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Efekt hem arginátu na akutní infekci HIV-1 a
na reaktivaci latentní infekce

Effects of heme arginate in HIV-1 acute infection and in latency reversal

Ph.D. Thesis

Supervisor: MUDr. Zora Mělková, PhD.

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Content:

1. Abstract

Abstract

The available antiretroviral compounds can effectively suppress the replication of HIV-1 and block the disease progression. However it is impossible to eradicate the virus from the organism as the HIV-1 integrated in the genome is not affected by the existing anti-HIV-1 drugs. Therefore, new latency reversing agents are being actively developed as part of “shock and kill” therapy to reactivate the provirus and clear the reservoir. Normosang (heme arginate; HA) is a human hemin-containing compound used to treat acute porphyria. Heme is physiologically catabolised by heme oxygenases to form iron (Fe^{2+}), carbon monoxide (CO) and biliverdin that is further converted to bilirubin by biliverdin reductase.

In this study, we have demonstrated that HA inhibited HIV-1 replication during the acute infection, which was accompanied by the inhibition of reverse transcription. On the other hand, HA synergised with phorbol myristyl acetate (PMA) and reactivated the HIV-1 provirus in ACH-2 cells and the HIV-1 “mini-virus” in Jurkat cell clones A2 and H12. HIV-1 ‘‘mini-virus’’ was reactivated also by HA-alone. Further, we have studied the effects of heme degradation products on latent HIV-1 reactivation when added individually. We employed addition of ascorbate to generate Fe^{2+}, resulting in an increased expression of both HIV-1 provirus and “mini-virus”. The other two heme degradation products, CO or bilirubin, decreased the provirus expression. Antioxidant N-acetyl cysteine as well as iron chelator desferrioxamine inhibited the reactivation of HIV-1 provirus stimulated by PMA alone and in combination with either HA or ascorbate, suggesting that the effects of HA were mediated by heme- and iron-induced redox stress. Additionally, the effective concentrations of HA did neither affect activation of a T-cell line with PMA nor induce activation of the unstimulated cells. Finally, we demonstrated the synergistic effects of HA and PMA on HIV-1 expression in peripheral blood mononuclear cells of HIV-infected patients effectively controlled by antiretrovirals cultured ex vivo. These results may point towards a new direction in the latent HIV-1 reactivation and therapy.

Key words: HIV-1, latency, reactivation, heme arginate, iron, CO, bilirubin, ascorbate, redox stress, latency reversal, therapeutic reactivation

Abstrakt

V současnosti dostupná antiretrovirotika efektivně potlačují replikaci HIV-1 a blokují progresi onemocnění. Virus však není možné eliminovat z organismu, neboť HIV-1 integrovaný do genomu není existujícími antiretrovirotiky ovlivňován. Aktivně se proto vyvíjejí nové látky schopné reaktivovat provirus a zlikvidovat latentní rezervoár, tzv. terapie "shock and kill". Normosang (hem arginát; HA) je preparát obsahující lidský hemin využívaný k léčbě akutní porfyrie. Hem je fyziologicky degradován hem oxygenasami na železo (Fe^{2+}), oxid uhelnatý (CO) a biliverdin, který je dále přeměňován biliverdin reduktasou na bilirubin.

V této práci jsme prokázali, že HA inhibuje replikaci HIV-1 při akutní infekci, což bylo doprovázeno inhibicí reversní transkripce. Naproti tomu HA působil synergicky s forbol myristyl acetátem (PMA) a reaktivoval HIV-1 provirus v buňkách ACH-2 a HIV-1 „minivirus" v klonech buněk Jurkat A2 a H12. HIV-1 „minivirus" byl reaktivován i samotným HA. Dále jsme studovali efekty degradačních produktů hemu na reaktivaci latentního HIV-1 přidaných jednotlivě. K tvorbě Fe^{2+} jsme využili askorbát, jehož přidání zvyšovalo expresi HIV-1 proviru i „miniviru". Další dva degradační produkty hemu, CO a bilirubin, expresi proviru inhibovaly. Antioxidans N-acetylcystein stejně jako chelátor železa desferrioxamin inhibovaly reaktivaci HIV-1 proviru stimulovanou samotným PMA nebo jeho kombinací s HA či askorbátem, což naznačuje, že efekty HA byly zprostředkovány redoxním stresem indukovaným hemem či železem. Navíc efektivní koncentrace HA neovlivnily aktivaci T-buněčné linie pomocí PMA ani neindukovaly aktivaci nestimulovaných buněk. Synergické působení HA a PMA na expresi HIV-1 se nám podařilo prokázat i v periferních mononukleárech HIV-infikovaných pacientů s antiretrovirovou terapií při kultivaci *ex vivo*. Tyto výsledky naznačují nový směr v reaktivaci latentního proviru a v terapii HIV infekce.

Klíčová slova: HIV-1, latence, reaktivace, hem arginát, železo, CO, bilirubin, askorbát, redoxní stres, reaktivace latentní infekce, terapeutická reaktivace.

2. Abbreviations

AIDS	-	Aquired immunodeficiency syndrome
APOBEC3G	-	Apolipoprotein B mRNA-editing enzyme 3G
ART	-	Antiretroviral therapy
Asc	-	Ascorbate
BVR	-	Biliverdin reductase
CA	-	Capsid protein
cART	-	Combined antiretroviral therapy
CDK9	-	Cyclin-dependent kinase 9
CO	-	Carbon monoxide
CORM-A1	-	Carbon monoxide releasing molecule –A1
ddPCR	-	digital droplet PCR
DFO	-	Desferrioxamine
DNMT	-	DNA methyltransferase
DNMTi	-	DNA methyltransferase inhibitor
EGFP	-	Enhanced green fluorescence protein
Env	-	Envelop
HA	-	Heme arginate
HAART	-	Highly active antiretroviral therapy
HAT	-	Histone acetyl transferase
HDAC	-	Histone deacetylase
HDACi	-	Histone deacetylase inhibitor
HIV	-	Human immunodeficiency virus
HKMT	-	Histone lysine methyltransferase
HMT	-	Histone methyltransferase
HO-1	-	Heme oxygenase-1
IN	-	Integrase

LRA	-	Latency reversing agent
LTR	-	Long terminal repeats
MA	-	Matrix protein
NAC	-	N-Acetyl cysteine
NC	-	Nucleocapsid
Nef	-	Negative factor
NFAT	-	Nuclear factor of activated T cells
NF-kB	-	Nuclear factor kappa B
Nrf2	-	Nuclear factor (erythroid-derived 2)-like 2
PBMC	-	peripheral blood mononuclear cell
PKC	-	Protein kinase C
PMA	-	Phorbol myristate acetate
PR	-	Protease
PRMT	-	Protein arginine methyltransferase
P-TEFb	-	Positive transcription elongation factor b
PTEN	-	Phosphatase and tensin homolog
RCI	-	Resting CD4+T cell infection
Rev	-	Regulator of viral transport
ROS	-	Reactive oxygen species
RT	-	Reverse transcriptase
SAHA	-	Suberoylanilide hydroxamic acid
SIV	-	Simian immunodeficiency virus
snPP IX	-	Tin protoporphyrin IX
TALE	-	Transcription activator-like effector
TAR	-	Transactivation response element
Tat	-	Transactivator of transcription

TNF-α	-	Tumor necrosis factor -α
Vif	-	Viral infectivity factor
Vpr	-	Viral protein R
Vpu	-	Viral protein U

3. Introduction

3.1 Human immunodeficiency virus

Depletion of CD4+ T cells and a resulting severe immunodeficiency was first observed in 1981 in groups of young homosexual men and injection drug users in the United States who had Kaposi's sarcoma or *Pneumocystis carinii* infection (CDC, 1981). The new disease was recognised and the name acquired immunodeficiency syndrome (AIDS) was designated by CDC in 1982 (CDC, 1982). Immediately in the following years, the virus that causes AIDS was isolated by various groups in France and in the USA. Luc Montagnier and colleagues isolated the virus in 1983 from France and named it lymphadenopathy-associated virus (LAV) (Barre-Sinoussi et al., 1983). In 1984, two groups from the USA independently isolated the virus; Robert Gallo and colleagues called it human T-lymphotropic virus III (HTLV III) (Gallo et al., 1984) and the Jay Levy and his co-workers named their isolate as AIDS-associated retrovirus (ARV) (Levy et al., 1984). The common name human immunodeficiency virus-1 (HIV-1) was assigned in 1986 by The International Committee on the Taxonomy of Viruses. Barre-Sinoussi and Luc Montagnier were awarded Nobel Prize in 2008 for their discovery.

3.1.1 HIV classification and phylogeny

HIV was renamed as HIV-1 when another virus related to HIV-1 but antigenically distinct, today known as HIV-2 was reported to cause AIDS in western Africa (Clavel et al., 1986). Both HIV-1 and HIV-2 belong to the genus lentivirus of retroviridae family. Also, both of them have originated in non human primates and as a result of zoonotic transfer they crossed the species barrier and became infectious viruses of human beings (Sharp and Hahn, 2011). HIV-1 is phylogenetically related to simian immunodeficiency virus that infects chimpanzees (SIVcpz), endemic to subspecies *Pan troglodytes troglodytes* (SIVcpz*ptt*) (Gao et al., 1999), while HIV-2 has its origin from SIV that infects sooty mangabeys (SIVsm). The four distinct lineages of HIV-1 are: Group M (Main), Group O (Outlier), Group N (non-M, non-O) and Group P (Pending). Group M is responsible for more than 95% of the global pandemic (Merson et al., 2008) and is further divided in to sub types or clades from A to K. Group O is endemic to west-central Africa (mostly in Cameroon) and accounts to less than 1% of HIV-1 infection globally (Mauclere et al., 1997). Group N is found only in few cases in Cameroon (Simon et al., 1998) and Group P was

isolated in two Cameroonian women living in France (Plantier et al., 2009, Vallari et al., 2011). Origin of HIV-1 Group O and P was also from SIVcpz*ptt*, but the primate reservoir of these viruses was found to be wild gorillas (Van Heuverswyn et al., 2006, Plantier et al., 2009). 78 million people have become infected with HIV and 35 million people have died from AIDS-related illnesses since the start of the epidemic. (http://www.unaids.org/en/resources/fact-sheet)

3.1.2 HIV-1 structure, genes and proteins

HIV-1 is a spherical, enveloped virus of about 120nm diameter. As shown in Fig. 1, the genetic material of HIV-1 is two single-stranded positive sense RNA, which is covered by a conical capsid formed by a capsid protein (p24). The single-stranded RNA is tightly bound to nucleocapsid (NC) protein (p7) and other functional enzymes like integrase, reverse transcriptase and protease. The capsid is surrounded by a matrix (MA) protein (p17). The outer bi-layered lipid envelope is studded with three glycoprotein cap molecules (gp120) and three transmembrane glycoproteins (gp41) that connect gp120 with the lipid envelope.

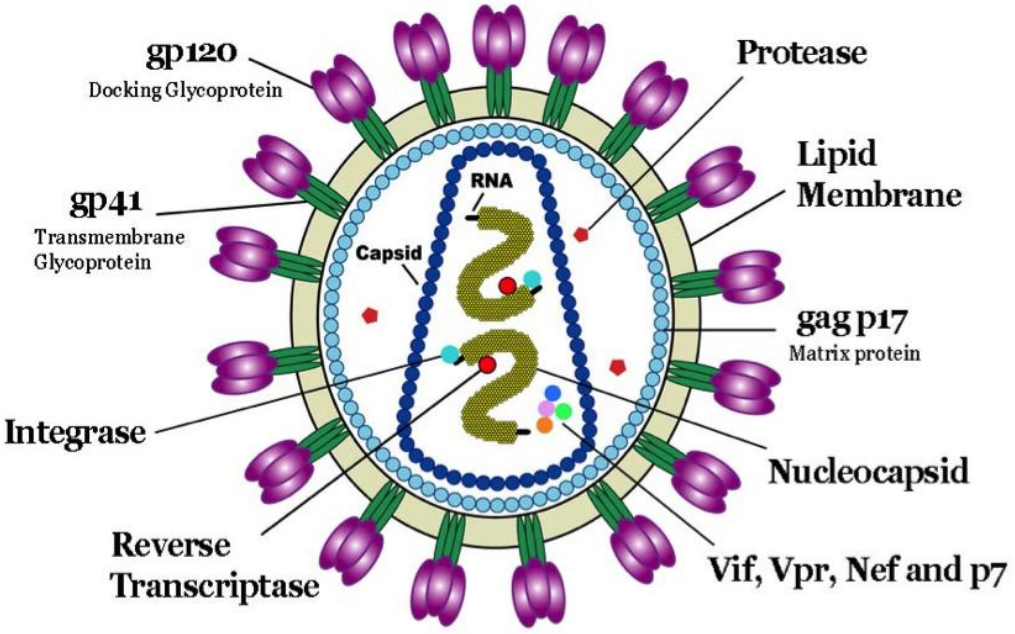


Fig. 1. Structure of HIV-1. The two copies of single stranded RNA genome and other functional enzymes are packed inside a conical capsid protein, which in turn is covered by a matrix protein. The bi-layered lipid membrane of HIV-1 contains gp120 and gp41 glycoproteins. (Image source: NIAID).

The genome of HIV-1 is approximately 9.8kb in length (Muesing et al., 1985) (Fig. 2). Both the 5' and 3' ends are flanked by long terminal repeats (LTR). Between the 5' and 3' LTR, the HIV-1 genes are coded in three open reading frames. Gag gene encodes the structural proteins like matrix, capsid and nucleocapsid proteins. Pol gene codes for the functional proteins like the protease, reverse transcriptase and integrase enzymes. Env gene encodes the envelope glycoproteins gp120 and gp41. Apart from these three major gene segments, the HIV-1 genome codes for other regulatory proteins like, transactivator of viral transcription (Tat), regulator of RNA transport (Rev), and accessory proteins such as viral infectivity factor (Vif), viral protein R (Vpr) viral protein U (Vpu), negative factor (Nef). 5' and 3' LTRs are necessary for the integration of HIV-1 into the host genome and 5' LTR acts as a promoter region.

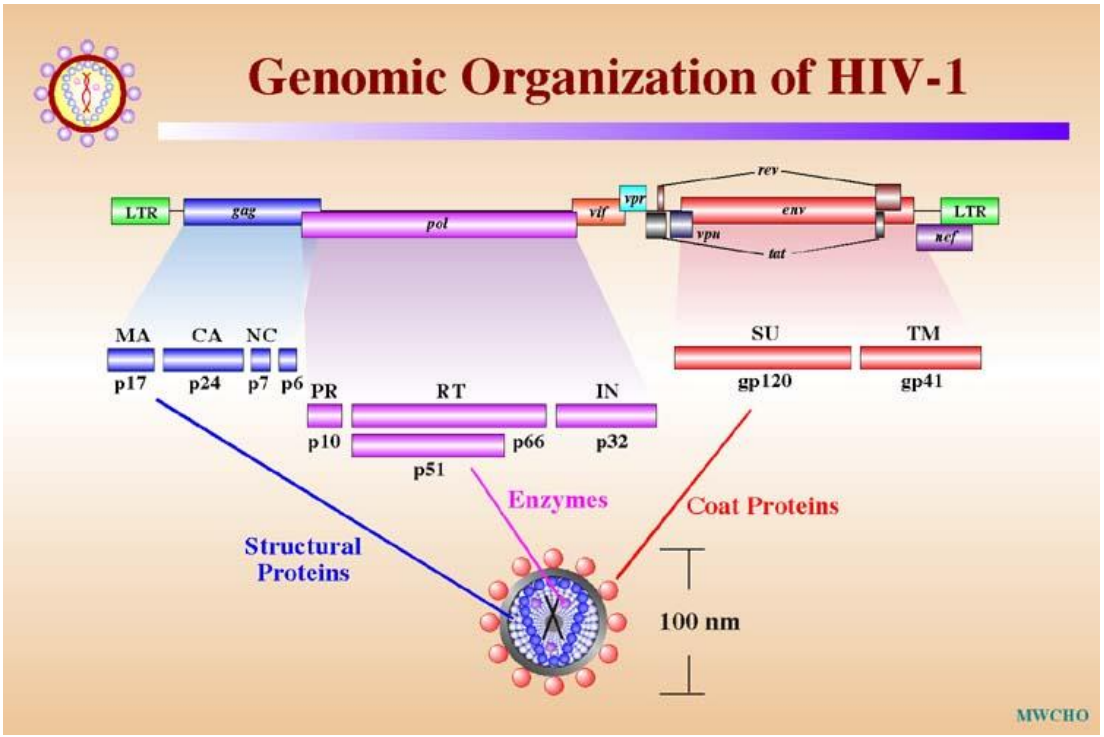


Fig. 2. Genomic organization of HIV-1. The three major genes Gag, Pol and Env and other genes encoding regulatory and accessory proteins are coded in three open reading frames in between 5' and 3' LTRs of HIV-1 genome.
(Image source: https://web.stanford.edu/group/virus/retro/2005gongishmail/HIV.html)

HIV-1 protease (PR) is an aspartyl protease and is necessary for gag and gag-pol precursor protein cleavage during HIV maturation. The 55kD gag precursor protein (p55) is cleaved after budding by the viral protease enzyme to yield capsid (p24), matrix (p17) and nucleocapsid (p9) and (p6) proteins (Göttlinger et al., 1989).

160kD env protein (gp160) is also cleaved by protease to gp120 and gp41 subunits that are present in the envelope and involved in the interaction between HIV-1 and target cells (Landau et al., 1988). The Reverse transcriptase (RT) is a RNA-dependent and DNA-dependent polymerase enzyme which produces double-stranded DNA from the positive-sense single-stranded viral RNA. Incorporation of HIV DNA into the host genome is mediated by integrase (IN). Tat is a RNA binding protein and is essential for transcriptional transactivation and full length elongation of HIV-1 transcription (Feinberg et al., 1991). Transition from early to late gene expression is induced by rev protein (Cochrane, 2014). CD4 and MHC class-I molecules on the host cell surface is down regulated by nef (Garcia and Miller, 1992), it also enhances infectivity of the virion (Miller et al., 1994; Pereira and daSilva, 2016). Vpr is found incorporated within the virions and has been shown to modulate the host and viral gene transcription, induction of cell cycle arrest and apoptosis, involved in importing viral DNA in to the nucleus of the non-dividing cells (Guenzel et al., 2014). Vpu down regulates CD4 molecules in the endoplasmic reticulum (Willey et al., 1992) and enhances virion release from plasma membrane of the infected cells (Schubert et al., 1996). Vif is a 23kD protein that enhances HIV-1 infectivity by preventing the action of host antiretroviral factors of APOBEC-3 family (Salter et al., 2014).

3.1.3 HIV-1 infection and life cycle

The HIV-1 replication cycle can be divided into two phases: the early phase starting from binding and fusion of virus at the cell surface to the integration of HIV-1 viral DNA into the host genome. and The late phase includes the events from expression of HIV-1 genes to the release and maturation of new virions (Freed, 2015). HIV-1 trimeric surface protein gp120 binds to the primary receptor CD4 molecule present in the target cell. This binding results in a conformational change in gp120 and enables it to attach to one of the two co-receptors - the chemokine receptor CCR5 or CXCR4. Macrophage tropic, non-syncytium-inducing (R5) viruses bind to CCR5 and the T-cell-tropic, syncytium-inducing (X4) viruses bind to the CXCR4. Binding of gp120 to cellular receptors changes the conformation of gp41 that results in gp41 penetrating the plasma membrane, inducing fusion of HIV-1 with target cell (Wilen et al., 2012).

Upon fusion and entry into the cytoplasm, the capsid protein is uncoated and the viral single-stranded RNA genetic material is reverse transcribed to double-stranded DNA by virus-encoded reverse transcriptase (RT) (Hu and Hughes, 2012). The pre-integration complex (PIC) containing the newly formed viral DNA and associated viral and cellular proteins migrates to the nucleus using the cellular dynein and microtubule network (McDonald et al., 2002) and integrase catalyses the integration of double-stranded viral DNA into the host chromosome (Craigie and Bushman, 2012) and establishes a functional provirus.

The integrated provirus can either enter into a latent form or be actively transcribed by the cellular RNA polymerase II transcription machinery that is promoted by the viral transcription transactivator protein (Tat) (Lucic and Lusic, 2016). From early multiply spliced viral mRNAs, the regulatory proteins Tat, Nef and rev are produced, later larger incompletely spliced mRNA encoding accessory proteins Vif, Vpr and Vpu is transcribed, and finally an unspliced transcript yielding the Gag-Pol polyprotein is produced; the same full length transcript is used as new viral genomic RNA (Karn and Stolzfus, 2012). The viral mRNAs are then exported to the cytoplasm, where the viral proteins are synthesised and the new virus particles are assembled at the plasma membrane, which is mediated by Gag-Pol polyprotein as well as Nef and Env. The ESCRT (endosomal sorting complexes required for transport) machinery plays a key role in budding and releasing of the newly formed virions. Virus maturation concomitantly takes place with budding and release. HIV-1 protease enzyme cleaves the Gag and Gag-Pol polyproteins to form fully processed MA, CA, NC, p6, PR, RT, and IN proteins (Sundquist and Krausslich, 2012; Freed, 2015) and the new HIV-1 particles are released by budding (Fig. 3).

