latency reactivation is still unclear. On one hand, AURK function might participate in HIV-1 latency thanks to its reported ability to phosphorylate histone H3[301], which leads to chromatin condensation (reviewed in[302]). Thus, inhibition of AURK would prevent this condensed and inaccessible chromatin state, allowing the reactivation and transcription of HIV-1 proviruses integrated in those areas. Alternatively, G2 arrest induced by the inhibition of AURK, and

the associated increase in LTR expression and virus production, could also influence HIV-1 reactivation, similar to previous reports on the role for Vpr-mediated G2 arrest of infected cells[303,304]. Finally, AURKi-mediated latency-reversal could also be directly linked to provirus integration site, that is, given the association of AURK and the cell centromere[241,242], AURK inhibition would result in the accessibility of the centromere heterochromatin and the reactivation of HIV-1 provirus that might be integrated there. Further investigations will unravel the specific mechanism explaining AURK role in HIV-1 latency reversal.

Despite their role in reversing HIV-1 latency, anticancer compounds have also been reported to impair HIV-1 replication, including the first antiretroviral compound, AZT[7]. AZT is a nucleoside analogue, one of the first class compounds used for cancer treatment (reviewed in[305]) and, soon after that, other classes of anticancer agents were also found to display antiviral effects, including non-nucleoside analogs, topoisomerase 1 enzyme inhibitors, estrogen receptor ligands and cyclin-dependent kinase inhibitors (CDKIs) (reviewed in[306]). In our study, we have also reported an antiviral effect for the AURKi, being able to decrease HIV-1 replication *in vitro* in both, primary macrophages and CD4+ T cells. This dual effect has also been reported in monocyte-derived macrophages for the well-known LRA vorinostat in a SAMHD1-dependent manner[283], through CDK1 depletion. The identification of an agent that is able to purge the latent HIV-1 reservoir but also harbors antiviral activity is highly relevant as, in theory, it might inhibit additional replication cycles consequence of the production of new infectious virus by the former latently infected cells.

Actually, our results regarding AURKi antiviral activity may have been previously reported too, but interpreted differently. In Vargas et al. study[260], they reported the ability of danusertib to block HIV-1 reactivation as the quantification of replication competent viruses following the reactivation of resting CD4+ T cells in a TZM-bl reporter cell-based assay[307]. However, this result could also be explained by the blocking of the infection of the reporter cells, which would agree with our observations.

In addition to what we have shown in HIV-1 latency and HIV-1 replication, other authors have also associated AURKs function with HIV-1 accessory proteins Vpr[308], Vif[309] and Nef[310], providing additional evidence of a link between HIV-1 and AURKs.

In summary, Aurora kinase inhibitors show great promise as putative future LRAs. However, up to now, and although lead compounds are in different stages of clinical trials, none of the inhibitors has been approved for use in humans, and therefore the repositioning for an alternative use might be delayed compared to an already approved drug. In this sense, another compound showed a significant and consistent capacity to induce HIV-1 latency reversal in our screening, the multi-kinase inhibitor midostaurin. Midostaurin is a staurosporine derivative that was initially identified as a PKC inhibitor[273] and that was approved for the treatment of AML in 2017[262]. In comparison to AURKi, midostaurin targets a significantly higher number of kinases and thus its role as LRA cannot be asociated to a single process. Ao et al.[277] reported the midostaurin-dependent phosphorylation of the Ser311 in the NF-κB transcription factor family member p65, which has been shown to be important for NF-κB transcriptional activity[311], thus suggesting that NF-κB pathway stimulation plays a role in midostaurin-mediated HIV-1 latency reversal activity. The NF-κB pathway takes part in the innate immune response, being activated by PRRs upon pathogen recognition, which leads to the production of IFN-I, inflammatory chemokines and regulation cellular responses including apoptosis and cell survival (reviewed in[312]). Therefore, midostaurin is also able to modulate the innate immunity which, in theory, should enhance the killing of the latently infected cells as it was hypothesized for acitretin. However, in agreement to what we already reported for the latter, midostaurin-treated cells did not exhibited a higher death rate. In fact, midostaurin showed less toxicity that the HDACi vorinostat and panobinostat in our primary CD4+ T cell model. However, midostaurin was potent enough to reactivate HIV-1 latency, suggesting that the lack of killing was not the result of a lack of potency. Nevertheless, given the broad target spectrum of midostaurin, contributing effects of the inhibition of additional kinases unrelated to the NF-κB pathway in the latency-reversal properties of midostaurin cannot be discarded.