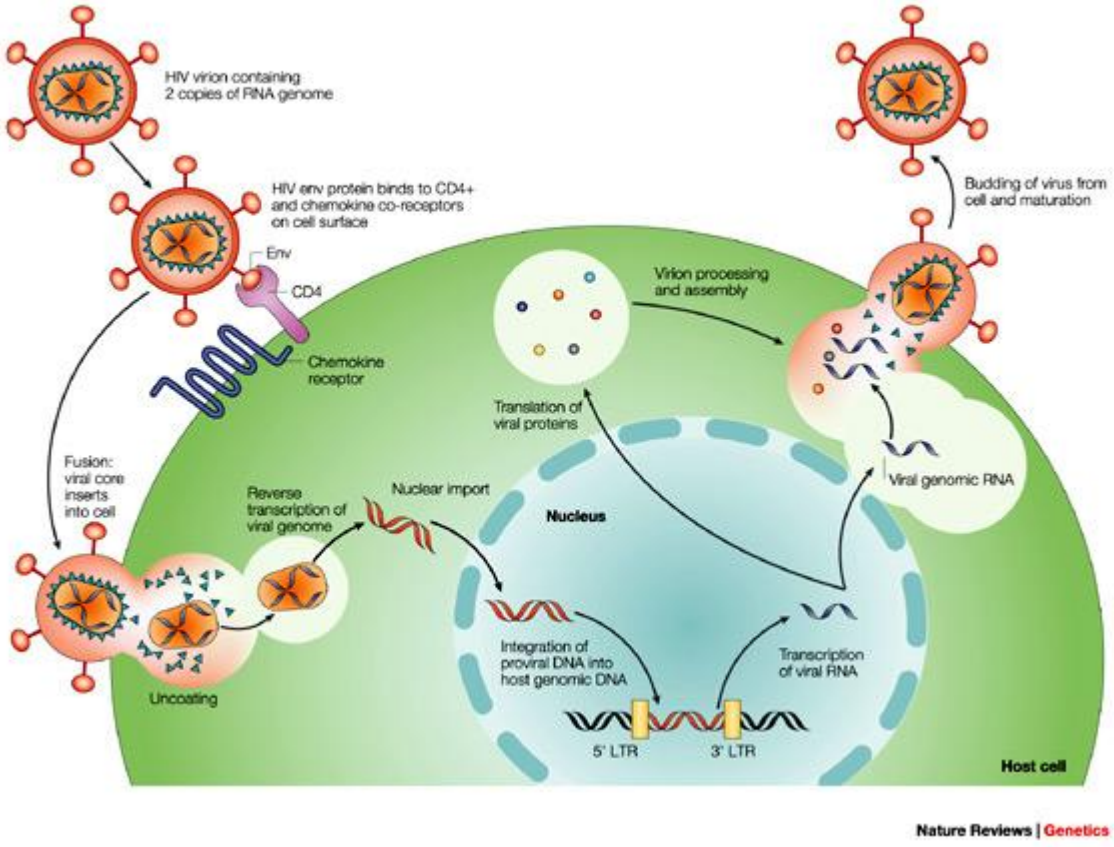


Fig. 3. HIV-1 replication and lifecycle. The early and late phase of HIV-1 life cycle beginning with fusion and entry of HIV-1 into target cell, reverse transcription of viral RNA to DNA, integration of viral DNA into host genome, transcription of viral genes, translation of viral proteins in the cytoplasm, assembly of viral proteins and finally budding and releasing of matured new HIV-1 particles. (Image source: Rambaut et al., 2004)

3.1.4 HIV-1 Latency and reactivation – a molecular insight

3.1.4.1 HIV-1 infection: Targets and latent reservoirs

Although HIV-1 can infect and provirus has been found in monocytes (McElrath et al., 1991), dendritic cells (Langhoff et al., 1991), CD8+ T cells (De Maria et al., 1991), endothelial cells (Steffan et al., 1992), astrocytes (Churchill et al., 2009), and hematopoetic stem cells (Carter et al., 2010), macrophages and CD4+ T lymphocytes are the major targets of HIV-1 (Zhang et al., 1999). Among these cells, resting CD4+ T cells, mean half-life of which can reach 44 months, are considered to be the most important reservoirs of latent HIV-1 in the body and it has been estimated that it would require over 60 years of cART to eradicate the latent reservoirs from the body (Finzi et al., 1999, Siliciano et al., 2003).

HIV-1 infection in the resting CD4+ T cells is blocked by several cellular restriction factors like apolipoprotein B mRNA-editing enzyme 3G (APOBEC3G), bone marrow stromal cell antigen 2 (BST-2), cyclophilin A, tripartite motif protein 5 alpha (Trim5α) and cellular microRNAs (miRNAs) (Chiu et al., 2005; Van Damme et al., 2008; Qi et al., 2008; Yap et al., 2006, Ahluwalia et al., 2008). These blocks prevent the integration of the virus into the host genome resulting in a relatively short latent state known as pre-integration latency. On the other hand, in active T cells the viral RNA is reverse transcribed efficiently and the pre-integration complex is imported into the nucleus and integrated within the host genome. Hence, when a small portion of infected active CD4+ T cells returns to a quiescent state, it results in a transcriptionally silent and more stable latent state known as post-integration latency (Chun et al., 1995; Marcello, 2006). However, a recent study has showed that latency can be established directly and early in both resting and activated primary T cells (Chavez et al., 2015).

The successful reactivation of latent virus from its reservoir requires both transcription initiation and its efficient elongation. Since the HIV-1 provirus is integrated into the host genome, these steps depend upon the effects of *cis*- and *trans*- acting factors of both the host and the virus (Marcelo, 2006). Understanding the factors that limit both initiation and elongation of HIV-1 provirus gene transcription is necessary for developing strategies to eliminate the latent reservoir.

3.1.4.2 Epigenetic regulation of transcriptional silence

Heritable changes that modify the gene expression and activity without altering the source DNA sequence are termed as epigenetic regulation (Goldberg et al., 2007). Covalent and reversible modifications of histone proteins (eg. Acetylation, methylation, phosphorylation, ubiquitination and sumoylation), DNA methylation, chromatin remodelling, RNA interference are the major epigenetic mechanisms by which the genes are regulated (Handel et al., 2010). Cell development, differentiation, stress response etc., can be influenced by epigenetic modifications. Both the host genome and the HIV-1 proviral DNA incorporated within it are susceptible to epigenetic modifications.

(A) Histone acetylation

Chromatin environment and integration site of HIV-1 is one of the factors determining transcriptional silence. The condensed chromatin in eukaryotes, known as heterochromatin, reduces the accessibility of DNA to transcriptional regulators and is associated with the transcriptionally silent regions of the genome. The relaxed chromatin region conducive for active transcription is called euchromatin (Craig, 2005). This condensation and relaxation of chromatin can be regulated by a variety of mechanisms, including ATP-dependent chromatin remodelling and post-translational modifications of histone proteins. Interaction of the negatively charged DNA and positively charged histone is decreased by acetylation of histone tail which results in relaxed, transcriptionally active chromatin. This can be reversed by deacetylation. Histone acetyl transferases (HATs) and histone deacetylases (HDACs) are the enzymes responsible for acetylation and deacetylation of histones in the nucleosomes (Haberland et al., 2009). *In vitro* infection of T-cell lines with HIV-1 showed a preferential integration in heterochromatin (Jordan, 2001). In contrast to this observation, HIV-1 has been found in introns of actively transcribed genes in the resting memory CD4+ cells derived from patient on HAART (Han et al., 2004). However, a genome-wide analysis of HIV-1 integration sites in T-cell lines showed virus latency could occur from integration into heterochromatin as well as in actively transcribed genes (Lewinski et al., 2005).

(B) Histone methylation

Methylation of histone and DNA is another important epigenetic mechanism that regulates gene expression in eukaryotes. Histones are methylated on arginine and lysine residues and the methylation is carried out by protein arginine methyltransferases (PRMTs) and histone lysine methyltransferases (HKMTs) respectively. PRMTs and HKMTs catalyze the transfer of one to three methyl groups from the cofactor S-adenosylmethionine (SAM) to lysine and arginine residues of histone tails. The methylation of histones is reversed by two families of demethylases : the lysine specific demethylase 1 (LSD1) family and the Jumonji C (JmjC) domain family. (Jenuwein and Allis, 2001; Kouzarides, 2007). Trimethylation of histone H3 lysine at position 9 (H3K9me3) by SUV39H1 and G9a

methyltransferases; and at position 27 (H3K27me3) by EZH2 or dimethylation at lysine 9 (H3K9me2) by G9a has been shown to suppress HIV-1 provirus transcription. (Kouzarides, 2002; Friedman, 2011; du Chene, 2007 and Imai, 2010). Also it has been showed that Suv39H1, heterochromatin protein 1 gamma (HP1γ) and histone H3K9 trimethylation play a major role in chromatin-mediated repression of integrated HIV-1 gene expression in different cell lines, including PBMCs isolated from HIV-1-infected donors (du Chene, 2007).

(C) DNA methylation

DNA methylation involves covalent transfer of methyl group to the carbon at the 5th position (C-5) of the cytosine ring of DNA by DNA methyltransferases (DNMTs) (Jin et al 2011). While over 98% of methylation occurs at CpG islands (CGIs) in the somatic cells, but in embryonic stem cells around 25% of all methylation occurs at non CpG context (Lister et al., 2009). DNA methylation, together with histone modification, regulates the gene transcription by condensing the chromatin and restricting the cellular transcription factors from accessing the DNA (Cedar and Bergman, 2009). The role of CpG methylation as an important factor in the maintenance and stability of HIV latency has been showed by many research groups (Bednarik et al., 1990, Schulze-Forster et al., 1990, Blazkova et al., 2009, Kauder et al., 2009). The two CpG islands flanking the transcription start site of HIV-1 5'LTR are methylated in latently infected J-Lat T cells and primary CD4+T cells and methyl-CpG binding domain protein 2 (MBD2) and HDAC2 are found at one of these CpG islands during latency. Also, MBD2 acts as a regulator of HIV-1 latency. (Kauder et al., 2009). However, results from memory CD4+T cells separated from HIV-1 infected aviremic individuals are contrasting as high (19 - 100%) CpG methylation at 5'LTR was observed in long term (over 11 years) cART treated indvidulas where as the methylated CpGs in individuals treated for less than 5 years are very low (only 1 - 2%) (Blazkova et al., 2009; Blazkova et al., 2012 and Trejbalova et al., 2016). The percentage of methylation in HIV-1 proviral promoters is higher in long-term nonprogressor and elite controller patients who control viremia than aviremic patients receiving HAART (Palacios et al., 2012).

(D) Chromatin remodelling of HIV promoter

Independent of the host integration site, the HIV-1 LTR has two nucleosomes, nuc-0 and nuc-1 upstream and downstream of transcription start site, separated by approximately 265 nt nucleosome-free region (Verdin et al., 1993). The nucleosomes are positioned near the *cis*- acting regulatory elements in the 5'LTR. The nuc-1 is positioned downstream and in close proximity of transcription initiation site. The cellular transcription factors such as late SV40 factor (LSF), ying yang 1 (YY1), activating protein 4 (AP-4), NFkB p50/50 homodimers, C-promoter binding factor-1 (CBF-1) etc., have been showed to recruit HDAC1 to the latent HIV-1 5'LTR (Romerio et al., 1997; Imai and Okamoto, 2006; Williams et al., 2006; Tyagi and Karn, 2007) which leads to deacetylation of nuc-1 and configuring nuc-1 to repressive state. Disruption and displacement of nuc-1 during transcriptional activation suggests the chromatin remodelling of nuc-1 and its crucial role in maintaining the provirus transcriptionally silent (Van Lint et al., 1996).

(E) RNA interference

The microRNAs (miRNA) are single-stranded noncoding RNAs approx. 19-25 nucleotides in length. They are ubiquitous, endogenously expressed and function as gene regulators by inducing degradation or inducing translational inhibition of their target mRNAs (Tan Gana et al., 2012). The primary-miRNA precursor is transcribed from the intergenic regions of the genome by RNA polymerase II (RNAP-II) which is cleaved to 70 nt long preliminary-miRNA by RNase III endonuclease Drosha and DiGeorge critical region gene 8 (DGCR8), a ribonuclease binding protein (RBP) and transported from nucleus to cytoplasm (Faller et al., 2010). In the cytoplasm the pre-miRNA is cleaved by Dicer (also an RNase III endonuclease) to an asymmetric duplex of mature-miRNA (Hammond, 2005). Post-transcriptional gene silencing (PTGS) of gene expression by miRNA is achieved by guiding the RNA-induced Silencing Complex (RISC), the effector complex of RNAi, to the target mRNA that results in endonucleolytic cleavage of the target.

Post transcriptional modification by RNA interference has been shown as one of the factors involved in inhibiting HIV-1 replication and silencing provirus gene expression (Sanchez-Del Cojo et al., 2011). Cellular miRNAs, including miR-28,

miR-125b, miR-150, miR-223 and miR-382 share sequence complementarity with the 3’ end of HIV mRNA and can inhibit HIV replication. Specific inhibitors of these miRNAs substantially increased virus production in resting CD4+T isolated from HIV-1-infected individuals on suppressive HAART and also in CD4+ T cells transfected with HIV-1 infectious clones. This finding indicates that cellular miRNAs are pivotal in HIV-1 latency maintenance (Huang et al., 2007). In general, the quiescent monocyte is not supportive for HIV-1 infection, while the monocyte-derived macrophages are highly susceptible (Bergamaschi and Pancino, 2010). Also these miRNAs (miRNA-28, miRNA-150, miRNA-223, and miRNA-382) are enriched in monocytes in comparison with monocyte-derived macrophages. The suppression of these anti-HIV-1 miRNAs in monocytes facilitates HIV-1 infectivity, whereas increase of the anti-HIV-1 miRNA expression in macrophages inhibited HIV-1 replication. These results support the anti-HIV-1 properties of the cellular miRNAs (Wang et al., 2009). Cellular miRNA, namely miR-15a, miR-15b, miR-16, miR-24, miR-29a, miR-29b, miR-150, and miR-223 that are directly targeting HIV-1 Nef-3′-LTR region, exhibiting significant inhibitory effects on HIV-1 replication and promoting latency (Sun et al., 2012, Patel et al., 2014). Recent studies suggest two more cellular microRNAs, miR-196b and miR-1290 target the 3' untranslated region of HIV-1 and affect its expression in latently infected and productively infected cells, again emphasizing the role of cellular miRNAs in HIV-1 latency regulation (Wang et al., 2015). In addition to binding to the HIV-1 mRNA and regulating its expression, cellular miRNAs also interfere with the cellular factors that are directly or indirectly required for HIV gene expression. For example, miR-198 inhibits HIV-1 gene expression and replication in monocytes via repression of cyclin T1 which is a regulatory subunit of a cellular RNA polymerase II elongation factor known as P-TEFb (positive transcription elongation factor b). Cyclin T1 is also required for Tat transactivation of HIV-1 LTR-directed gene expression (Sung and Rice, 2009). When the virus replication is inhibited by type III RNases Dicer and Drosha, the HIV-1 in turn, actively suppressed the expression of the polycistronic miRNA cluster miR-17/92 to support its own replication. As miR-17/92 can target and block cellular proteins such as p300-CREB binding protein-associated factor (PCAF) that are necessary for HIV gene expression, the counter attack on miR-17/92 by HIV becomes important. These

results highlight the involvement of the miRNA-silencing pathway in HIV-1 replication and latency (Triboulet et al., 2007).

On the other hand, though very low in number, HIV-1 encoded viral micoRNAs (vmiRNA) also interact with viral and cellular mRNAs and proteins (Yeung et al., 2009). HIV-1 TAR microRNA (hiv1-miR-TAR) transcribed from the TAR element in the 5' LTR, H1 microRNA (hiv1-miR-H1) originated from 3′ U3 LTR region, Nef microRNA (hiv1-miR-N367) and HIV-1 anti-sene microRNAs are examples of the vmiRNA families that were shown to interact with various potent cellular and viral targets to enhance HIV-1 replication or to maintain latency (Tan Gana et al., 2012, Swaminathan et al., 2014).

3.1.4.3 LTR and Transcription factors: the *cis* and *trans* elements in latency and reversal

The HIV 5'LTR is functionally divided into four elements; the Tat activating region (TAR), the promoter, the enhancer and the modulatory element (Schiralli Lester and Henderson, 2012). The U3 region of 5'LTR contains binding sites for several transcription factors that are ubiquitous or tissue specific and play a key role in HIV gene expression (Verdin et al., 1993; Van Lint et al., 1996; Rohr et al., 2003). The key sites of the promoter sequence are TATA box, a potent initiator sequence and the GC-rich binding sites for the Sp family of transcription factors including Sp-1 (specificity protein 1) (Jones and Peterlin, 1994). The enhancer element contains two binding sites for nuclear factor kappa B (NF-kB) and related factors (Nabel and Baltimore, 1987). The modulatory region, which is upstream of the NF-kB sites, contains binding sites for numerous factors, including CCAAT/enhancer binding protein (C/EBP) factors (Henderson et al., 1995), activating transcription factor/cyclic AMP response element binding (ATF/CREB) factors (Krebs et al., 1997), lymphocyte enhancer factor (LEF-1), nuclear factor of activated T cells (NFAT) (Shaw et al., 1988), CD28 responsive element (CD28RE) (Duverger et al., 2013) (Fig. 4) . Since the proviral reservoir is established within resting memory CD4+T cell in which the important cellular transcription factors such as NF-κB and NFAT are sequestered in the cytoplasm and not active, the transcription initiation is inhibited (Colin and Van Lint, 2009). Upon T-cell activation by external stimulants, the transcription factors are

freed from their inhibitors and are transported into the nucleus where they bind to their respective binding sites in the enhancer region of 5'LTR, resulting in provirus expression.

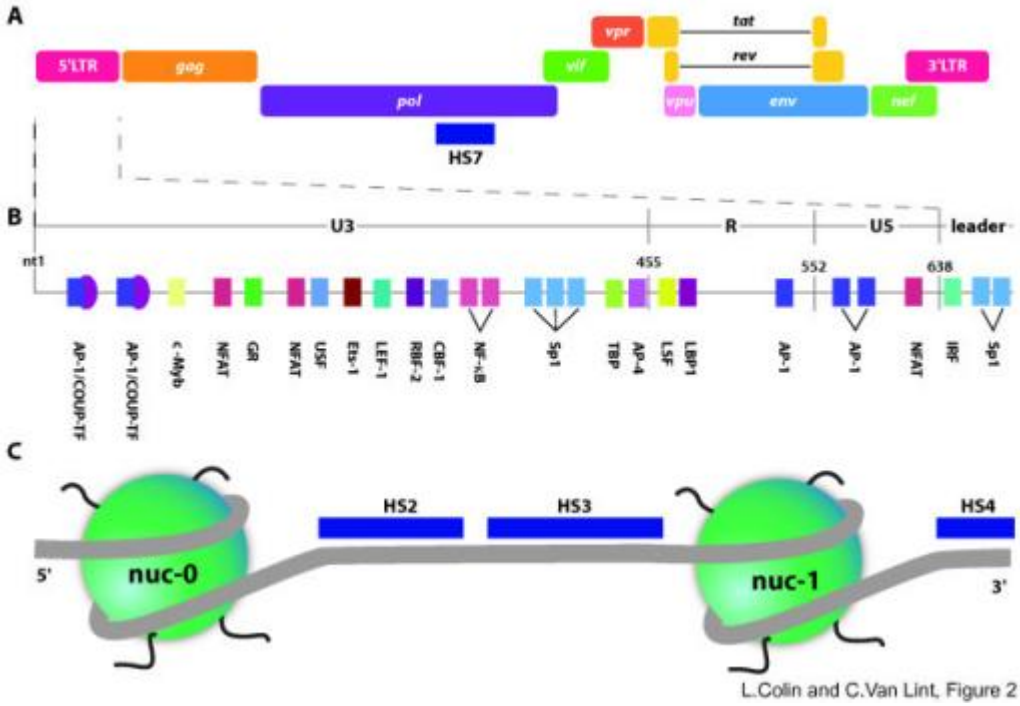


Fig. 4. HIV-1 LTR promoter contains multiple binding sites for several key transcription factors. (Image source: Colin and Van Lint, 2009)

Even though the host transcription factors binding to 5'LTR can initiate latent virus transcription, the expression level is very low. The basal transcription is boosted by a viral *trans*-activating protein Tat. Tat interacts with the *cis*-acting RNA element TAR (Transactivation response element) that forms a RNA stem loop structure, present at the 5'end of each newly formed viral transcripts (Berkhout et al., 1989). Tat plays a pivotal role in HIV transcription through the number of protein-protein and protein-RNA interactions that pave the way for the transcriptionally active complexes to assemble at the LTR. HATs, such as the CREB-binding protein (CBP)/p300 complex, are recruited to the HIV-1 promoter region by Tat. These enzymes then acetylate the nucleosomes on the promoter and make the viral LTR accessible for transcription initiation (Pumfery et al., 2003). The host transcription factor Sp1 is phosphorylated and activated by Tat induced dsDNA-dependent protein kinase (DNA-PK) and consequently, Sp1 proteins bind to LTR to upregulate the HIV-1 promoter (Chun et al., 1998). The tight regulation of Tat by ubiquitination reveals

the close interplay between the viral trans-activator and the host cell (Bres et al., 2003).

Together with the above described viral and cellular *cis*- and *trans*- factors that are necessary for transcription initiation, other factors that contribute to the transcription elongation of full length viral mRNA also determine the successful prolongation of latency and/or its reversal and gene expression.

The HIV-1 proviral DNA is transcribed by cellular RNA polymerase II enzyme (RNAPII). Transcription initiated by RNAPII in response to various cell stimulation signals produces the early regulatory protein Tat which is essential for the elongation phase of HIV-1 transcription (Colin and Van Lint, 2009; Feinberg et al., 1991). Since the RNAPII is associated with two negative elongation factors, DRB sensitivity-inducing factor (DSIF) and negative elongation factor (NELF) that inhibit RNAPII processivity, the progress of transcription initiation to elongation phase is impeded (Yamaguchi et al., 2002). The cellular positive transcription elongation factor b (P-TEFb) complex recruited by Tat helps to overcome this block (Peterlin and Price, 2006). P-TEFb complex is composed of regulatory subunit cyclin T1 and catalytic subunit cyclin-dependent kinase 9 (CDK9) proteins (Price, 2000). P-TEFb complex phosphorylates serine residues in the C-terminal domain (CTD) of RNA polymerase II and also it releases RNAPII from promoter-proximal pausing by phosphorylating NELF and DSIF. These events potentiate RNA polymerase II to synthesize full-length HIV transcripts (Williams et al., 2006). In activated cells, the P-TEFb is associated either with a 7SK RNP complex or with a chromatin-binding bromodomain protein BRD4. While the association of 7SK RNP complex inhibits P-TEFb activity (Yang et al., 2001), the BRD4 activates P-TEFb and stimulates transcriptional elongation of a wide range of genes (Yang et al., 2008; Itzen et al., 2014).

Recruitment of P-TEFb to the TAR RNA element by Tat is a crucial step in HIV-1 transcriptional activation. TAR hairpin contains a highly conserved 3-nucleotide (nt) pyrimidine bulge that binds the Tat protein and an apical 6-nt loop to which the transcriptional elongation factor P-TEFb binds in a Tat-dependent manner (Wei et al., 1998; Rana and Jeang, 1999; Richter et al., 2002). P-TEFb also directs

the recruitment of TATA box binding protein to the LTR promoter and thus stimulates the assembly of new transcription complexes (Raha et al., 2005). Recent studies show that P-TEFb is not a discrete elongation factor, rather it is an integral part of a much bigger “super elongation complex” (SEC) where several other proteins such as AF4/FMR2 family proteins AFF1 and AFF4 and ELL family of elongation stimulatory factors ELL1 or ELL2 are also playing a part in RNAPII elongation (He et al., 2010; Sobhian et al., 2010; Chou et al., 2013).

3.1.5. ‘Shock and Kill’ - Latency reversing agents

Combined antiretroviral therapy (cART) can successfully suppress the viraemia to undetectable levels and can facilitate the prolongation of the infected individual’s lifespan. Nevertheless, it cannot completely cure the infection, due to the inaccessible reservoirs of latent HIV-1 (Richman et al., 2009). If cART is interrupted, the persistent latent virus in the reservoirs like the resting CD+T cells would re-emerge rapidly and produce infectious virions, resulting in new episodes of active infection (Wong et al., 1997, Davey et al., 1999, Siliciano and Greene, 2011). Hence, the infected person must stay on therapy for the rest of the life, while cART reveals many adverse effects associated with metabolic, cardiovascular and renal diseases and toxicities (de Gaetano Donati et al., 2010, Fortuny et al., 2015).

Dieffenbach and Fauci have defined two types of cures – the sterilizing cure and the functional cure. A complete eradication of all replication-competent forms of the virus and the viral reservoirs from the infected person means a sterilizing cure, whereas a permanent control of virus replication without therapy represents a functional cure (Dieffenbach and Fauci, 2011). To achieve a sterilizing cure, the “shock and kill” strategy is currently the focus of many research groups. Reactivating the latent virus by stimulating the resting CD4+T cells is the “shock”, while “kill” is purging the activated reservoir cells as a result of induced viral cytopathic effects, immune clearance by cytolytic T lymphocytes (CTL) and cell death. To prevent the newly produced viruses from infecting other uninfected cells, the reactivation of latent virus must be carried out in conjunction with cART (Deeks et al, 2012; Shan et al., 2012).

In the beginning, attempts were made to reactivate the latent HIV-1 through activating T cells globally by combination of cytokines like interlukin-6 (IL-6), tumour necrosis factor alpha (TNF-α) or immunoregulatory cytokine interlukin-2 (IL-2) in conjunction with ART (Chun et al., 1998; Chun et al., 1999a). This strategy was termed as immune activation therapy (IAT). This combination of cytokines reactivated latent virus *ex vivo* in resting CD4+T cells obtained from HIV-1 infected individuals with or without HAART treatment. But *in vivo*, when the treatment was interrupted, within 2-3 weeks a detectable plasma viremia re-emerged, suggesting an only partial purge of the reservoir (Chun et al., 1999b; Dybul et al., 2002). Also, global T cell activation might result in increased number of uninfected target cells and increased virus production beyond the controlling threshold of antiretroviral therapy. Later strategies harnessed the molecules that selectively reactivate the latent provirus with minimal effect on the host cells. The compounds that can reactivate the latent virus are termed as 'latency reversing agents' (LRA). Blocking or activating the factors involved in the establishment of latency, maintenance of transcriptional silence, reactivation, initiation and elongation of provirus transcription are the targets of LRA.

(A) HDAC inhibitors

Inhibition of HDAC activity, thereby forming euchromatin, and increasing the accessibility of transcription factors for proviral DNA to facilitate the transcription is the principle of this strategy. All the four classes of HDAC inhibitors (HDACi): short-chain aliphatic acids, hydroxamic acid, benzamides, and cyclic tetrapeptides and despeptides have been showed to reactivate the provirus in latently infected T cell lines and in resting CD4+ T cells isolated from HIV-1 infected, cART-treated patients (Shang et al., 2015). Valproic acid (VPA), a short chain aliphatic acid and inhibitor of HDAC, was shown to induce the expression of quiescent provirus without fully activating the resting CD4+T cells of aviremic patients *ex vivo* (Ylisastigui et al., 2004). But in vivo, it depleted resting CD4+T cell infection (RCI) only in patients with intensified ART (Lehrman et al., 2005) and failed to deplete RCI in patients with standard ART (Archin et al., 2008). Later it was found out that inhibition of HDAC1 isoform alone is not sufficient to induce provirus gene expression and an additional inhibition of another isoform HDAC3 is necessary to reactivate the latent virus. In

case of valproic acid, its potency to inhibit HDAC3 is weak relative to HDAC1 (Huber et al., 2011). Hydroxamic acid HDAC inhibitor vorinostat (suberoylanilide hydroxamic acid; SAHA), is another well studied HDAC inhibitor. SAHA has been shown to induce provirus expression and to increase the unspliced cellular HIV-1 RNA levels in resting CD4+ T cells (Archin et al., 2012), but only a very small percentage (0.079%) of proviruses in resting CD4+ T cells were reactivated to produce virions (Cillo et al., 2014). Panobinostat is another potent HDACi that showed comparatively higher latency reversal activity than many other HDACis (Rasmussen et al., 2013). Loss of intestinal T helper 17 cells (Th17) in HIV-1 infection leads to damage in the muscosal barrier and to disease progression. In a recent clinical study, it was observed that while reactivating the latent provirus, panobinostat also increased Th17 cell activity and improved mucosal barrier function (Bjerg Christensen, 2015). Many more studies are exploring the potency of various new HDACi's to reactivate the latent HIV-1. To name a few, Oxamflatin (Yin et al., 2011), natural cyclic peptide Romidepsin (Istodax) (Wei et al., 2014), thiol-based HDACi ST7612AA1 (Badia et al., 2015).

(B) HMT inhibitors

As described earlier, histone methyltransferases like SUV39H1 and G9a methyltransferases play a role in transcriptional silencing of latent HIV-1 and consequently, the compounds that inhibit these enzymes are actively searched for exploiting them as potent latency reversing agents (Kumar et al, 2015). Chaetocin, a fungal mycotoxin from Chaetomium minutum belongs to 3–6-epi-dithio-diketopiperazines, acts as a specific inhibitor of Suv39H1 (Bernhard et al., 2011) has been shown to induce latent HIV-1 without producing a T cell response. Another molecule BIX-01294, a diazepin-quinazolin-amine derivative, functions as a specific inhibitor of G9a (Kubicek et al., 2007) also reactivates the provirus from latency (Imai et al., 2010). Later a detailed study reported that chaetocin induced HIV-1 recovery in 50% of CD8+-depleted PBMCs cultures and in 86% of resting CD4+ T-cell isolated from HIV-1-infected, HAART-treated patients, whereas BIX-01294 reactivated HIV-1 expression in 80% of resting CD4+ T-cell cultures isolated from similar patients. This study also showed that the synergistic action of both these compounds with prostratin have even stronger reactivation potential (Bouchat et al.,

2012). HMT inhibitors and HDAC inhibitors are reported to synergistically induce the reactivation of latent HIV-1. An example to this is, enhancer of zeste 2 Polycomb repressive complex 2 subunit 1 or 2 EZH1/EZH2 (a HMTi) demethylates H3K27. This epigenetic change does not induce provirus gene expression, but a subsequent exposure to HDACi SAHA resulted in an increased expression of HIV-1 provirus from resting CD4+T cells (Tripathy et al, 2015).

(C) DNMT inhibitors

Along with HDACis and HMTis, DNA methyltransferase inhibitors are also considered as epigenetic therapeutics for HIV-1 infection. DNMT inhibitor 5-aza-2′deoxycytidine (aza-CdR) was shown to inhibit cytosine methylation and abrogate recruitment of MBD2 and HDAC2 at the CpG islands flanking the HIV-1 transcription start site. The reactivation of latent HIV-1 by aza-CdR was significantly enhanced in synergy with the NF-κB activators prostratin or TNF-α (Kauder et al., 2009). However, when examining the effect of aza-CdR with TNF-α more closely, a syngeristic action was found to be different for different cell lines. In Jurkat T cell-derived J-Lat cell lines (6.3, 8.4 and 9.2) the aza-CdR plus TNF-α combination increased the reactivation of latent HIV-1 at least two fold when compared to TNF- α alone. In contrast it decreased the reactivation in J-Lat 10.6, ACH-2, J1.1 and U1 cell lines (Fernandez and Zeichner, 2010). Overall, there is a need to explore more new DNMTis with synergising compound combinations to develop DNMT inhibitors as a possible molecule for HIV-1 latency reversal.

(D) Protein kinase C activators

The protein kinase C (PKC) signal cascade is one of the well known cellular signal transduction pathways, affecting most important cellular functions such as cell growth, differentiation, maturation and death (Spitaler and Cantrell, 2004). Phorbol esters promote activation of monocytes and T cells, through the action of PKC. As mentioned in earlier sections, reactivation and expression of latent HIV-1 is highly dependent on the availability of host transcription factors, such as NF-κB, NFAT and AP1, and transcriptional elongation factor P-TEFb. PKC family serine-threonine kinases are important for degradation of IκB-α (an inhibitor of NF-κB) and nuclear translocation of the activated NF-κB that is required for latent HIV reactivation.

Phorbol esters such as phorbol-12-myristate 13-acetate (PMA) and prostratin, and lactones like bryostatin-1 can act as analogues of DAG (an important second messenger molecule in the PKC pathway) and induce latent proviral expression (McKernan et al., 2012). Bryostatin-1 showed a significant ex vivo potency to revert HIV-1 latency, compared to other LRA, but in a recent phase I trial the drug did not show any effect on PKC activity or on the transcription of latent HIV, probably due to low plasma concentrations (Gutiérrez et al., 2016). Even though phorbol esters and DAG analogue lactones activate PKC family kinases, the PKC isoforms they target are different. For example, PMA or prostratin induce latent HIV-1 transcription by the sequential action of PKC α and PKC θ isoforms (Trushin et al., 2005), while bryostatin-1 reactivates through PKC α and PKC δ (Mehla, 2010). PMA is a known T cell activator and tumor promoting phorbol ester, while prostratin is a non-tumorigenic phorbol ester, extracted from the barks of Samoan medicinal plant, *Homalanthus nutans*. Prostratin inhibits HIV-1 infection and viral spread (Gustafson et al., 1992) and also reactivates latent provirus from cells (Gulakowski, 1997). The anti-HIV-1 effects of prostratin is associated with its ability to down regulate the expression of CD4 receptor, the primary receptor for HIV-1, and also it down-regulates the HIV-1 co-receptors CXCR4 and CCR5. Non-tumorigenic property, inhibition of HIV-1 infection and yet reactivation of the latent provirus, all these qualities suggest that prostratin may be a potential inductive adjuvant for cART (Kulkosky et al., 2001).

Ingenol, another PKC inducer, is also a natural compound isolated from the succulent plant *Euphorbia tirucalli.* Derivatives of ingenol inhibit HIV-1 infection by down-regulating CD4 and CXCR4 receptors (Hong et al., 2011). Another ingenol derivate, 3-caproyl-ingenol (ingenol B, ING B) reactivates the latent HIV-1 gene transcription through PKC activation and NF-κB nuclear translocation. Notably, in both HIV latency models and HIV infected primary resting cells, ING B induces higher levels of reactivation when compared to TNF-α, PMA, SAHA and HMBA (Jose et al., 2014). A novel ingenol synthetic derivative (ISD) plays a dual role in HIV-1 infection, inhibiting the de novo infection while reactivating provirus from latency (Abreu, 2014). In general, growing numbers of natural and synthetic PKC inducers that exhibit anti-HIV-1 activity, lower toxicity and potent reactivation property, with

some of which being dual players in HIV-1 infection, are among the most interesting molecules in the realm of functional and sterilizing therapies of HIV-1 infection.

(E) Other unclassified LRAs

BRG-Brahma associated factors (BAF) are ATP-dependent chromatin remodelling complexes. Two small molecule inhibitors of BAF (BAFi), caffeic acid phenethylester and pyrimethamine, have been shown to reactivate latent HIV-1 through the removal of a repressive nucleosome nuc-1, without inducing a general T cell activation. These BAF inhibitors synergistically enhanced the induction of latency reversal with HDAC inhibitor SAHA and with PKC agonist prostratin in latently infected T cell lines, ex vivo infected primary cells and cells from aviremic HIV-1 infected individuals (Stoszko et al., 2016).

Disulfiram, an inhibitor of acetaldehyde dehydrogenase used to treat alcoholism, has been shown to reactivate latent HIV-1 (Xing et al., 2011) through depleting phosphatase and tensin homolog (PTEN) protein (Doyon et al., 2013) . A recent pilot study showed that a short-term administration of disulfiram increased cell-associated unspliced HIV RNA, consistent with activating HIV latency and the study suggested that disulfiram might be suited for future studies of combination and prolonged therapy to activate latent HIV (Elliott et al., 2014).

Another recent and novel approach to reactivate latent HIV-1 provirus is based on exploiting the customizable DNA-binding platforms such as engineered zinc-finger, transcription activator-like effector (TALE) proteins, as well as CRISPR-Cas9. Wang and colleagues, for the first time demonstrated the reactivation of HIV-1 expression from latently infected cells using engineered zinc finger transcription factors. The custom zinc finger transcription factor named as ZF-VP64 is composed of designer zinc-finger proteins and the herpes simplex virus-based transcriptional activator VP64 domain. The reactivation of HIV-1 by ZF-VP64 occurs through specific binding to the 5′-LTR promoter and without altering cell proliferation or cell cycle progression (Wang et al., 2014).

Transcription activator-like effectors (TALEs), originating from Xanthomonas plant pathogens, are a class of transcriptional activators (Miller et al., 2011).

Artificially designed TALE fusion proteins, containing a DNA-binding domain specifically targeting the HIV-1 promoter and the herpes simplex virus-based transcriptional activator VP64 domain has been shown to reactivate latent HIV-1 via a specific binding to the HIV-LTR promoter. Also, the TALE-VP64 fusion protein did not affect cell proliferation or cell cycle distribution while reactivating the latent provirus (Wang et al., 2015). Moreover, complementing TALE transcription factors with HDAC inhibitors can further enhance TALE-induced activation of latent HIV-1 expression (Perdigao et al., 2016).

RNA-guided CRISPR-Cas9 system comprises single guide RNAs (sgRNAs) with a nuclease-deficient Cas9 mutant (dCas9) fused to the VP64 transactivation domain (dCas9-VP64). A CRISPR-Cas9 system engineered to target a “hotspot” in the enhancer sequence of HIV-1 5’LTR consistently and effectively reactivated latent HIV-1 mediated by activator sgRNAs, across multiple different in vitro latency cell models (Saayman et al., 2015). Another transcriptional system, dCas9-SunTag-VP64, with guide RNA (sgRNA) targeting specific sequences upstream of the transcription start sites of HIV-1 was also shown to reactive latent HIV-1, without exhibiting genotoxicity and global T-cell activation (Ji et al., 2016). These novel approaches provide new means for reactivating the virus from the latent reservoirs and could contribute to the development of next-generation HIV-1 therapies.

3.1.6. Model systems to study HIV-1 latency

In vitro and *in vivo* HIV-1 latency model systems have greatly helped in understanding the molecular mechanisms underlying latency establishment, maintenance and reactivation. Also, primary screening and efficacy evaluation of latency reversing drugs are routinely conducted in these *in vitro* and *in vivo* models. The cell- and animal-based models are the two main model systems to study the HIV-1 latency (Archin et al., 2014) (Fig. 5).

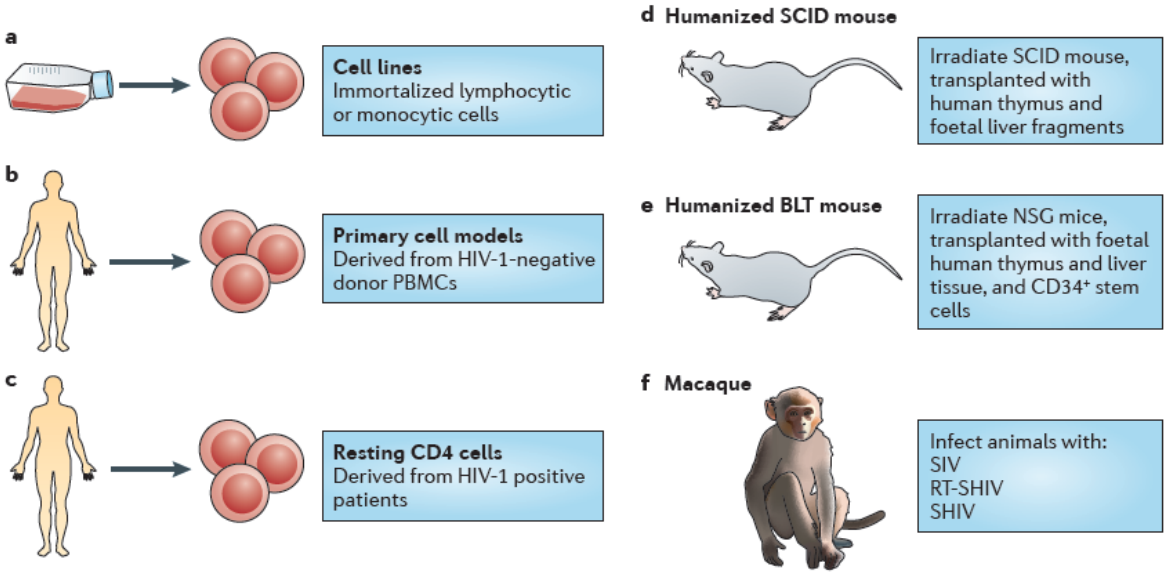


Fig. 5. Model systems to study HIV-1 latency. Cell based models (a, b and c) and animal based models (d, e and f) (Image source: Archin et al., 2014).