In addition to its activity as a LRA, we have also shown the antiviral properties of midostaurin, by preventing the inhibition of the HIV-1 restriction factor SAMHD1 through CDK2. In this regard, the activity of midostaurin is similar to that reported for the anticancer compound palbociclib[266], a CDK4/6 inhibitor that has been used for the treatment of mantle cell lymphoma[313] and is also able to inhibit SAMHD1 phosphorylation and viral replication. As discussed for its latency reversal function, antiviral activity of midostaurin could also be the result of several contributing factors besides SAMHD1 activation. However, in this case, these hypothetical contributions should not be as important as the inhibition of SAMHD1 phosphorylation and inactivation, given that the midostaurin-mediated antiviral activity was completely lost upon SAMHD1 degradation. Nevertheless, and although it proved to be an interesting dual agent, the multiple targets of midostaurin make it a less than ideal compound due to the off-target effects and possible associated toxicities. Thus, further investigation is needed in order to verify which are the targets responsible for the midostaurin-mediated HIV-1 latency reversal and antiviral activity and then, ideally, develop a compound able to specifically inhibit the target.

As mentioned earlier, current data does not support the existence of a single agent able to efficiently reactivate all integrated provirus irrespective of their genome location[186]. Thus, we have also *in vitro* assayed the putative effect of combining the newly described LRAs with the extensively studied HDAC inhibitors vorinostat and panobinostat. Interestingly, both AURK inhibitors and midostaurin enhanced HIV-1 reactivation when used in combination with the HDACi vorinostat and panobinostat, supporting the idea that different drug classes target distinct integrated provirus. Thus, both AURKi and midostaurin represent interesting candidates for the LRAs cocktails as they are able to target distinct proviruses than those reactivated by the HDACi. As a whole, these observations support the relevance of the anticancer compounds in the pursuing of the HIV-1 cure through the shock and kill strategy, given their roles in HIV-1 latency reactivation and replication inhibition.

In this dissertation we assessed the effect of the innate immune modulator acitretin and the anticancer compounds midostaurin and aurora kinase inhibitors in the “shock and

kill" strategy. Our results support the idea of Chen et al.[186] regarding the specificity of LRAs for proviral integration sites and the suggestion made by several authors[178,186] about the importance of an LRA combination for a global HIV-1 reactivation. In the future, LRA research should focus not on a holy grail able to reactivate the whole latent reservoir by itself, but the right combination of agents that grant the full coverage of the genome, being able to make accessible and reactivate any provirus regardless of its location, and that exhibit good synergism with other LRAs in the absence of global T cell activation. This global activation, result of the lack of selectivity of current LRAs, could cause toxicity[176], LRA-associated side-effects and/or even induce the reactivation of other latent proviruses present in the genome[314]. Moreover, research should also focus in the study of novel LRAs able to target not only the resting memory T cells of the latent reservoir, but also all the cells, tissues and organs that contribute to HIV-1 persistence, like the central nervous system (CNS), where limited drug penetration and immune privilege could have a negative impact in the shock and kill strategy[129,315,316]. That being said, another aspect that should gain more attention are the killing properties of LRAs. With the observation that the immune system is not able to effectively kill the reactivated cells and that some current LRAs could actually impair the killing of the reactivated cells, some authors have started to turn to additional strategies like the use of antibodies, CAR engineered T cells, therapeutic vaccination and pro-apoptotic compounds[177,190]. Although further investigation in those approaches could eventually improve the shock and kill strategy, it should also be noted that the additional treatments, which would be administrated together with antiretrovirals and LRAs, could result in a less than ideal situation for the patients. The side-effects, cumulative toxicities from the multiple drugs, the multiple interventions that would be needed for administration of antibodies or engineered cells infusion, and the increase in the cost and complexity of the procedures could minimize the advantages that the "shock and kill" strategy has compared to the other sterilizing strategies, and have a negative impact in the comfort of the individuals and the availability of the treatment. Therefore, a better and more ideal alternative would be the study, identification and application of novel LRAs able to decrease the reservoir by themselves that could, eventually, be used in tablet or capsule formats similar to ART.

However, and despite the ideal scenario depicted above, we scientists need to be aware that this situation may never take place. One of the major hurdles for the "shock and kill" strategy, as well as for any other strategy aiming for a sterilizing cure, is the inability of the current methodology to accurately identify and quantify the real size of the HIV-1 latent reservoir. Only after solving how to accurately measure the viral reservoir and being able to confirm the complete reactivation and elimination of infected cells, would it be possible to know if a sterilizing strategy has indeed eradicated the virus from the individual. Until then, the "apparent HIV-1 remission cases" will instead remain as "HIV-1 cure cases". Moreover, reports on a population of latently infected cells that do not respond to LRA treatment[286] suggests the possibility that the cocktails may not, and maybe never will, reactivate the whole latent reservoir, regardless of the combinations of LRAs. In this regard, we have to realize and accept the possibility that the" shock and kill" strategy, as it is right now, may never lead to a complete elimination of the reservoir, and for that reason an alternative would be needed. Following this suggestion, an approach that could still involve the original "shock and kill" strategy would be the use of the latter to reactivate the reactivatable, or shallow, reservoir and then pair it with the block and lock strategy, in order to silence this non-reactivatable, or deep, reservoir and render it innocuous, ideally (**Figure 29**). However, this "shock and kill/block and lock" strategy would not induce the killing of the whole HIV-1 reservoir and, therefore, it would not be considered a sterilizing cure but a functional one.