(A) Cell-based latency models

Immortalised T cell and monocyte cell lines that contain either a complete HIV-1 DNA or a minimal HIV-1 DNA promoter with a reporter gene for readout, integrated into the cellular chromosomes are widely used to study the HIV-1 latency and reactivation. ACH-2 cells derived from parental A3.01 T cell line containing one integrated HIV-1 proviral copy (Clouse et al., 1989, Folks et al., 1989), J-Lat cells derived from Jurkat T cell clones containing full length HIV-1 without nef and env (Jordan et al., 2003), U1 cell line, a sub clone of pro-monocytic cell line U937 that has been chronically infected with HIV-1 (Folks, et al., 1987), several Jurkat cell clones that harbour HIV-1 “mini-virus” (containing EGFP reporter cloned under HIV-1 LTR) (Jordan et al., 2001) are a few examples of such transformed cell lines that serve as HIV-1 latency models. Despite of the usefulness in basic and applied studies, none of these model system could fully recapitulate the *in vivo* latency environment of an infected person. In a study where a panel of thirteen molecules known to reactivate HIV-1 were tested across different latent cell models including five primary T cell models, four J-Lat cell models and resting CD4+ T cells from aviremic patients, to compare the responsive properties of these different cell models to latency reactivating drugs. The results indicated that no single *in vitro* cell model

alone was able to recapitulate accurately the *ex vivo* response characteristics of latently infected T cells from patients. Also, it made clear that the latency reactivation by most drugs is not uniform across the different cell models (Spina et al., 2013).

Despite the fact that the frequency of latent CD4+ memory T cells is very low (1 in 10^6 memory CD4+ T cells), isolating resting CD4+ T cells from HIV-1 infected, cART-treated aviraemic patients is a valuable method to study HIV latency or the effect of putative LRAs *ex vivo* (Archin et al, 2014). To mimic the resting stage of latent CD4+ T cells, active CD4+ T cells isolated from uninfected healthy donors are infected with HIV or HIV-derived vectors, followed by the induction of a resting state. This *in vitro* generation of memory T cells is a tedious process and only few cells transform to quiescent stage (MacLeod et al., 2010). Direct infection of resting CD4+ T cells in the absence of any stimuli (Swiggard et al., 2005) ; transducing active CD4+ T cells isolated from uninfected donors with Bcl-2, to increase the lifespan of the cells in culture and to allow them to return back to the resting stage and to establish latency by infecting with HIV-1 vector (Yang et al., 2009) are other ways to study latency in primary cell models.

(B) Animal models of latency

SCID (severe combined immune deficiency) mice are the base for the development of many humanised mouse models to study HIV-1 pathogenesis. *scid*-hu-Thy/Liv mice are generated by transplanting human foetus thymus and liver cells into *scid* mice. They produce human haematopoietic progenitor stem cells (CD34+) and mature human lymphocytes and HIV-1 latency could be established during thymopoiesis (Brooks et al., 2001). A novel variant of scid-hu-Thy/Liv mouse named NSG-hu-Thy/Liv (TOM, T cell only mice) has been developed and shown to be a promising tool in studying HIV-1 latency establishment, with no graft-versus-host disease (Honeycutt et al., 2013). BLT mice generated by transplanting human bone marrow-liver-thymus are superior model over *scid*-hu-Thy/Liv mice, as they can provide peripheral reconstitution with multiple haematopoietic lineages. HIV-1 latency has been established in BLT mice with integrated, activation-inducible, replication-competent virus infection (Marsden et al., 2012).

Recent advances in HAART that made possible a successful control of SIV in non-human primates have open the doors to study HIV-1 latency in these system (North et al., 2010). The possibility of studying different organs in non-human primates makes them more advantageous models to study virus latency. Non-human primates have immune responses that resemble human immune response but the HIV-1 latency in human lasts for many years while latency in non-human primates is only few months (Pace et al., 2011).

3.1.7. Assays to quantify latent reservoir

Quantifying the latent reservoir is important to evaluate the efficacy of latency reversal-based strategies to eradicate the reservoir. Quantitative viral outgrowth assay (Q-VOA) (Finzi et al, 1997; Siliciano and Siliciano, 2005, Laird et al., 2013) is considered as a golden standard to quantify the number of latently infected cells carrying replication competent virus. In this method highly purified latently infected resting CD4+ T cells isolated from an HIV patient are serially diluted and maximally stimulated to reactivate the latent provirus. To expand the virus produced from the reactivated latent reservoirs, activated PBMCs from healthy donors are added. After the incubation, HIV-1 p24 is quantified by ELISA or PCR to estimate the number of original latently infected cells. However, Q-VOA is laborious, expensive, requiring large volume of blood, highly time consuming and possibly underestimating the size of the latent reservoir (Ho et al., 2013). qPCR-based methods are being developed as an alternative to Q-VOA. However, qPCR-based assays could overestimate the latent reservoir as they measure all cell-associated DNA or different RNA species, including both functional and abortive transcription (Pasternak et al., 2013). Prime Flow RNA is a latest development in quantification of latency reservoirs. It combines cell-based detection of proteins with antibodies and detection of intracellular RNA with specific probes. It was reported to detect 1 infected cell/10^4-10^5 (Romerio and Zapata, 2015).

3.2 Heme Arginate

Hematin, used in the treatment of acute hepatic porphyrias is highly unstable. Consequently, a search for a stable heme preparation led to the development of heme arginate by Tenhunen and his colleagues in 1987 (Tenhunen et al., 1987).

Heme arginate is a product of pure hemin isolated from human blood complexed with amino acid L-arginine (Fig.6). The stability of heme arginate is better than hematin, the degradation rates in 4 hours is 1% for heme arginate, whereas it is 61% for hematin. Due to the increased stability, heme arginate has lesser side effects in homeostasis than hematin (Volin et al., 1988). It is an approved drug (Normosang, Orphan Europe) used to treat acute hepatic porphyrias and available outside the USA. In addition several positive effects of heme arginate has been reported, for example, HA decreases the blood pressure in spontaneously hypertensive rats (Levere et al., 1990), ameliorates murine type 2 diabetes (Choudhary et al., 2013), improves reperfusion patterns after ischemia (Andreas et al., 2012).

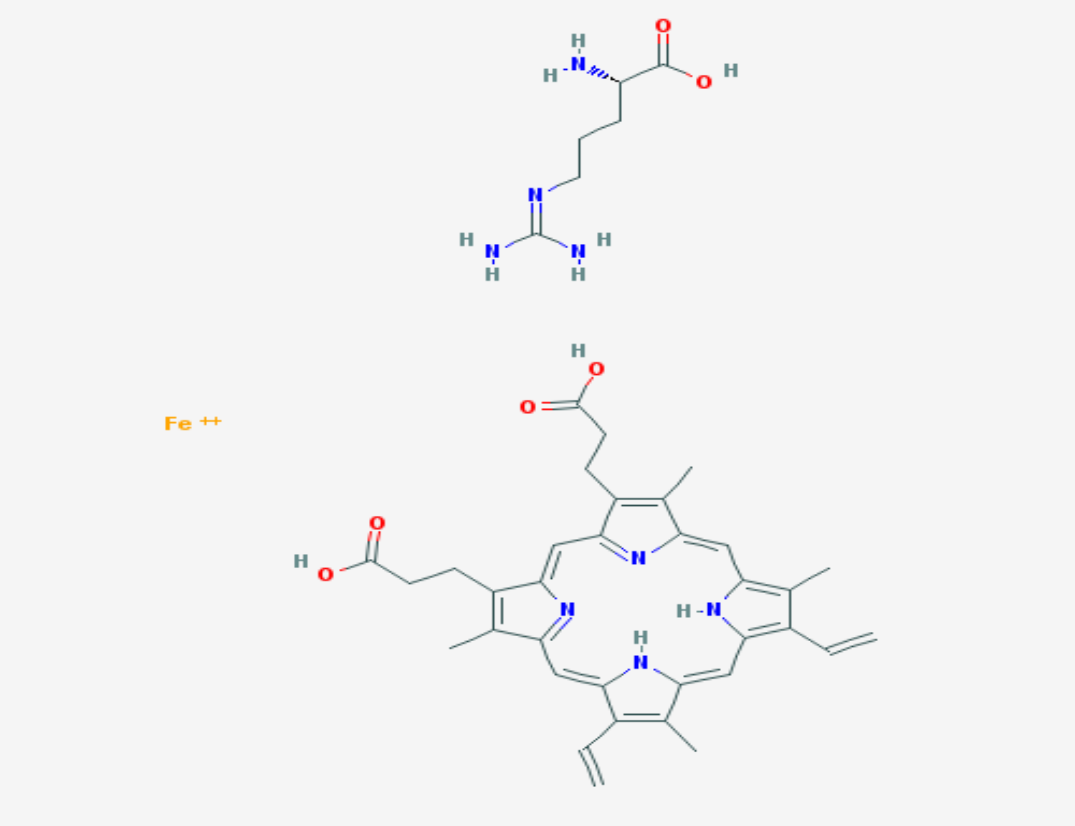


Fig. 6. Two dimensional structure of heme arginate (Image source: PubChem)

3.2.1 Heme catabolism

Heme is catabolised by heme oxygenases (HO-1,2), leading to formation of equimolar amounts of biliverdin, free iron (Fe^{2+}) and carbon monoxide. The formed biliverdin is rapidly reduced to bilirubin by biliverdin reductase (Tenhunen et al., 1968). There are three isoforms of heme oxygenase, heme oxygenase-1 (HO-1)

which is inducible and heme oxygenase-2 that is expressed constitutively. HO-1 is inducible by its natural substrate heme and a large number of structurally unrelated agents and variety of circumstances like oxidative stress, hypoxia, heat shock, reactive oxygen species (ROS), nitric oxide (NO) etc., (Fig. 7) (Abraham and Kappas, 2008). HO is considered as an important antioxidant enzyme and its cytoprotective effects are defined by the antioxidant potencies of CO and bilirubin (Parfenova et al., 2006). There are many research articles reporting a therapeutic role of HO in metabolic diseases (Son et al., 2013), cardiovascular diseases (Immenschuh and Schroder, 2006), lung diseases (Raval and Lee, 2010), renal diseases (Jarmi and Agarwal, 2009), cancer (Chau, 2015), neurodegenerative diseases (Cuadrado and Rojo, 2008), diabetes (Tiwari and Ndisang, 2014).

In vitro, heme (Fe2+, ferroprotoporphyrin IX) has been demonstrated as very efficient in inhibiting HIV-1 reverse transcription (Argyris et al., 2001; Levere et al., 1991). Further, hemin (Fe3+, ferriprotoporphyrin IX) ameliorated HIV-1 infection in humanized mice, and heme oxygenase-1 (HO-1) was suggested to be responsible for the inhibitory effect (Devadas and Dhawan, 2006).

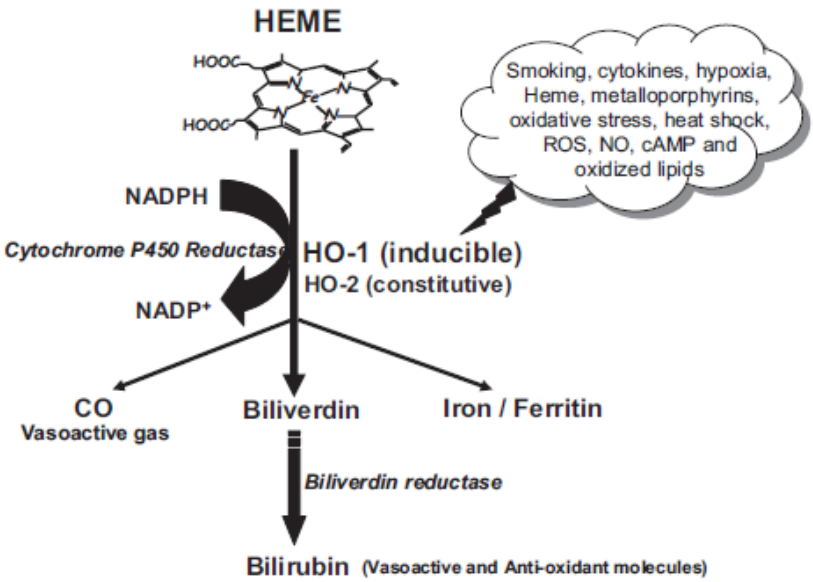


Fig. 7. Heme degradation by heme oxygenase. HO-1 and HO-2 degrades heme to yield Biliverdin, iron and CO. Biliverdin is reduced immediately by biliverdin reductase to bilirubin. HO-1 is inducible by variety of stress including heat shock, oxidative stress, ROS, UV light etc., (Image source: Abraham and Kappas, 2008)

3.2.1.1 Iron

Iron is an essential trace element present in biological systems in either ferrous (Fe^{2+}) or ferric (Fe^{3+}) state. Iron is also an essential co-factor for many important biological activities, including transport of oxygen via red blood cells (Galaris and Pantopoulos, 2008). Iron generated by HO-catalysed degradation of heme is sequestered by ferritin protein and ferritin expression is induced by increased iron concentration (Paller and Jacob, 1994). Free iron (Fe^{2+}) produced from heme degradation is pro-oxidative and was shown to generate ROS. Iron and ROS are recognised as initiators and mediators of cell death in a variety of pathological situations (Dixon and Stockwell, 2014).

Iron metabolism plays a central role in HIV-1 replication. The decline in iron stores due to poor adsorption in early HIV-1 infection may however increase later due to iron accumulation in macrophages and other cells as the disease progress (Drakesmith and Prentice, 2008). There are many reports correlating the high iron status with increased HIV-1 infection and replication (McDermid et al., 2007; Rawat et al., 2009; Chang et al., 2014). Four different clinical observations have shown the possibility that high iron status may adversely affect the outcome of HIV-1 infection (Gordeuk et al., 2001). In addition, plasma iron burden has been shown to increase due to release of a bound iron from the apoproteins as a result of increased oxidative stress (Award, 2006). Anaemia or iron deficiency (ID) is common in chronic HIV-1 infection (Redig and Berliner, 2013). The liver produced hormone hepcidin regulates systemic iron homeostasis by inhibiting ferroportin. Increased hepcidin and decreased plasma iron levels has been observed in HIV-1 acute infection, also hepcidin remained elevated in untreated or cART-treated persons with chronic HIV-1 infection (Armitage et al., 2014). Anaemia is a clinical problem in HIV-infected patients in both pre- and post- cART era. This suggests that HIV infection could result in a fundamental change in the regulation of erythropoiesis. Several studies have also reported a decrease in CD34+ progenitor cells and serum erythropoietin levels in HIV infected patients when compared to uninfected persons, demonstrating a direct effect of HIV infection on haematopoietic progenitor cells and erythropoietin responsiveness (Costantini et al., 2009; Calis et al., 2010).

3.2.1.2 Carbon monoxide

Evidence for a vital role of carbon monoxide (CO) in a number of biological and physiological functions have changed the evil image of CO as a pollutant and intoxicant (Wu and Wang, 2005). CO can mimic certain effects of nitric oxide (NO); the action of certain neurotransmitters and muscle relaxants can be regulated by both molecules (Abraham and Kappas, 2008). Carbon monoxide is an odourless, colourless, tasteless gas and the major source of endogenous CO in animals is heme degradation by HO (Abraham et al., 1983). CO can bind to ferrous (Fe II) heme and inhibit heme proteins, for example cytochrome p450 (Dulak and Jozkowicz, 2003). CO has been shown to inhibit cytochrome-c oxidase in mitochondria by competing with oxygen for binding to the reduced form of the enzyme (Alonso et al., 2003). CO acts as a vasodilator and the CO-induced vascular relaxation is ubiquitous (Wang, 1998). CO has been showed to protect against ischemia-reperfusion injury *in vitro* via its antioxidant properties (Berne et al., 2012). Several CO-releasing molecules (CORM) are being evaluated for their therapeutic potential for cardiovascular disease, inflammatory disorders and organ transplantation (Motterlini, 2007; Motterlini and Otterbein, 2010).

3.2.1.3 Biliverdin-Bilirubin

Biliverdin, a bile pigment, is also one of end the products of heme catabolism by HO. Biliverdin is rapidly reduced to bilirubin by biliverdin reductase (BVR). Bilirubin is a major physiologic antioxidant and cytoprotectant (Baranano et al., 2002). Bilirubin is oxidized by ROS to biliverdin that is then reduced back to bilirubin by the action of BVR in a process known as ‘BVR-amplified redox cycle’ (Maghzal et al., 2009). Biliverdin reductase A (BVR-A) is also a serine/threonine/tyrosine kinase involved in regulation of glucose metabolism and cell growth (Kapitulnik and Maines, 2009). In addition, BVR-A is a leucine zipper-like DNA binding protein and acts as a transcription factor for activator protein 1 (AP-1)-regulated genes such as HO-1 (Kravets et al., 2004). Due to all these properties (cytoprotective BVR-amplified redox cycle, kinase and transcription factor activity) BVR becomes a vital enzyme in the heme degradation pathway. Biiliverdin and bilirubin have been shown to inhibit HIV-1, HIV-2 and SIV protease (McPhee, 1996). Biliverdin has been shown to induce

tolerance to cardiac allograft by inhibiting the activation of ROS-sensitive transcription factors NFAT and NF-kB (Yamashita et al., 2004; Gibbs and Maines, 2007).

3.3 HIV infection and oxidative stress

Oxidative stress, defined as a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defences (Betteridge, 2000). Oxidative stress can cause harm to the biological system by damaging biomolecules such as lipids, proteins and DNA, but at the same time they can play a useful role in physiologic adaptation and also in the regulation of intracellular signal transduction (Yoshikawa and Naito, 2002). The most important reactive oxygen species are superoxide radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), singlet oxygen ($^1O_2$), hydroperoxyl radical (HOO), alkylhydroperoxide (LOOH), alkylperoxyl radical ($LOO^{\cdot}$), alkoxyl radical ($LO^{\cdot}$), hypochlorite ion (ClO^-), ferryl ion ($Fe^{4+}O$), periferryl ion ($Fe^{5+}O$) and nitric oxide (NO). Superoxide anion is considered as a ‘primary’ ROS, because it can form ‘secondary’ ROS via enzyme or metal-catalysed reactions. Superoxide dismutase (SOD) reduces $O_2^{\cdot -}$ to hydrogen peroxide (H_2O_2) that can be converted to hydroxyl radical ($^{\cdot}OH$) through the Fenton reaction (Juarez et al., 2008). Inflammatory cells (neutrophils, eosinophils and macrophages), peroxisomes and complex I and III of mitochondrial respiratory chain are sources of endogenous cellular ROS generation (Sandalio et al., 2013; Sabharwal and Schumacker, 2014).

Oxidative stress appears to affect the HIV-1 infection in two ways: HIV-1 infection generates oxidative stress in the cells and oxidative stress drives HIV-1 disease progression. Oxidative stress may contribute to several aspects of HIV disease pathogenesis, including viral replication, inflammatory response, decreased immune cell proliferation, loss of immune function, apoptosis, chronic weight loss, and increased sensitivity to drug toxicities (Pace and Leaf, 1995). Oxidative stress due to a persistent immune activation associated with uncontrolled HIV-1 replication leads to excessive ROS generation and metabolic acidosis (Salmen and Berrueta, 2012, Gil del Valle, 2011). HIV-1 increases oxidative stress in the infected patients

by disturbing the cellular antioxidant defense system and progressively depleting the antioxidant system (Pasupathi et al., 2009). The low CD4+ T cell count in HIV positive patients is also attributed to the HIV-1 induced oxidative stress. The ROS produced in the cell due to HIV-1 replication triggers apoptosis that leads to cell death (Patki et al., 1997; Aires et al., 1995). Also in an astrocytoma cell line, HIV-1 induced oxidative stress resulted in telomere shortening and apoptosis (Pollicita et al., 2009). HIV-1 Tat has been shown to induce enterocyte apoptosis through a redox-mediated mechanism (Buccigrossi et al., 2011). HIV-1 Nef, another regulatory protein also increases oxidative stress in primary human astrocytes and led to their rapid cell death (Masanetz and Lehman, 2011).

Oxidative stress affects the intracellular redox status that results in the activation of protein kinases, including a series of receptor and non-receptor tyrosine kinases, protein kinase C and the MAP kinase cascade (Chen et al, 2016; Son et al., 2011; Serras, 2016) leading to the induction of various cellular responses. NF-kB is an important transcription factor that regulates the expression of hundreds of genes that are involved in regulating most functions at all stages of the cell growth is affected by the inhibitory/stimulatory effects of ROS. Genes regulated by NF-kB in turn play a role in regulating ROS levels in the cell (Morgan and Liu, 2011). The ‘bad’ and ‘good’ effects of ROS are determined by their concentration. A high concentration of ROS may lead to tissue damage or cell death, whereas a low concentration of ROS would lead to a cytoprotective response such as activating the Nrf2-antioxidant response element signalling pathway (Ma, 2013).

4. Aims of the study

The overall aim of the thesis has been to evaluate heme arginate as a possible HIV-1 latency reversing agent. The specifics objectives were:

1. To assess the effect of heme arginate on HIV-1 acute infection.
2. To study the effect of heme arginate on HIV-1 latency reversal.
3. To find the effect of heme degradation compounds on HIV-1 latency reversal.
4. To evaluate the effect of heme arginate on PBMCs of HIV-1-infected cART-treated patients.