In summary, further research is still necessary in order to know whether the shock and kill strategy could eventually lead to a sterilizing cure, either by itself or in combination with other strategies, or to a functional cure. As researchers for an HIV-1 cure, our most logical goal is to eradicate the virus from infected patients and return them to a healthy condition, which means that we may not want to "settle" for anything less, i.e. a functional cure. Nonetheless, from the point of view of an HIV-1+ individual that has to live with a potentially dangerous and infectious agent, being able to remain undetectable and untransmittable in the absence of ART, and consequently their associated toxicities, could be a more than desirable situation. Hence, it will not matter whether all our efforts in the improvement and implementation of the shock and kill

strategy end up resulting in the formulation of a sterilizing or a functional cure, as none of them would be considered a failure, but indeed a cure.

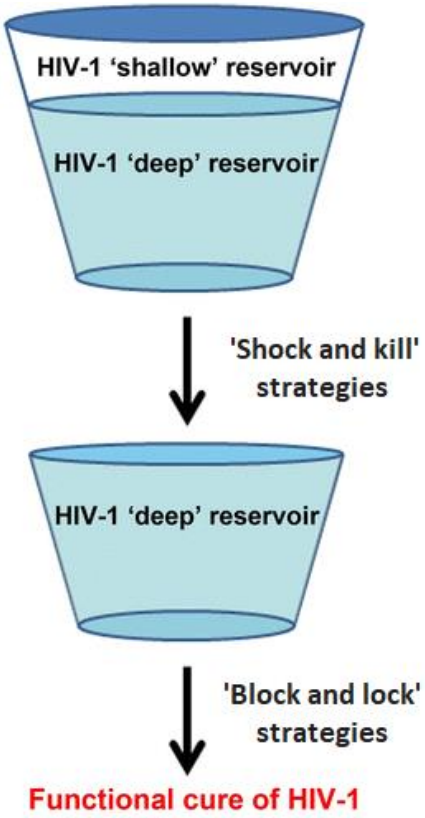


Figure 29. Shock and kill/Block and lock strategy. Scheme of the strategy in which LRAs would reactivate and eliminate the reactivatable, or "shallow", reservoir while the non-reactivatable, or "deep" reservoir, is kept silenced, so there will not be a viral rebound even in the absence of ART (Modified image from[163]).

CONCLUSIONS

1. Boosting of the innate immunity has been proposed as an alternative mechanism to that of current LRAs. The proposed model for acitretin to enhance both viral transcription in a p300-dependent manner and apoptosis of infected cells through activation of the RIG-I innate immune pathway is promising. However, acitretin proved to be not potent enough to validate the model.

2. Targeting and inhibition of cell cycle-related proteins is a source of novel and potential LRAs, as demonstrated by the LRA screening with anti-tumoral compounds, suggesting a link between HIV-1 infection and oncogenic processes.

3. Aurora kinases inhibitors reactivate HIV-1 latency through an unknown mechanism. Nevertheless, the aurora kinase family represents a potential target for the development of novel LRAs.

4. Midostaurin is a multi-kinase inhibitor that acts as an LRA. The mechanism of action of midostaurin depends on SAMHD1 function as enhancing of viral transcription occurs only in SAMHD1-depleted cells.

5. Aurora kinase inhibitors and midostaurin enhance the HIV-1 reactivation mediated by HDAC inhibitors, suggesting that each agent has a different specificity towards HIV-1 proviral integration sites. Therefore, cocktails of LRAs affecting distinct pathways and with different HIV-1 integration site specificities, like AURKi and midostaurin, could represent an option for the complete reactivation and eradication of the HIV-1 latent reservoir.

6. Non-clonal models of latent HIV infection characterized by a significant heterogeneity in proviral integration sites, like J-Hig cells, seemed to be more prone to HIV-1 reactivation than clonal models. This suggests that non-clonal models for HIV-1 latency may be more sensitive and, therefore, a better option to test the latency-reversing capacity of new compounds.

7. Aurora kinase inhibitors and midostaurin exhibit a dual function in acute and latent HIV-1 infection that would allow HIV-1 latency reversal while preventing the *de novo* infection mediated by the reactivated virus.

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