5. Materials and Methods

5.1. Chemicals

All the media and growth supplements were purchased from Thermo Scientific, USA, Invitrogen Corporation (Carlsbad, CA) or PAA Laboratories GmbH (Pasching, Austria). Other chemicals used, including phorbol myristate acetate (PMA), ferric nitrate, ascorbic acid, carbon monoxide releasing molecule-A1 (CORM-A1), bilirubin, desferrioxamine mesylate salt (DFO) and N-Acetyl cysteine (NAC) were purchased from Sigma-Aldrich (Germany) unless otherwise specified. Heme arginate (Normosang) was purchased from Orphan Europe (Paris, France), tin protoporphyrin IX (SnPP) from Frontier Scientific (Logan, UT), TNF-a from Peprotech (London, United Kingdom), and RETRO-TEK HIV-1 p24 Antigen ELISA from ZeptoMetrix Corp. (Buffalo, NY). The chemicals for RNA and DNA isolation, PCR and real-time PCR were purchased from Top-Bio (Czech Republic), for ddPCR from Bio-Rad (Hercules, USA), primers and probes from IDT (Belgium) and Life Technologies (Carlsbad, CA, USA). TURBO DNA-free kit, Ambion, was from Life Technologies (Carlsbad, CA, USA).

5.2. Cell lines

Human T-cell lines A3.01 and Jurkat (a clone with high expression of CD4), ACH-2 cells harboring an integrated HIV-1 provirus (clone #4; Clouse et al., 1989), and A2 and H12 clones of Jurkat cells latently infected with a ‘‘mini-virus’’ containing the HIV-1 LTR-Tat-IRES-EGFP-LTR (Blazkova et al., 2009; Jordan et al., 2001) were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 12.5 mM Hepes, and antibiotics (penicillin $1X10^5$ U/l, streptomycin 100 mg/l; 10% FBS-RPMI). The cells were treated with increasing concentrations of HA (1.25 and 2.5 µl/ml of HA correspond to 31.25 and 62.5 µg/ml of hemin or 48 and 96 µM hemin, respectively). ACH-2, A2 and H12 cells were stimulated with phorbol myristate acetate (PMA; final concentration 0.5 ng/ml was used throughout the experiments) to express HIV-1 or EGFP, respectively. The cells were also treated with N-acetyl cysteine (final concentration 5 and 10 mM), SnPP (final concentration 6.25 µM), TNF-a (final concentration 1 and 10 U/ml), PHA (final concentration 0.5 and 1 µg/ml) Stock solutions of PMA and bilirubin were prepared in DMSO and freshly diluted in culture medium to 20x working solutions. Other working solutions

were freshly prepared as follows: 100x Fe^{3+} (1 mM $FeNO_3$ in 50 mM Hepes pH=8 and 20 mM EDTA), 100x ascorbate (25 mM ascorbic acid in H_2O), 100x CORM-A1 (5 mM CORM-A1 in H_2O), 20x NAC (100mM in RPMI), 100x DFO (12.5 mM in H_2O). The compounds were added at final concentrations specified in each experiment.

5.3. Primary cells

Peripheral blood mononuclear cells (PBMCs) of HIV-infected patients on a combined anti-retroviral therapy (cART) with viremia < 50 copies/ml were isolated by Ficoll gradient centrifugation (Histopaq, Sigma-Aldrich, Germany), differential blood count was determined by Advia 60 Hematology System (Bayer Healthcare, country) and PBMC's were resuspended at final concentration of 5 x 10^6 lymphocytes/ml in 10% FBS-RPMI. PBMC's were plated at 1 ml/well in a 24-well plate and subjected to treatment with 2.5 µl of HA/ml and different concentrations of PMA for 18 h.

5.4. Viruses and infection

The stock of HIV-1 was prepared using a transient transfection of Jurkat cells with pNL4–3 (Adachi et al., 1986). The culture supernatant was collected at day 7 after transfection and virus titer was estimated as 4.8 $X10^{10}$ TU/ml (transducing units/ml) based on levels of p24 antigen determined by RETRO-TEK HIV-1 p24 antigen ELISA according to the manufacturer's protocol. For time course experiments, 0.2X 10^6 cells in 0.2 ml of 10% FBS-RPMI were infected with 2 µl of the stock; after 4 h of adsorption of inoculum, 0.8 ml of 10% FBS-RPMI was added and supplemented with HA (final concentration 1.25 and 2.5 µl/ml). The cells were split 1:4 at the indicated times after infection and the media was supplemented with HA to keep the final concentrations as indicated. The growth of HIV-1 was characterized by levels of p24 antigen in culture supernatants. For detection of HIV-1 reverse transcripts, virus stock was treated with RNAse-free DNase I (Sigma, Germany; final concentration 300 U/100 µl of virus stock) and incubated at room temperature for 45 min to remove plasmid and cellular DNA present in the inoculum. 0.5 x 10^6 A3.01 and Jurkat cells in 0.2 ml of 10% FBS-RPMI were infected with 100 µl of the DNase I-treated virus stock, and after 4 h of adsorption of inoculum, 0.8 ml of 10% FBS-RPMI was added and supplemented with HA (final concentration 2.5 µl/ml) or

Azidothymidine (AZT; final concentration 10 μM) as a control. Forty eight hours after infection, the cells were collected in PBS, trypsinized and used for DNA isolation.

5.5. Western blot analysis

Samples from all experiments that were analysed by western blot, were performed by seeding an initial concentration of $0.5x10^6$ cells/ml (ACH-2, A2 and H12 Jurkat cells) unless otherwise specified. Cells were collected and lysed in Laemmli reducing sample buffer, boiled and analyzed by SDS–PAGE and western blotting as previously described (Harlow and Lane, 1988; Laemmli, 1970), using chemiluminescence (West Femto, Thermo Fisher Scientific –Pierce, Rockford, IL). For p24 antigen, the cell lysates were resolved on a 14% SDS–PAGE and detected using a monoclonal antibody ND-1 (dilution 1:500; Exbio, Prague, Czech Republic) and a peroxidise conjugated goat anti-mouse IgG (dilution 1:20,000; Sigma Co., St.Louis, MO). EGFP was detected using a 12% SDS–PAGE, a rabbit polyclonal antibody (dilution 1:1000; Exbio, Prague, Czech Republic) and a peroxidase-conjugated goat anti-rabbit IgG (dilution 1:20,000; MP Biomedicals – Cappel, Solon, OH), HO-1 was detected using a 10% SDS–PAGE, a rabbit polyclonal antibody (dilution 1:20,000; Abcam, Cambridge, United Kingdom) and a peroxidise-conjugated goat anti-rabbit IgG (dilution 1:20,000). β-Actin was detected on a 10% gel, using either a goat polyclonal antibody (dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and a peroxidase-conjugated donkey anti-goat IgG (dilution 1:20,000; Jackson ImmunoResearch Laboratories, West Grove, PA) or using a rabbit polyclonal antibody (dilution 1:7500; Abcam, Cambridge, United Kingdom) and a peroxidase-conjugated goat anti-rabbit IgG (dilution 1:20,000). Wherever indicated, the chemiluminescence was recorded using ChemiDoc MP system (BioRad, Hercules, USA) and the densitometry analysis was performed with ImageLab software version 5.0 (BioRad, Hercules, USA).

5.6. ELISA for HIV-1 p24 quantification

Samples from all experiments that were analysed by p24 ELISA, were performed by seeding an initial concentration of $0.5x10^6$ cells/ml of ACH-2 cells unless otherwise specified. HIV-1 capsid protein p24 accumulation in the culture supernatant was quantified using RETRO-TEK HIV-1 p24 Antigen ELISA

(ZeptoMetrix Corp. Buffalo, NY) according to the manufacturer's protocol. Cell culture supernatant was lysed in lysis buffer, diluted and incubated in HIV-1 p24 antibody coated microplate for 2 hours at 37ºC. Followed by subsequent incubation with HIV-1 p24 detector antibody, streptavidin-peroxidase, substrate (containing tetramenthylbenzidine (TMB), and dimethyl sulfoxide (DMSO)) in the order respectively with intermittent washings between each step. Finally, optical density of the colour produced by enzyme-substrate reaction was measured at 450nm using a microplate reader (Victor, PerkinElmer, USA) and the concentration of HIV-1 p24 (pg/ml) is calculated by interpolating the OD values of samples from the standard curve plotted with antigen standard.

5.7. Flow cytometry analysis

Flow cytometer Canto II (Becton Dickinson) equipped with 3 lasers emitting at 488, 405 and 633 nm, and with 8 detectors was used. Flow cytometry measurements were performed using the Diva 6 software (Becton Dickinson, Franklin Lakes, NJ). Subsequent analyses of the flow cytometric data were performed using Diva 6 and/or FlowJo (Tree Star, Inc., Ashland, OR). At each time point, cells were collected, stained with a fluorochrome, and used for further analysis in the appropriate detecting channel. Ten thousand cells were collected upon gating on a FSC-A x SSC-A dot plot. The region used for further analysis contained live cells, as well as their apoptotic counterparts (Fig. 8). Discrimination of apoptotic cells was performed on a FSC-A x SSC-A dot plot and/or using staining with Hoechst 33342 (Intergen; final concentration 0.1µg/ml; Kalbacova et al., 2002; Lizard et al., 1996) and 7-AAD (final concentration (1µg/ml) followed by flow cytometry analysis in FL5 (detecting at 474–496 nm) and FL4 (detecting at 750–810 nm), respectively. Percentage of apoptotic cells determined on a FSC-A x SSC-A dot plot correlated with the percentage of apoptotic cells determined on a Hoechst 33342 x 7-AAD dot plot. For assessment of cell viability of the infected cells during the time course experiment, the cells were first fixed with 1% paraformaldehyde, and then analyzed as described above. EGFP fluorescence was characterized by a flow cytometry analysis in FL1 (detecting at 515–545 nm). EGFP expression was assessed as the arithmetic mean of green fluorescence of green cell population x percentage of all EGFP-positive cells. EGFP fluorescence intensity was characterized by the median

fluorescence of live green cells. Detection of CD69 expression was performed using a mouse monoclonal antibody against human CD69 labelled with Alexa Fluor-700 (dilution 1:50; Exbio, Prague, Czech Republic) followed by flow cytometry analysis in FL7 (detecting at 700–720 nm).

In all the experiments were EGFP expression in A2 and H12 cells were analysed by flow cytometry, an initial concentration of $0.1x10^6$/200 µl cells was seeded, except for the experiments with iron and ascorbate (Fig. 23), where it was $0.5x10^6$/ml. Also, in all the experiments with A3.01 cells were the cell viability and apoptotic cell percentage were analysed by FACS, an intital concentration of $0.5x10^6$/ml cells was seeded, unless otherwise specified.

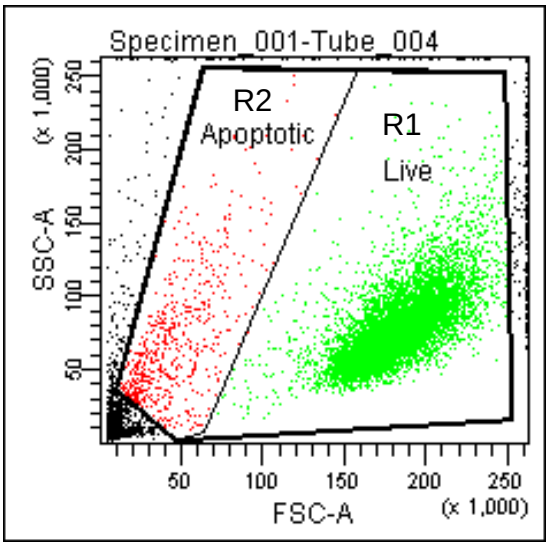


Fig. 8. Example of a flow cytometric analysis. Gating was done based on size and granularity of the cells. Cell populations in Regions 1 and 2 were used for further analysis; the two regions contained live and apoptotic cells, respectively. The samples were collected, incubated with individual fluorescent indicators as indicated, and analyzed using flow cytometry and Diva 6 or FlowJo software.

5.8. Cytotoxicity assays and determination of CC50

Cytotoxicity of heme arginate was characterized by determination of induction of apoptosis using flow cytometry (see above) and by the effects on cell viability and growth using a protocol adapted according to TOX-1 kit (Sigma Co., St. Louis, MO). Briefly, A3.01 and Jurkat cells were diluted with fresh culture medium and 24 h later, they were plated in 24-well plates at a density of 0.06 $x10^6$/ml/well in culture medium containing increasing concentrations of HA. In parallel, wells with culture medium

and HA were incubated to be used as individual blanks for each particular concentration of HA. After 2 days of incubation, cell growth and viability were characterized by activity of mitochondrial dehydrogenases using the MTT assay. The conversion of MTT to formazan was determined photometrically at 540 nm after dissolving the product in the acidified isopropanol. The cytotoxic concentration was expressed as CC50, the concentration of the tested compound that reduced cell growth to 50% compared to vehiculum-treated controls.

5.9. DNA and RNA isolation and quantification

Total cellular DNA was isolated using a modified method of Miller's salting-out procedure, without proteinase K and with addition of a chloroform extraction phase (Olerup and Zetterquist, 1992). Ethanol-precipitated DNA was dissolved in TE buffer and quantified by measuring the absorbance at 260 nm using UV spectrophotometer BioPhotometer (Eppendorff AG, Germany).

RNA was isolated using RNA Blue reagent by precipitation of the aqueous phase according to the manufacturer's protocol (Top-Bio, Czech Republic); RNA was solubilized in nuclease- and protease-free molecular biology grade water (Sigma-Aldrich, Germany) with the addition of RiboLock RNase inhibitor (Thermo Scientific, USA; final concentration 1 U/µl) and stored in -80ºC for further use. The concentration and purity of RNA was determined by measuring the absorbance at 260 and 280 nm using UV spectrophotometer Eppendorf BioSpectrometer (Eppendorff AG, Germany). The isolated RNA was then treated with TURBO DNase (TURBO DNA-free kit, Ambion, Life Technologies) to remove contaminant genomic DNA according to the manufacturer's protocol.

5.10. PCR detection of HIV-1 reverse transcripts

HIV-1 reverse transcripts were determined by PCR using primers specific for LTR/gag (Schmidtmayerova et al., 1998) and for GAPDH (sense 5'-TTC TGT CTT CCA CTC ACT CC-3', antisense 5'-GTA TTC CCC CAG GTT TAC ATG-3') in a 50 µl reaction volume containing 1U of Taq DNA polymerase (Top-Bio, Czech Republic), 1x PCR buffer (10 mM Tris–HCl, pH 8.8; 50 mM KCl; 0.1% Triton X-100), 200 nM each primer, 200 lM dNTPs, MgCl2 (1 mM for LTR/gag; 0.75 mM for

GAPDH) and sample DNA (1000 ng for LTR/gag; 200 ng for GAPDH. PCR conditions: initial denaturation 94ºC/4 min and 35 cycles of 94ºC/30 s, 52ºC/30 s for LTR/gag or 57ºC/30 s for GAPDH, 72ºC/60 s, with final extension 72ºC/10 min in a Mastercycler Gradient thermocycler (Eppendorf AG, Hamburg, Germany) . The PCR products were resolved using a 1.5% agarose gel electrophoresis in 1x TBE buffer and 0.5 µg/ml ethidium bromide, and visualized under UV transilluminator.

5.11. Quantification of HIV-1 RNA by droplet digital PCR

Absolute quantification of the HIV-1 RNA expressed in ACH-2 cells was performed using the QX100 droplet digital PCR system (Bio Rad, Hercules, USA) and One-Step RT ddPCR kit for probes (Bio Rad, Hercules, USA) according to manufacturer's protocol. Twenty µl of the reaction mixture consisted of 10 µl of 2x one-step RT ddPCR super mix, 0.8 µl of 25 mM manganese acetate, 0.5 or 5 ng of DNase treated RNA and primers and probes for HIV Gag or human GAPDH. Primers and probes used: HIV-1 Gag – forward primer GAG1 (5'-TCAGCCCAGAAGTAATACCCATGT-3') and and reverse primer GAG2 (5'-CACTGTGTTTAGCATGGTGTTT-3'), final concentration 200 nM each, probe GAG3 (FAM-5'-ATTATCAGAAGGAGCCACCCCACAAGA-3'-BHQ1), final concentration 250 nM (Pasternak et al., 2008; Kiselinova et al., 2014); human GAPDH – 1x primer-probe Hs02758991_g1 VIC-MGB labelled (Life Technologies, Calsbard, CA, USA). The droplets were generated using the QX100 droplet generator (Bio Rad, Hercules, USA) by mixing 70µl of droplet generating oil (Bio Rad, Hercules, USA) with 20 µl of ddPCR reaction mixture and PCR amplification was performed on a T100 thermal cycler (Bio Rad, Hercules, USA). The thermal cycling conditions were as follows: reverse transcription at 60ºC for 30 minutes, 95ºC for 5 min, 40 cycles consisting of denaturation at 94ºC for 30 s and annealing/extension at 60ºC for 60 s, followed by a terminal extension at 98ºC for 10 min. The amplified droplets were read by the QX100 droplet reader (Bio Rad, Hercules, USA) and the data were analysed by QuantaSoft analysis software 1.6.6.0320 (Bio Rad, Hercules, USA). Copies of Gag RNA were standardized to human GAPDH as a reference gene.

5.12. Quantification of HIV-1 RNA by real-time PCR

RNA isolated from cultured and stimulated PBMC's of HIV-infected patients treated with cART (viremia < 50 copies/ml) was used for detection of cell-associated HIV-1 RNA using semi-nested 2-step RT-qPCR adapted from (Kiselinova et al., 2014; Pasternak et al., 2008). Sixteen µl of DNase-treated RNA was used for reverse transcription in a final volume of 40 µl using random hexamer primers (Premium Reverse Transcriptase, Thermo Scientific). Then, 20 µl of cDNA was amplified in a total volume of 100 µl using GAG1 and SK431 primers (final concentration 250 nM each; SK431 sequence: 5'-TGCTATGTCAGTTCCCCTTGGTTCTCT-3'; (Pasternak et al., 2008)) and 0.05 U/µl of Blood Taq DNA polymerase (Top-Bio, Czech Republic) in 1x PCR Blue buffer (Top-Bio, Czech Republic) containing 2 mM $MgCl_2$. The thermal cycling conditions were as follows: initial denaturation at 95ºC for 5 min, 15 cycles consisting of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s, and extension at 72ºC for 60 s, followed by terminal extension at 72ºC for 10 min using the Mastercycler Gradient (Eppendorf AG, Hamburg, Germany). Finally, 10 µl of the first PCR product was used in duplicate for real-time PCR in a total volume of 50 µl containing GAG1 and GAG2 primers, GAG3 probe (final concentration 200 nM each; GAG3 sequence: FAM-5'-ATTATCAGAAGGAGCCACCCCACAAGA-3'-TAMRA; (Pasternak et al., 2008)), and 1x qPCR Blue buffer (Top-Bio, Czech Republic) using the Applied Biosystems 7300 Real-time PCR System (Applied Biosystems, Foster City, CA) and the universal cycling conditions. Four µl of the first PCR product was used for quantification of a reference gene in a 20 µl reaction also in duplicate. Levels of HIV Gag RNA were standardized to GAPDH. Four µl of DNase-treated RNA was used for a no-RT control and further processed in the way analogous to cDNA. The mean of technical duplicates was used for relative quantification of HIV Gag compared to GAPDH. Changes in expression of 10 additional house-keeping genes in healthy donors PBMCs after the individual treatments were determined using Real-time PCR kit for Human Reference Genes (Generi Biotech, Czech Republic).

5.13. Statistical analysis

Results are presented as means ± SD (standard deviation) or SEM (standard error of mean). The data were analysed and graphically represented using GraphPad Prism 5.04 software. Statistical differences between each group and control or between two groups were determined using a two-sample two-tailed Student's t-test for either equal or unequal variances. Equality of variances was tested with F-test.

6. Results

6.1 Heme arginate inhibited HIV-1 acute infection

Based on preliminary experiments and on the estimated distribution volume of heme arginate in vivo, 1.25 and 2.5 µl/ml of HA (31.25 and 62.5 µg/ml of hemin or 48 and 96 µM hemin, respectively) were selected and used to assess the effect of HA on HIV-1 acute infection. In a time course experiment, T-cell lines A3.01 and Jurkat were acutely infected with HIV-1 or mock infected in the presence of HA. Aliquots of culture supernatant were collected at three days interval (days 4, 7, 10 and 13 p.i.), to determine the HIV-1 p24 by ELISA. Throughout the incubation period, HA strongly inhibited the growth of HIV-1 in both cell lines and the levels of the p24 antigen in the culture supernatants were significantly decreased when compared to levels of the untreated controls in both cell lines (Fig. 9). In untreated cells, the HIV-1 replication and p24 levels kept increasing during the experiment period and were found to reach a maximum on day 10, with a decrease on day 13 as there remained only few cells alive (Fig. 10A). Treatment with HA 1.25 µl/ml decreased the levels of the p24 antigen several folds in comparison with untreated cells, while HA 2.5 µl/ml was even more effective in inhibiting HIV-1 infection and kept the levels of p24 antigen very low, close to the detection limit of the assay.

A

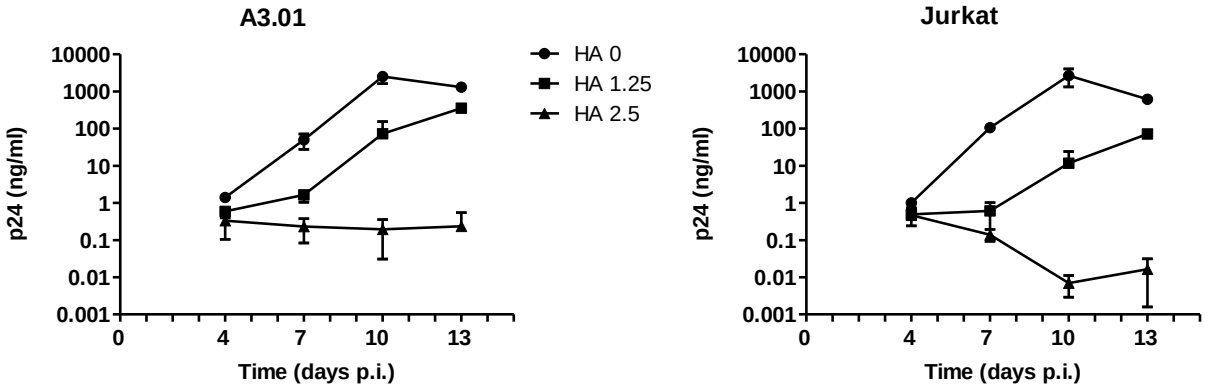


Fig. 9. Heme arginate inhibits HIV-1 acute infection. A3.01 and Jurkat cells were infected with HIV-1 and treated with HA 1.25 and 2.5 µl/ml. HIV-1 p24 levels in the culture supernatant were quantified by ELISA at indicated days after infection (p.i.). Graph represents the results of two independent experiments performed in duplicates.

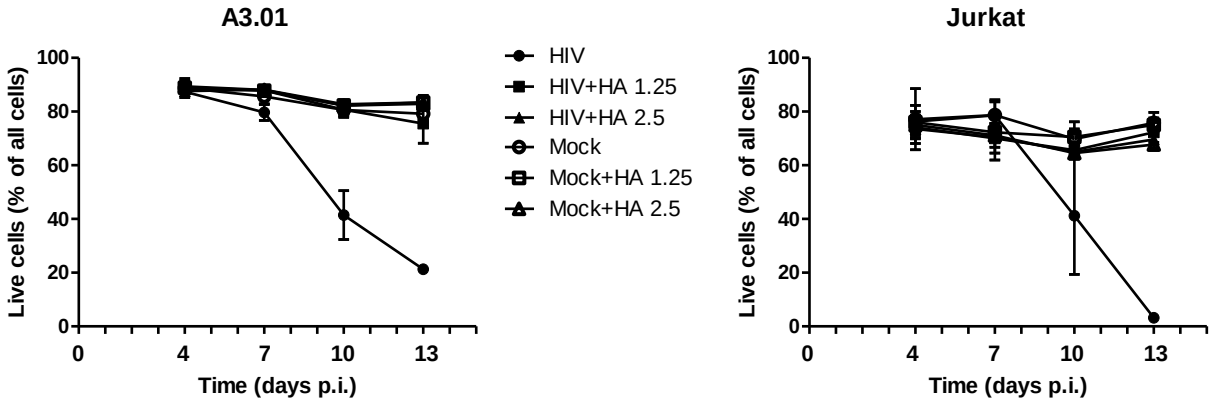
6.2 Effect of HA on T cell viability

In a parallel time course experiment with the same setup as above, the cells collected at different times p.i. were fixed with 1% paraformaldehyde and analysed by flow cytometry to determine the viability. The apoptotic and live cells were determined based on the size and granulosity of the cells as analysed by FSC-A X SSC-A. Viability of the HIV-infected, untreated cells sharply decreased during the incubation period. In contrast, viability of both HA-treated infected and mock-infected cells were comparable to untreated mock-infected cells up to the 13 days p.i. (Fig. 10A).

Additionally, uninfected A3.01 and Jurkat cells were treated with increasing concentrations of HA for 48 h to characterize the effects of HA on T-cell viability, growth, and cytotoxicity (Table 10B). Percentage of apoptotic cells was analysed by flow cytometry based on cell size and granulosity (FSC-A X SSC-A). HA 1.25 and 2.5 µl/ml that inhibited HIV-1 growth did not induce any increased apoptosis of A3.01 cells, while 2.5 µl/ml of HA increased apoptosis of Jurkat cells slightly. The live and apoptotic cells analyzed by flow cytometry with Hoechst 33342 and 7-AAD labelling, yielded similar results (data not shown). The cytotoxicity and growth inhibitory properties of HA were further characterized by the activity of mitochondrial dehydrogenases using the MTT assay. The results indicated that 1.25 µl/ml of HA did not induce any significant decrease of this activity, while 2.5 µl/ml of HA somewhat decreased it in both cell lines.

The CC50 of HA in A3.01 and Jurkat cells was determined by both flow cytometry and MTT assay. Based on flow cytometry assays, CC50 was determined as 42 and 17 µl/ml of HA (1612 and 636 µM hemin) in A3.01 and Jurkat cells, respectively, while it was 10.7 and 6.4 µl/ml of HA (412 and 244 µM hemin) in A3.01 and Jurkat cells, respectively, based on MTT test.

A



B

A3.01

HA (µl/ml)	0	1.25	2.5	5.0	7.5
Apoptosis (%)	7.40 ± 0.84	8.24 ± 1.09	9.55 ± 1.09	11.90 ± 1.11	15.00 ± 1.32
MTT (%)	100.00 ± 0.00	97.04 ± 4.12	92.00 ± 7.27	82.48 ± 13.66	62.54 ± 9.45

Jurkat

HA (µl/ml)	0	1.25	2.5	5.0	7.5
Apoptosis (%)	9.95 ± 2.59	10.32 ± 2.71	13.47 ± 2.93	21.18 ± 0.85	27.57 ± 1.42
MTT (%)	100.00 ± 0.00	96.08 ± 3.75	81.02 ± 9.02	67.83 ± 7.37	36.18 ± 4.22

Fig. 10. Effect of HA on T cell viability. (A) Viability of HIV-1-infected, untreated and HA-treated A3.01 and Jurkat cells as characterized by flow cytometry. Graphs represent the results of two independent experiments performed in duplicate. **(B)** Apoptosis and cytotoxicity of HA in A3.01 and Jurkat cells as determined by flow cytometry and MTT assay. The results represent means of three (A3.01) or two experiments (Jurkat) performed in duplicates ±SD.

6.3. Heme arginate inhibited reverse transcription of HIV-1

Inhibiton of reverse transcriptase by heme has been demonstrated previously (Levere et al., 1991; Staudinger et al., 1996; Argyris et al., 2001). To test the effects of HA on HIV-1 reverse transcription in A3.01 and Jurkat cells, the cells were infected with DNase-treated inocula of HIV-1 or mock-infected and treated with HA 2.5 µl/ml. Treatment with a nucleoside analogue inhibitor of reverse transcriptase,

azidothymidine (AZT, was included as a control for RT inhibition. After 48 h incubation, cellular DNA was isolated and PCR was performed using primers specific for HIV LTR/gag (Fig. 11). The results showed a decrease in HIV-1 DNA demonstrating the inhibitory effects of HA on levels of reverse transcripts that were comparable to those of AZT. On the other hand, levels of a house-keeping gene GAPDH were found comparable in all samples.

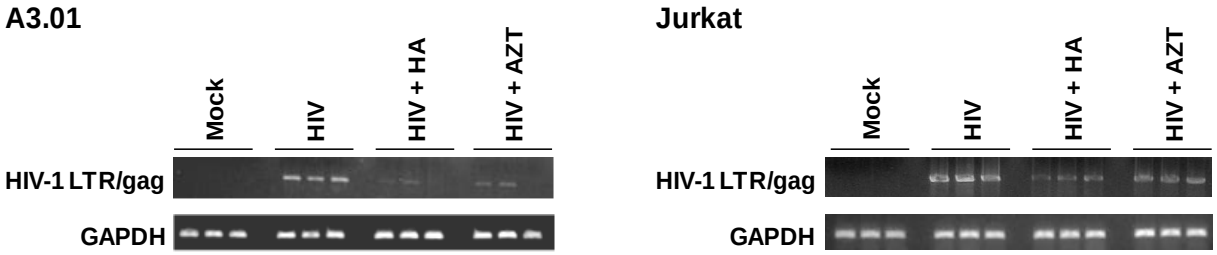


Fig. 11. HA inhibits reverse transcription of HIV-1. HIV-1 LTR/Gag specific PCR in DNA isolated from HIV-1 infected, HA2.5 µl/ml treated A3.01 and Jurkat cells demonstrated the inhibitory activity of heme arginate on HIV-1 RT. Representative results of two independent experiments performed in triplicates.

6.4. Determination of PMA concentration

To determine the minimum concentration of PMA required to activate the T cell lines, $0.5x10^6$/ml A3.01 cells were treated with different concentrations of PMA (0.05, 0.1, 0.5 and 1 ng/ml) for 24 h. After incubation, cells were collected and the expression of CD69 on the cell surface was analysed by flow cytometry using anti-CD69 antibody labelled with AlexaFluor 700. PMA 0.5 ng/ml was found as the lowest concentration to activate A3.01 cells as determined by the expression of CD69 molecule – a marker of activated T cells (Fig.12). The stimulatory effect of PMA observed in A3.01 cells was confirmed also in ACH-2 cells (data non shown).

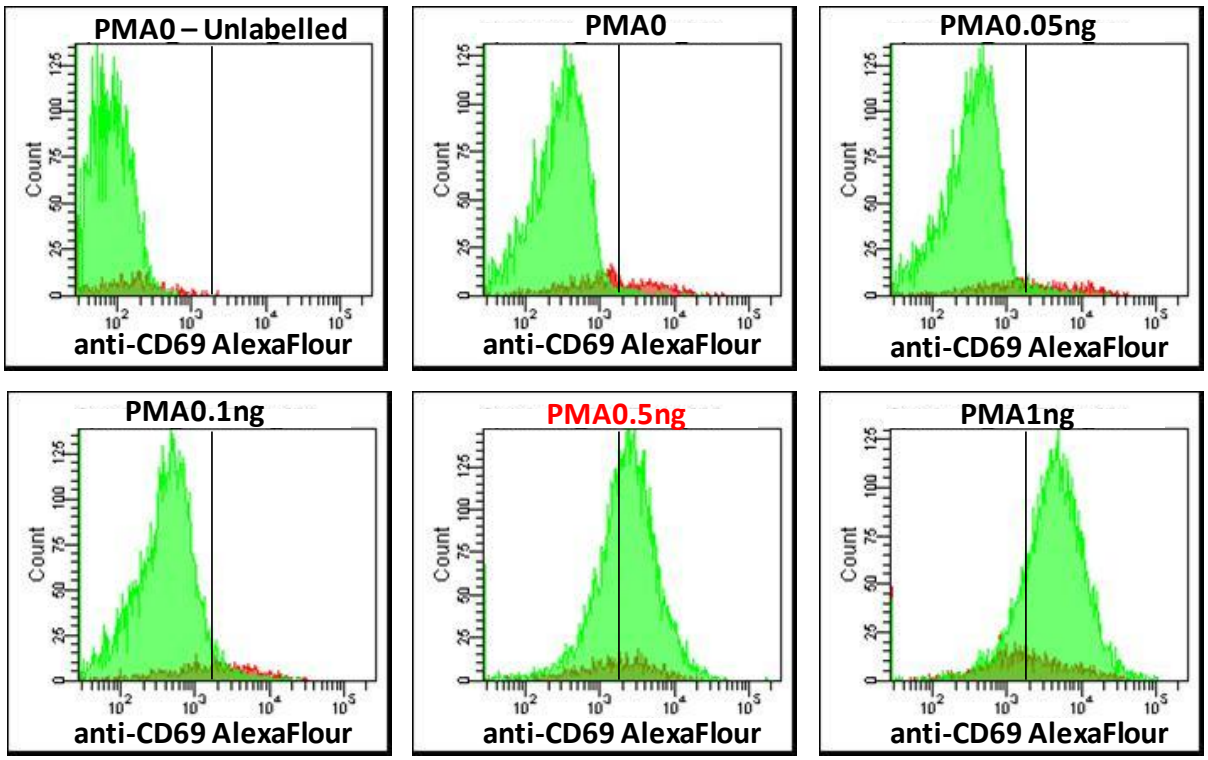


Fig. 12. PMA 0.5 ng/ml was the lowest concentration required to activate T cell lines. A3.01 cells were treated with increasing concentrations (0.05, 0.1, 0.5 and 1 ng/ml) of PMA for 24 and analysed by flow cytometry to detect the expression of CD69 molecule. Representative results of two independent experiments.

6.5. Effect of HA on reactivation of HIV-1 provirus

Reactivation of the latent HIV-1 is a hot topic in HIV therapy research. The effects of heme or hemin on reactivation of the HIV-1 provirus has not been previously studied. Therefore, we decided to study the effect of HA treatment in HIV-1 latency reversal. ACH-2 cells, harboring an integrated HIV-1 provirus, were pre-treated with different concentration of HA (2.5, 5.0 and 7.5 µl/ml) for 24 h and stimulated with PMA. The cells and culture supernatants were collected 24 h after PMA stimulation and analysed for HIV-1 p24 antigen determined by western blot analysis and ELISA, respectively. Interestingly, the results showed that HA dose-dependently increased reactivation of the provirus in PMA-stimulated ACH-2 cells. The levels of the p24 antigen and its precursor p55 were found increased both in cells (Fig. 13A), and culture supernatants (Fig. 13B). It was clear from the quantitative ELISA results that the basal induction of HIV-1 reactivation by PMA was strongly potentiated by HA and as a result p24 production was increased by several

hundred-folds. These results indicated that HA dose-dependently reactivated HIV-1 provirus in PMA-stimulated ACH-2 cells. On the other hand, HA alone was not found to stimulate reactivation of the HIV-1 provirus at any concentration tested (data not shown).

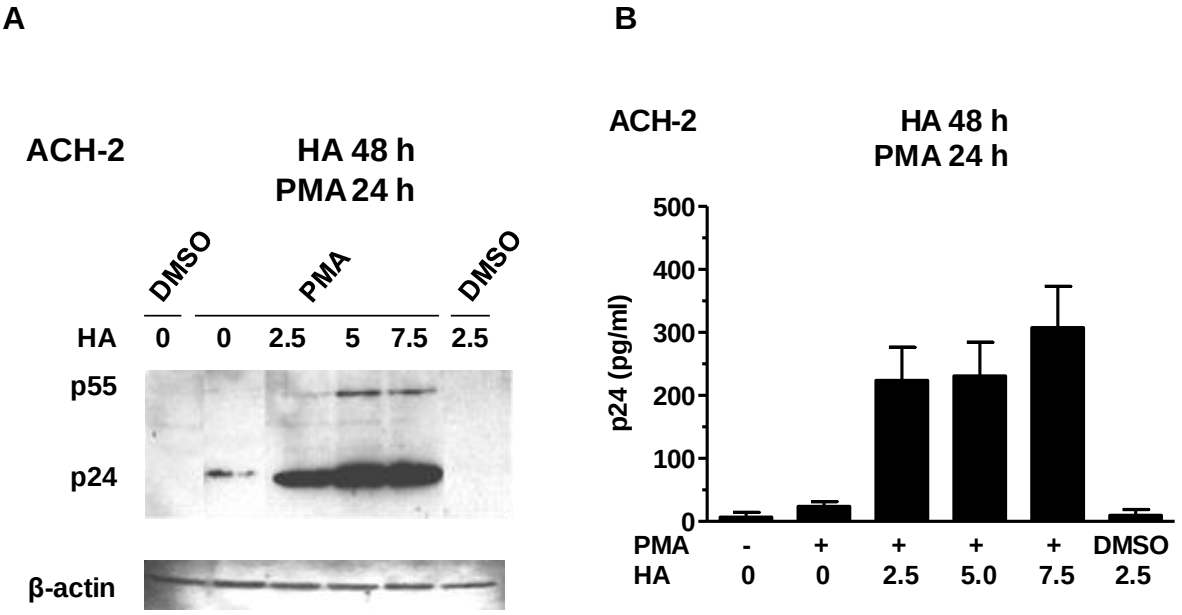


Fig. 13. Effects of HA on reactivation of the latent HIV-1 provirus in ACH-2 cells. ACH-2 cells were pretreated with increasing concentrations of HA for 24 h, and then stimulated with (PMA; final concentration 0.5 ng/ml) for 24 h. **(A)** Western blot analysis of p24 antigen in the cells. Representative results of two independent experiments. **(B)** p24 antigen in culture supernatants. Levels of p24 antigen were determined by ELISA. Graphs represent mean of two independent experiments performed in duplicates ± SD.

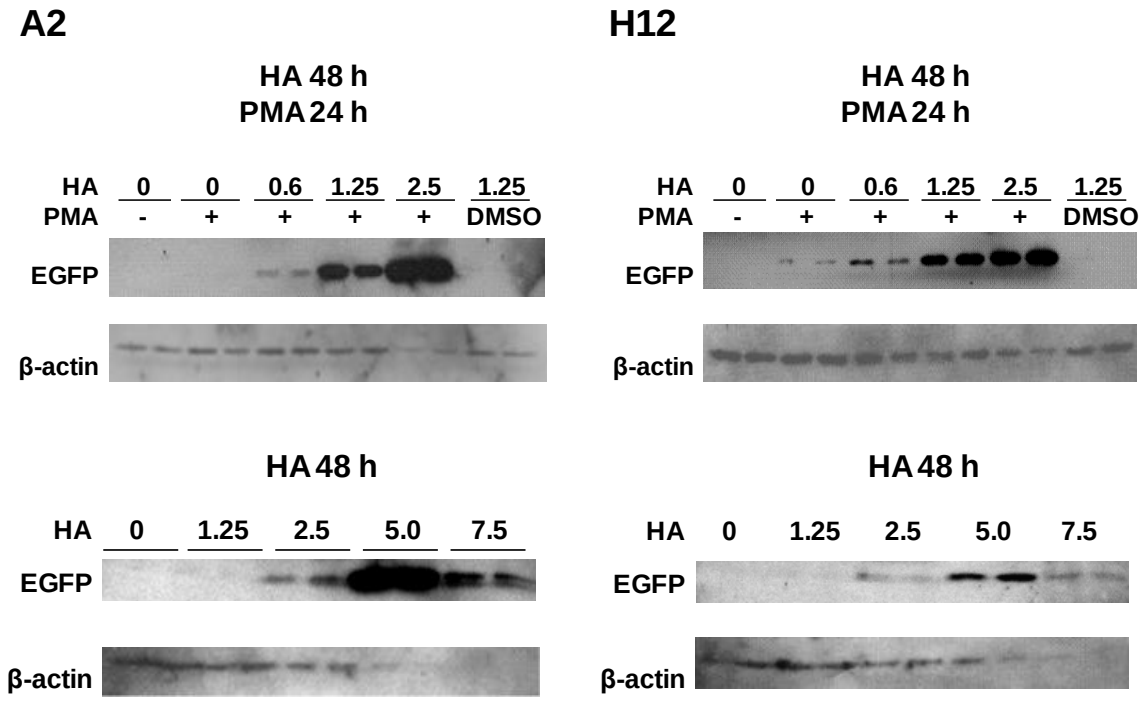
6.6. Effect of HA on reactivation of the latent HIV-1 ‘‘mini-virus’’

A2 and H12 clones of Jurkat cells that harbour a HIV-1 ‘‘mini-virus’’ containing EGFP reporter gene cloned under the HIV-1 LTR promoter serve as an *in vitro* model of reversible latency. Reactivation and EGFP expression in these Jurkat clones differs based on the site of HIV-1 “mini-virus” integration (Jordan et al., 2001) and modifications in the promoter/enhancer region (Blazkova et al., 2009). To study and confirm further the effect of HA on reactivation of the latent provirus, we treated these Jurkat clones with increasing concentrations of HA either for 48 h or pretreated the cells with HA for 24 h and stimulated them with PMA for another 24 h. The cells were collected after the incubation and the EGFP expression was analyzed by western blot and flow cytometry. HA induced the PMA-stimulated reactivation of

latent HIV-1 mini-virus and EGFP expression in A2 and H12 Jurkat cells (Fig. 14A and B) and the result was in agreement with the results obtained in ACH-2 cells. Interestingly, EGFP expression also revealed a stimulatory effect of HA-alone, but the effect was weaker than that in combination with PMA (Fig. 14A and B). Though, H12 cells revealed a higher background expression of EGFP than A2 cells, while EGFP expression upon treatment with HA alone or with PMA was somewhat higher in A2 cells than H12 cells.

The concentrations of HA 2.5 µl of HA/ml and higher were cytotoxic in A2 and H12 cells, as indicated by decreased levels of the house-keeping gene b-actin (Fig. 14A). Also, the increased expression of EGFP inversely correlated with cell viability, with a significant increase of apoptosis at concentrations of HA 2.5 µl/ml and higher (Table 14C). The stimulatory effects of HA-alone and in combination with PMA on the reactivation of HIV-1 mini-virus was comparable in western blot and flow cytometry analysis.

A



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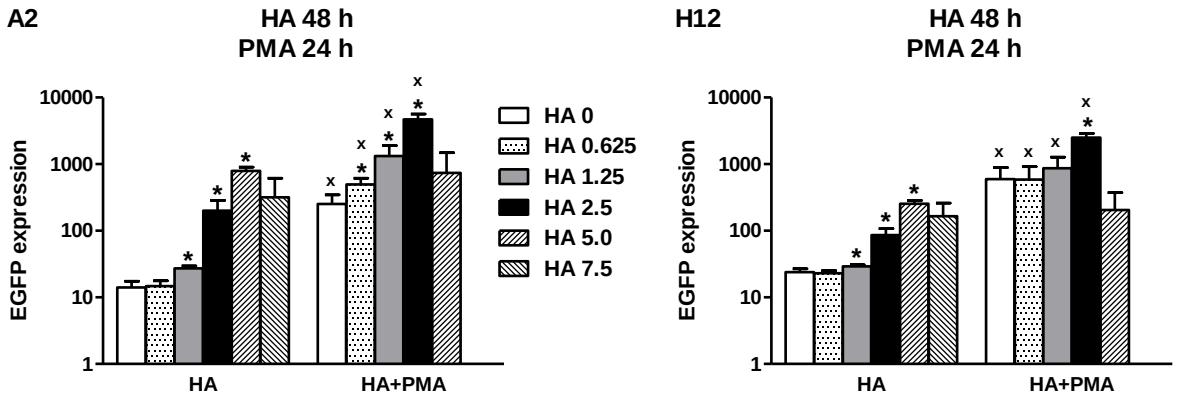


C

	EGFP+ Cells (%)			
HA	**A2**		**H12**	
	0	PMA	0	PMA
0	2.68	10.33	4.90	22.04
0.63	2.13	13.02	4.15	21.02
1.25	3.13	24.85	4.75	25.42
2.5	11.87	49.38	9.08	42.33
5	31.60	11.90	22.73	8.78
7.5	14.33	-	12.40	-
	SD		SD	
0	0.57	2.77	0.29	6.59
0.63	0.51	2.81	0.13	6.22
1.25	0.43	11.01	0.29	4.98
2.5	2.38	5.29	1.27	7.20
5	4.41	9.03	2.03	3.56
7.5	14.34	-	11.04	-

	Fluorescence Intensity (EGFP+ Live Cells Median)			
HA	**A2**		**H12**	
	0	PMA	0	PMA
0	386	1,917	411	2,132
0.63	432	2,644	435	2,062
1.25	464	4,145	454	2,611
2.5	1,035	9,405	657	7,008
5	3,722	23,493	1,438	16,351
7.5	5,933	-	2,533	-
	SD		SD	
0	73	437	22	790
0.63	58	303	22	870
1.25	79	628	24	996
2.5	280	3,130	70	1,184
5	1,659	8,909	622	9,638
7.5	3,887	-	1,971	-

	Cell Viability (% of Live Cells)			
HA	**A2**		**H12**	
	0	PMA	0	PMA
0	87.53	88.32	90.18	83.56
0.63	87.80	87.55	88.70	84.83
1.25	83.75	83.57	85.10	78.47
2.5	72.67	55.53	73.13	33.90
5	18.15	0.70	15.55	0.25
7.5	3.43	-	16.03	-
	SD		SD	
0	4.10	4.33	2.70	9.15
0.63	3.06	5.05	2.43	5.70
1.25	4.92	8.44	2.90	4.73
2.5	5.14	28.13	4.34	15.95
5	14.23	0.57	10.90	0.13
7.5	3.99	-	27.04	-

Fig. 14. Effect of HA on reactivation of the latent HIV-1 ''mini-virus''. Pretreatment with increasing concentrations of HA alone or in combination with PMA (final concentration 0.5 ng/ml) reactivated latent HIV-1 "mini-virus". **(A)** Western blot analysis of EGFP expression from $0.5x10^6$/ml cultured. Representative results of two independent experiments. **(B)** FACS analysis of EGFP from $0.1x10^6$/200 µl cells cultured. The graphs show a quantification of EGFP expression calculated as the arithmetic mean of green fluorescence of green cell population x percentage of all EGFP-positive cells. *Increase in EGFP expression is statistically significant when compared to HA 0 in each treatment ($p < 0.05$). x, Increase in EGFP expression in the presence of PMA is statistically significant when compared to the same concentration of HA without PMA ($p < 0.05$). **(C)**Table summarize percentage of all EGFP+ cells, fluorescence intensity of EGFP+ live cells (median of green fluorescence), and cell viability (% of live cells) presented in Fig. 14B. Live and apoptotic cells were distinguished based on their size and granulosity (FSC-A x SSC-A; see Fig. 8). The results represent means of two independent experiments performed in duplicates ± S.D. The experiments were performed with a kind help of Zora Melkova and Jana Liskova.

6.7. Induction of HO-1 expression by HA with PMA in ACH-2 cells

Heme oxygenase-1 (HO-1) is an inducible isoform of Heme oxygenase enzyme family that catabolise heme into carbon monoxide, biliverdin and Fe^{2+} (Tenhunen et al., 1969). Heme is a natural substrate of heme oxygenases, and it induces the expression of the HO-1 (Maines et al., 1986; Wu and Wang, 2005). Reactive oxygen species produced by the Fe^{2+} released by heme degradation (Kruszewski, 2003) could mediate reactivation HIV-1 provirus through the activation of transcription factor NF-kB. Hence, we first determined the expression of HO-1 in ACH-2 cells pre-treated with increasing concentration of heme arginate (1.25, 2.5, 5.0 and 7.5 µl/ml) for 24 h and then stimulated with PMA (final concentration 0.5 ng/ml) for another 24 hours. The cells were collected and analysed by western blotting for the expression of HO-1 enzyme using specific primary antibody against HO-1. We observed an increase in HO-1 levels with increasing HA concentrations in the presence of PMA i.e. under the conditions leading to the reactivation of HIV-1 provirus (Fig. 15). The low background level of HO-1 expression was not increased by treatment with PMA alone.

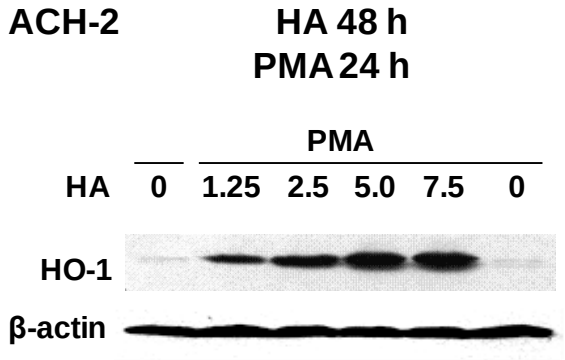


Fig. 15. HA with PMA stimulate expression of heme oxygenase-1. ACH-2 cells were pretreated with increasing concentrations of HA (24 h) and stimulated with PMA for another 24 h. Cell lysates were used to detect the HO-1 by western blotting. Representative result of two independent experiments performed in duplicates.

6.8 Anti-oxidant NAC decreased HA- and PMA-induced reactivation of HIV-1

In order to examine the involvement of heme arginate-mediated ROS production in reactivating the latent HIV-1, we pre-treated the cells with an anti-oxidant agent N-acetyl cysteine (NAC; final concentration 5mM), precursor of the

reduced glutathione (GSH) for 4 h and then treated with HA (2.5 µl/ml) for 24 h and another 24 h with PMA (0.5 ng/ml). HIV-1 p24 detected from the cell lysates by western blot analysis showed a decrease in reactivation of the provirus in HA-pre-treated, PMA-stimulated ACH-2 cells by the addition of NAC (Fig. 16A). In addition, PMA-alone stimulated expression of p24 was also decreased by NAC. On the other hand, ELISA analysis of culture supernatants (Fig. 16B) revealed that pre-treatment with NAC decreased the levels of p24 antigen released by PMA-stimulated ACH-2 cells, while it was not sufficient to decrease significantly the p24 release in HA-pre-treated, PMA-stimulated cells.

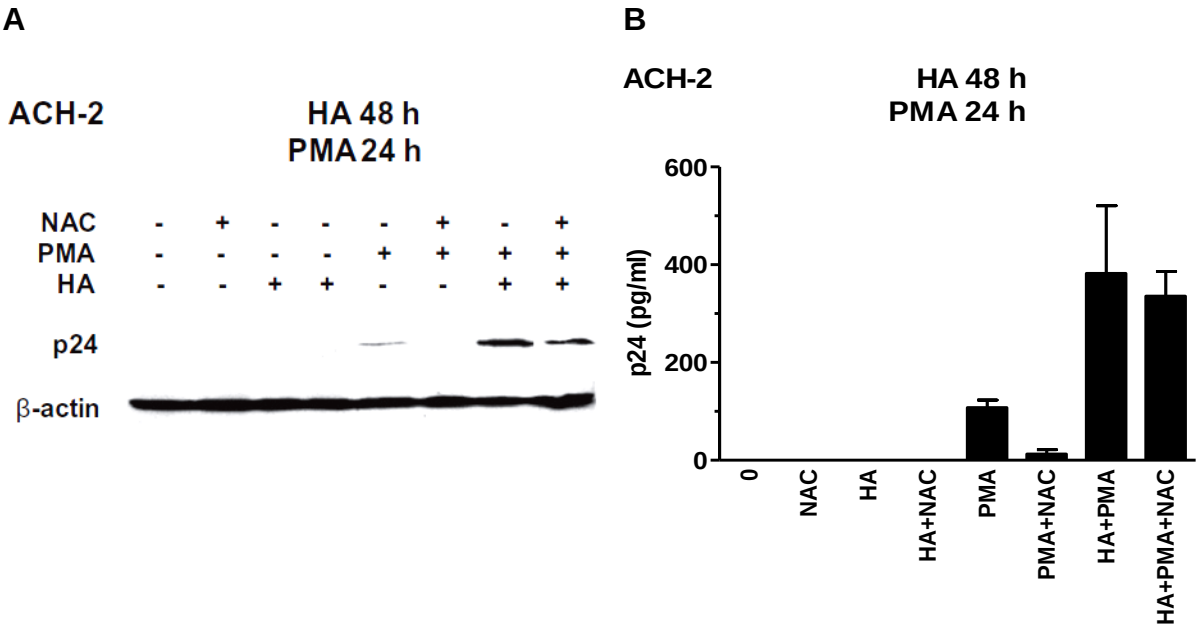


Fig. 16. N-acetyl cysteine decreased HA- and PMA-induced reactivation of HIV-1. ACH-2 cells were pretreated with anti-oxidant NAC and treated with HA for 24h and then with PMA. **(A)** Levels of p24 and b-actin were characterized by western blot. Representative result from two independent experiments performed in duplicate, **(B)** Levels of p24 antigen in culture supernatants were determined by ELISA. Representative results of two independent experiments ±SD.

6.9. HA did not induce expression of HO-1 in A2 and H12 cells

A2 and H12 Jurkat cells were treated with different concentrations of HA (1.25, 2.5 and 5.0 µl/ml) and incubated for 48 h. Expression of HO-1 was analysed by western blotting. In contrast to the dose-dependent increase in HO-1 expression by HA with PMA in ACH-2 cells (Fig. 15), the background HO-1 expression in A2 and H12 Jurkat cells was quite high already in untreated cells and there was no further

increase induced by HA (Fig. 17). HA and PMA treatment did not increase HO-1 levels in A2 and H12 cells either (data not shown). Also, HA at higher concentrations decreased HO-1 levels and was cytotoxic to A2 and H12 cells as evidenced by the decrease in β-actin level.

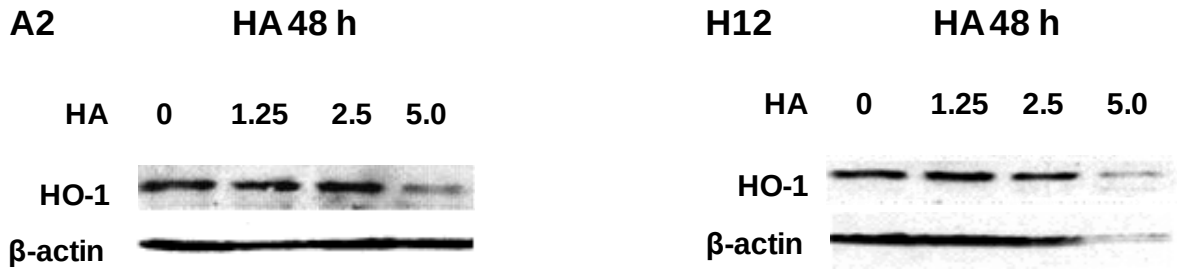


Fig. 17. HA did not induce the expression of HO-1 in A2 and H12 Jurkat cells. HO-1 was found expressed already in untreated cells and treatment with increasing concentrations (1.25, 2.5 and 5.0 µl/ml) of HA for 48 h did not increase HO-1 in A2 and H12 cells.

6.10. Effect of NAC and HO-1 inhibitor SnPP IX in A2 and H12 cells

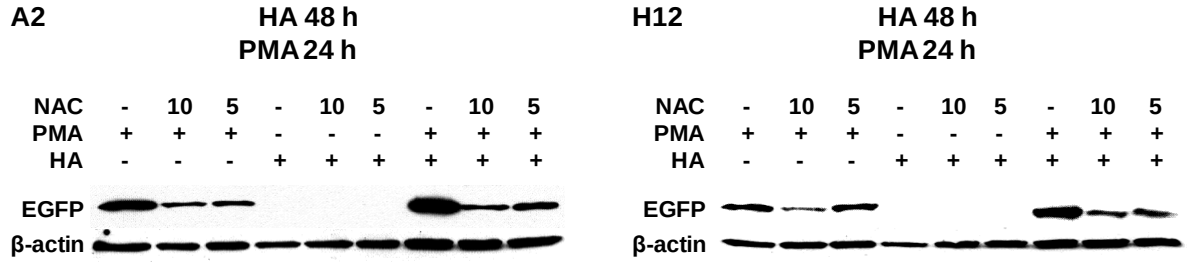
Following the inhibitory effect of anti-oxidant NAC observed in HA- and PMA-stimulated reactivation of HIV-1 provirus in ACH-2 cells (Fig. 16), a similar experiment was performed to explore the involvement of HA-mediated ROS production in HIV-1 “mini-virus” reactivation in A2 and H12 Jurkat clones. A2 and H12 cells were pre-treated with two different concentrations of NAC (10 and 5 mM) and treated with HA (2.5 µl/ml) for 24 h and stimulated with PMA (0.5 ng/ml) for another 24 h. Expression of EGFP in the cells was analysed by western blotting and flow cytometry. Anti-oxidant NAC, similar to the effects observed in ACH-2 cells, prevented the HIV-1 “mini-virus” reactivation, demonstrated by the decreased levels of EGFP in A2 and H12 cells treated with both HA and PMA, as well as in cells treated with PMA only (Fig. 18A, EGFP induced by HA only could be observed in longer exposures).

Tin protoporphyrin IX (SnPP) is an inhibitor of HO-1 activity. Therefore, the effects of SnPP on reactivation of HIV-1 in ACH-2 cells and on reactivation of HIV-1 “mini-virus” in Jurkat clones were studied. In short, either SnPP (6.25 µM) was added to untreated cells or after 4 h pretreatment with NAC (5 mM). Consequently, the cells were treated with HA and PMA as described above. The results of western blot

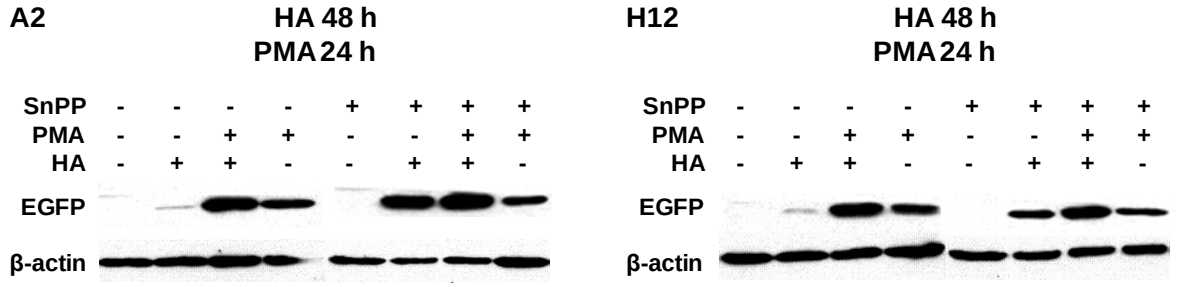
analysis revealed that inhibition of HO-1 activity by SnPP led to a strong increase in EGFP expression in A2 and H12 cells treated with HA alone (Fig. 18B), whereas the increase in EGFP levels in HA- and PMA-treated cells was not significant. SnPP did not affect or somewhat decreased the levels of EGFP in PMA-stimulated cells. SnPP alone did not stimulate any expression of EGFP in untreated cells. NAC pretreatment decreased the stimulation of EGFP expression in all combinations of SnPP, HA- and PMA-treated cells (Fig. 18C) suggesting that these effects were mediated by an increased redox stress. Interestingly, while decreasing the EGFP expression, NAC restored the cell viability in all treatments. EGFP expression analysed using flow cytometry from a same experimental set-up was comparable to the pattern of results obtained by western blot analysis (Fig. 18C, Table 18D).

The background expression of EGFP in untreated H12 cells was higher than in A2 cells, and in general, the response was lower than in A2 cells. In untreated control A2 cells the basal EGFP expression was apparently decreased by SnPP but it was not affected by NAC. In contrast, the basal EGFP expression in untreated control H12 cells was not affected by SnPP, whereas it was decreased by NAC. The stimulatory effects of SnPP observed in A2 and H12 cells were confirmed also in ACH-2 cells (data not shown).

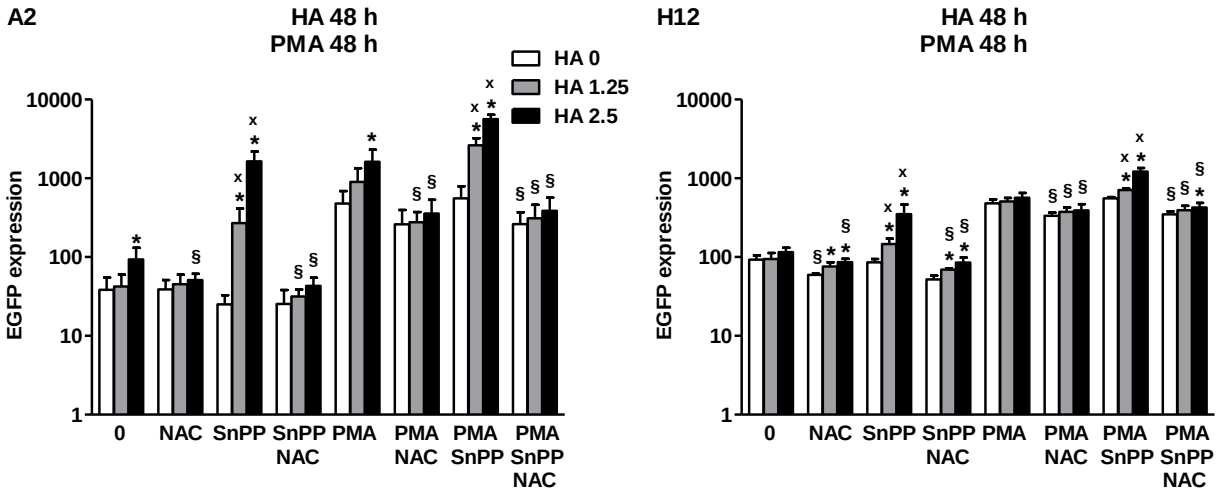
A


A2
HA 48 h
PMA 24 h
NAC - 10 5 - 10 5 - 10 5
PMA + + + - - - + + +
HA - - - + + + + + +
EGFP
β-actin
H12
HA 48 h
PMA 24 h
NAC - 10 5 - 10 5 - 10 5
PMA + + + - - - + + +
HA - - - + + + + + +
EGFP
β-actin

B


A2
HA 48 h
PMA 24 h
SnPP - - - - + + + +
PMA - - + + - - + +
HA - + + - - + + -
EGFP
β-actin
H12
HA 48 h
PMA 24 h
SnPP - - - - + + + +
PMA - - + + - - + +
HA - + + - - + + -
EGFP
β-actin

C


A2
HA 48 h
PMA 48 h
HA 0
HA 1.25
HA 2.5
EGFP expression
10000
1000
100
10
1
0
NAC
SnPP
SnPP NAC
PMA
PMA NAC
PMA SnPP
PMA SnPP NAC
H12
HA 48 h
PMA 48 h
EGFP expression
10000
1000
100
10
1
0
NAC
SnPP
SnPP NAC
PMA
PMA NAC
PMA SnPP
PMA SnPP NAC

D

A2	EGFP+ Cells (%)							
	0	NAC	SnPP	NAC SnPP	PMA	PMA NAC	PMA SnPP	PMA NAC SnPP
HA 0	2.65	2.97	1.46	1.30	12.38	8.37	12.60	6.92
HA 1.25	1.75	3.35	6.77	2.72	16.58	8.70	34.12	8.97
HA 2.5	3.03	3.78	23.67	3.38	24.43	10.68	50.98	11.18
	SD							
HA 0	0.67	0.93	0.30	0.39	3.96	2.72	4.49	2.51
HA 1.25	0.58	0.77	2.25	0.31	6.31	1.90	6.13	2.76
HA 2.5	0.84	0.50	3.51	0.36	8.64	3.82	4.79	3.17

H12	EGFP+ Cells (%)							
	0	NAC	SnPP	NAC SnPP	PMA	PMA NAC	PMA SnPP	PMA NAC SnPP
HA 0	7.03	5.33	6.40	4.38	18.70	15.93	19.00	16.25
HA 1.25	6.83	6.80	8.75	6.20	17.18	17.03	20.45	17.12
HA 2.5	7.25	7.48	13.05	7.40	17.77	17.65	25.30	17.60
	SD							
HA 0	0.21	0.05	0.47	0.33	1.46	1.83	0.30	2.78
HA 1.25	0.71	0.39	0.81	0.22	1.08	1.89	0.81	2.00
HA 2.5	0.42	0.53	1.91	0.60	2.14	3.26	1.23	2.10

A2	Fluorescence Intensity (EGFP+ Live Cells Median)							
	0	NAC	SnPP	NAC SnPP	PMA	PMA NAC	PMA SnPP	PMA NAC SnPP
HA 0	1,143	996	1,206	1,058	2,482	2,116	2,626	2,315
HA 1.25	1,279	857	2,227	824	3,459	1,808	5,758	2,062
HA 2.5	1,665	763	5,122	729	4,488	1,774	9,812	1,951
	SD							
HA 0	279	93	176	192	307	231	396	215
HA 1.25	158	118	388	100	772	179	681	316
HA 2.5	193	77	826	25	965	462	1,170	496

H12	Fluorescence Intensity (EGFP+ Live Cells Median)							
	0	NAC	SnPP	NAC SnPP	PMA	PMA NAC	PMA SnPP	PMA NAC SnPP
HA 0	1,070	914	1,035	899	2,002	1,598	2,183	1,786
HA 1.25	1,060	905	1,218	905	2,136	1,698	2,533	1,734
HA 2.5	1,141	886	1,744	876	2,286	1,627	3,445	1,788
	SD							
HA 0	118	55	56	88	255	75	145	277
HA 1.25	117	79	78	56	186	87	173	103
HA 2.5	89	47	304	82	190	117	253	92

A2	Cell Viability (% of Live Cells)							
	0	NAC	SnPP	NAC SnPP	PMA	PMA NAC	PMA SnPP	PMA NAC SnPP
HA 0	84.63	89.97	83.70	89.68	87.07	90.85	87.28	91.20
HA 1.25	82.47	88.83	76.30	87.20	84.23	90.17	79.32	90.33
HA 2.5	78.37	87.60	68.23	87.10	81.33	89.80	67.55	89.50
	SD							
HA 0	0.54	1.44	0.56	1.56	1.51	2.28	2.07	1.92
HA 1.25	2.08	1.22	1.14	2.60	2.28	2.19	1.17	1.25
HA 2.5	0.85	1.33	1.65	1.21	2.23	1.73	3.58	1.61

H12	Cell Viability (% of Live Cells)							
	0	NAC	SnPP	NAC SnPP	PMA	PMA NAC	PMA SnPP	PMA NAC SnPP
HA 0	86.60	94.33	84.18	93.68	88.85	93.45	88.43	93.50
HA 1.25	83.35	91.68	80.10	91.50	88.30	92.65	87.48	93.10
HA 2.5	80.00	91.98	74.73	91.10	87.37	92.58	84.60	92.18
	SD							
HA 0	1.63	0.43	1.60	0.68	1.40	2.44	2.32	2.74
HA 1.25	2.25	1.58	2.15	1.45	2.16	2.73	2.39	2.27
HA 2.5	3.70	1.36	4.19	0.82	2.27	2.58	2.81	2.51

Fig. 18. Effect of NAC and HO-1 inhibitor SnPP IX in A2 and H12 cells. (A) N-acetyl cysteine prevented the reactivation of HIV-1 'mini-virus'. Pretreatment of NAC (10 and 5 mM) decreased EGFP expression in HA (2.5 µl/ml) and PMA (0.5 ng/ml) stimulated cells as detected by western blot analysis. Representative results of two independent experiments performed in duplicates. **(B)** HO-1 inhibitor SnPP (6.25 µM) strongly stimulated HIV-1 mini-virus reactivation and EGFP expression in HA-alone treated A2 and H12 cells and the stimulation was not significant enough in HA and PMA treated cells. EGFP was detected by western blot. Representative results of two independent experiments performed in duplicate. **(C)** NAC decreased the stimulation of EGFP expression in A2 and H12 cells treated with HA-alone, PMA-alone, HA and PMA, and +/- SnPP treatments. The cells were analyzed by flow cytometry in FL1. The graphs show a quantification of EGFP expression calculated as the arithmetic mean of green fluorescence of green cell population X percentage of all EGFP-positive cells. *Increase in EGFP expression is statistically significant when compared to HA 0 in each treatment ($p < 0.05$). X, Increase in EGFP expression in the presence of SnPP is statistically significant when compared to the same treatment without SnPP ($p < 0.05$). [§]Decrease in EGFP expression in the presence of NAC is statistically significant when compared to the same treatment without NAC ($p < 0.05$). **(D)** Tables summarize percentage of all EGFP-positive cells, fluorescence intensity of EGFP+ live cells (median of green fluorescence), and cell viability (% of life cells) presented in Fig. 18C. The results represent means of two independent experiments performed in duplicates ± S.D.

6.11. Effect of Iron and ascorbate on PMA-stimulated reactivation of the latent HIV-1

As described earlier, heme is catabolized by heme oxygenases to release Iron (Fe^{2+}), CO and biliverdin. Free iron (Fe^{2+}) is pro-oxidative and was shown to generate ROS. ROS mediated reactivation of HIV-1 provirus from latency through the activation of transcription factor NF-kB has been previously demonstrated (Pyo et al., 2008). Since our experiments clearly demonstrated the potentiation by heme arginate in PMA-stimulated HIV-1 provirus reactivation, we decided to explore the effects individual products of heme degradation to understand the HA mediated reactivation of latent HIV-1 in more detail.

ACH-2 cells were treated for 24 h with ferric nitrate ($Fe(NO_3)_3$) (10 µM), ascorbate (0.25 mM) and PMA (0.5 ng) either alone or in combinations as labelled in Fig.6.10A and B. Fe^{2+} is unstable and readily oxidized to Fe^{3+} in aqueous environment. Ferric nitrate used in the experiments served as a source of Fe^{3+} and the ascorbate was added to reduce Fe^{3+} to Fe^{2+} (Hermes-Lima et al., 2000; Zhang et al., 2008). Reactivation of HIV-1 provirus and p24 expression was analyzed by western blotting and chemiluminescence, recorded and analyzed digitally by the ChemiDoc system. Without PMA stimulation, none of the treatment namely, Fe^{3+}-alone, ascorbate-alone or Fe^{3+} and ascorbate together – showed any effect on p24 expression in ACH-2 cells. Interestingly, the low levels of p24 expression induced by PMA stimulation were increased by ascorbate by several folds. Unexpectedly, Fe^{3+} decreased both the PMA-alone stimulated and PMA and ascorbate together stimulated reactivation of HIV-1 and p24 expression (Fig. 19A and B).

Total cellular RNA was isolated from the ACH-2 cells treated in the same way as above, and HIV-1 Gag mRNA was quantified using 1-step RT-ddPCR to characterize the mechanism of iron and ascorbate action in ACH-2 cells at gene expression level. A house-keeping gene (human GAPDH) was also quantified to normalize the HIV-1 Gag mRNA expression. In general, the mRNA expression pattern was similar to the trend observed in digital quantification of the p24 protein detected by western blots. As shown in Fig. 19C, the background Gag mRNA levels were increased 34-times by PMA-alone, while the addition of ascorbate increased

the PMA-stimulated levels about 6-fold and Fe^{3+} inhibited PMA alone and PMA with ascorbate stimulated HIV-1 Gag mRNA expression.

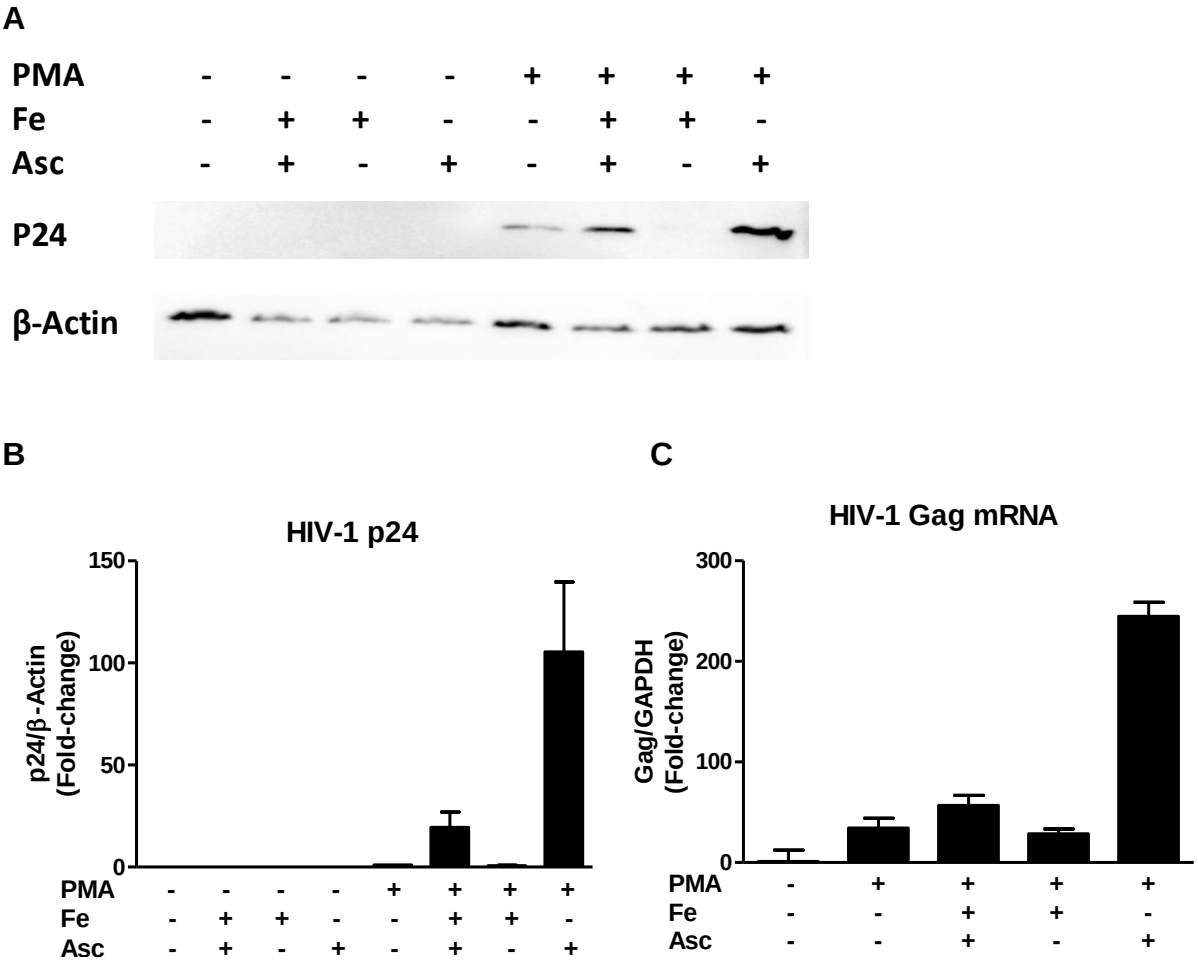


Fig. 19. Effect of Fe^{3+} and ascorbate on PMA-stimulated HIV-1 provirus reactivation in ACH-2 cells. ACH-2 cells treated with ferric nitrate (Fe; 10 μM), ascorbate (Asc; 0.25mM) and PMA either alone or in combination for 24 h. **(A)** HIV-1 p24 expression analyzed by western blot. Representative result of two independent experiments performed in duplicate **(B)** Densitometric analysis of samples presented in panel (A). Graph presented as mean of the values of two experiments performed in duplicate ± SEM. **(C)** HIV-1 Gag mRNA was quantified by 1-step RT-ddPCR and standardised to GAPDH gene. Graph presented as mean of the values of two experiments performed in duplicates ± SEM.

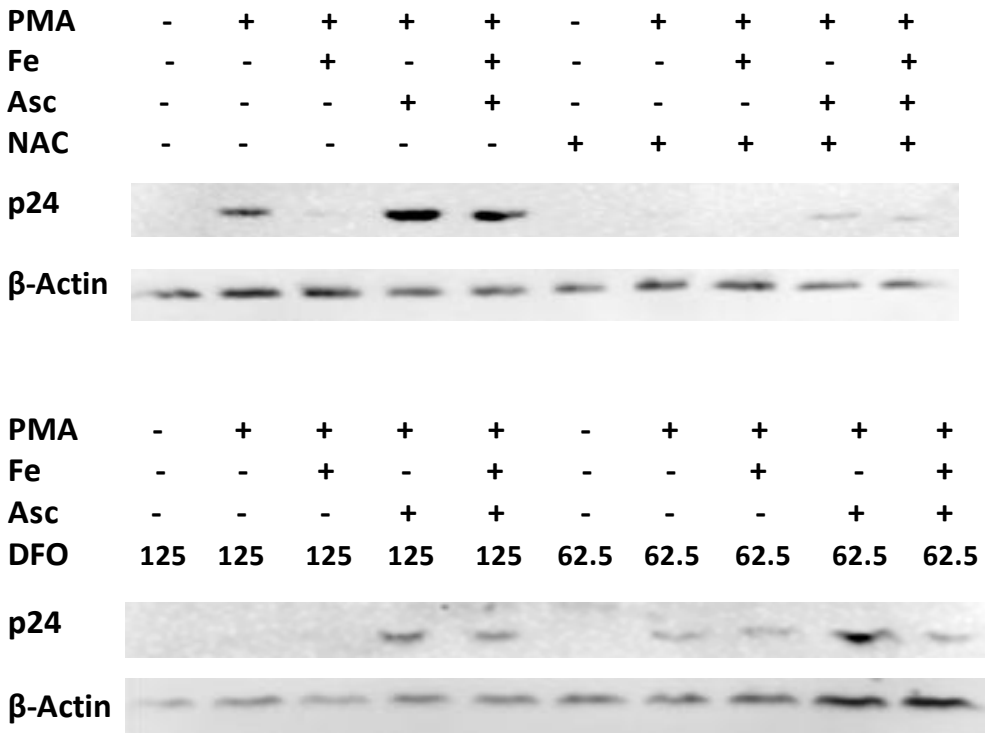
6.12. NAC and iron chelator DFO decreased PMA- and ascorbate-stimulated HIV-1 provirus reactivation

To determine the involvement of iron and ROS in PMA and ascorbate stimulated reactivation of latent HIV-1, ACH-2 cells were pretreated with either NAC (5 mM) or with iron chelator, desferrioxamine (DFO; 125 or 62.5 μM) for 4 h and

then treated with ferric nitrate (10 μM), ascorbate (0.25 mM) and PMA (0.5 ng) either alone or in combinations for 24 h, as above.

Western blot analysis of p24 in cell lysates demonstrated the stimulatory effects of PMA alone or with ascorbate in reactivation, while the expression of latent HIV-1 was decreased by both NAC and DFO (Fig. 20A and B), suggesting the involvement of iron and/or free radicals in the reactivation of the latent HIV-1 by these compounds. Fe^{3+} reduced the HIV-1 reactivation characterized by p24 expression by PMA-alone or with ascorbate in a way similar to NAC pointing that Fe^{3+} could act as an antioxidant in this system.

A



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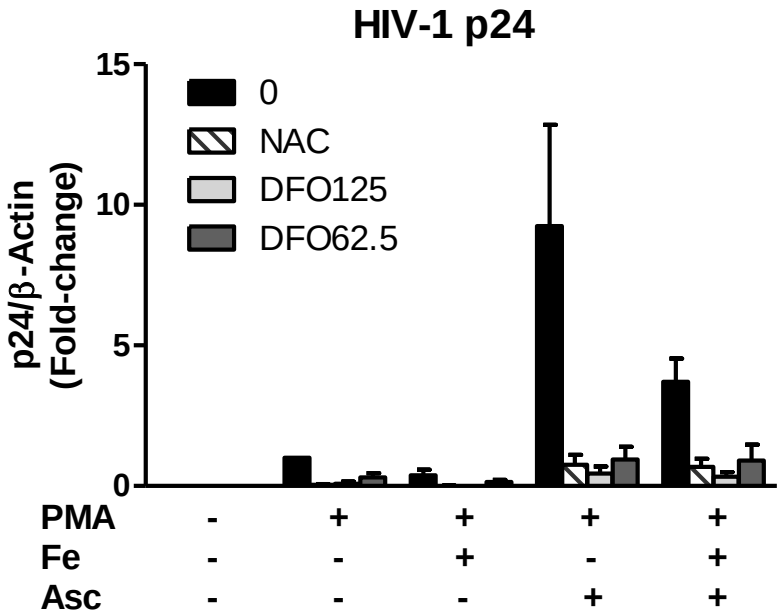


Fig. 20. NAC and iron chelator DFO decreased PMA and ascorbate stimulated HIV-1 provirus reactivation. ACH-2 cells pre-treated with either NAC (5 mM) or DFO (125 or 62.5 µM) for 4 h and treated with ferric nitrate (Fe; 10 µM), ascorbate (Asc; 0.25mM) and PMA either alone or in combination for 24 h. **(A)** HIV-1 p24 expression analyzed by western blot. Representative result of two independent experiments performed in duplicates **(B)** Densitometric analysis of samples presented in panel (A). Graph presented as mean of the values of two experiments performed in duplicates ± SEM.

6.13. Effect of iron and ascorbate on cell viability

A3.01 cells, the parental T cell line of ACH-2, were treated with ferric nitrate (Fe; 10 µM), ascorbate (2, 1, 0.5 and 0.25 mM) with or without PMA (0.5 ng) either alone or in combinations for 48 h. Induction of apoptosis by the effects of individual treatments was determined by analyzing the cell size and granulosity using flow cytometry (Fig. 21). Apoptosis in PMA-treated cells was found to increase in comparison with cells without PMA addition. Ascorbate 2 and 1 mM alone and with iron increased percentage of apoptosis 2-4-times when compared to the basal apoptosis percentage in cells with or without PMA treatment. Importantly, 0.25 mM ascorbate that was used in ACH-2 cells, did not induce any increase in apoptosis.

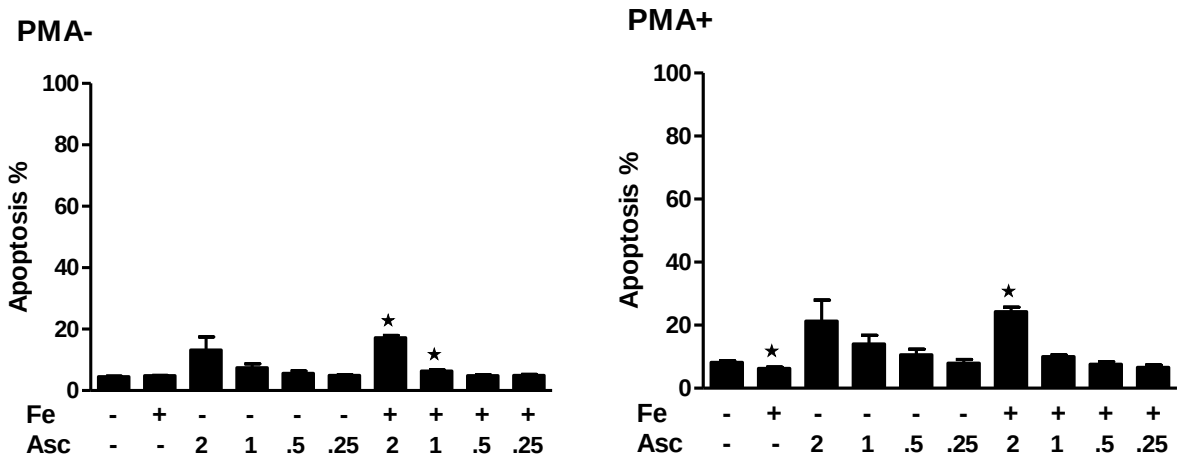
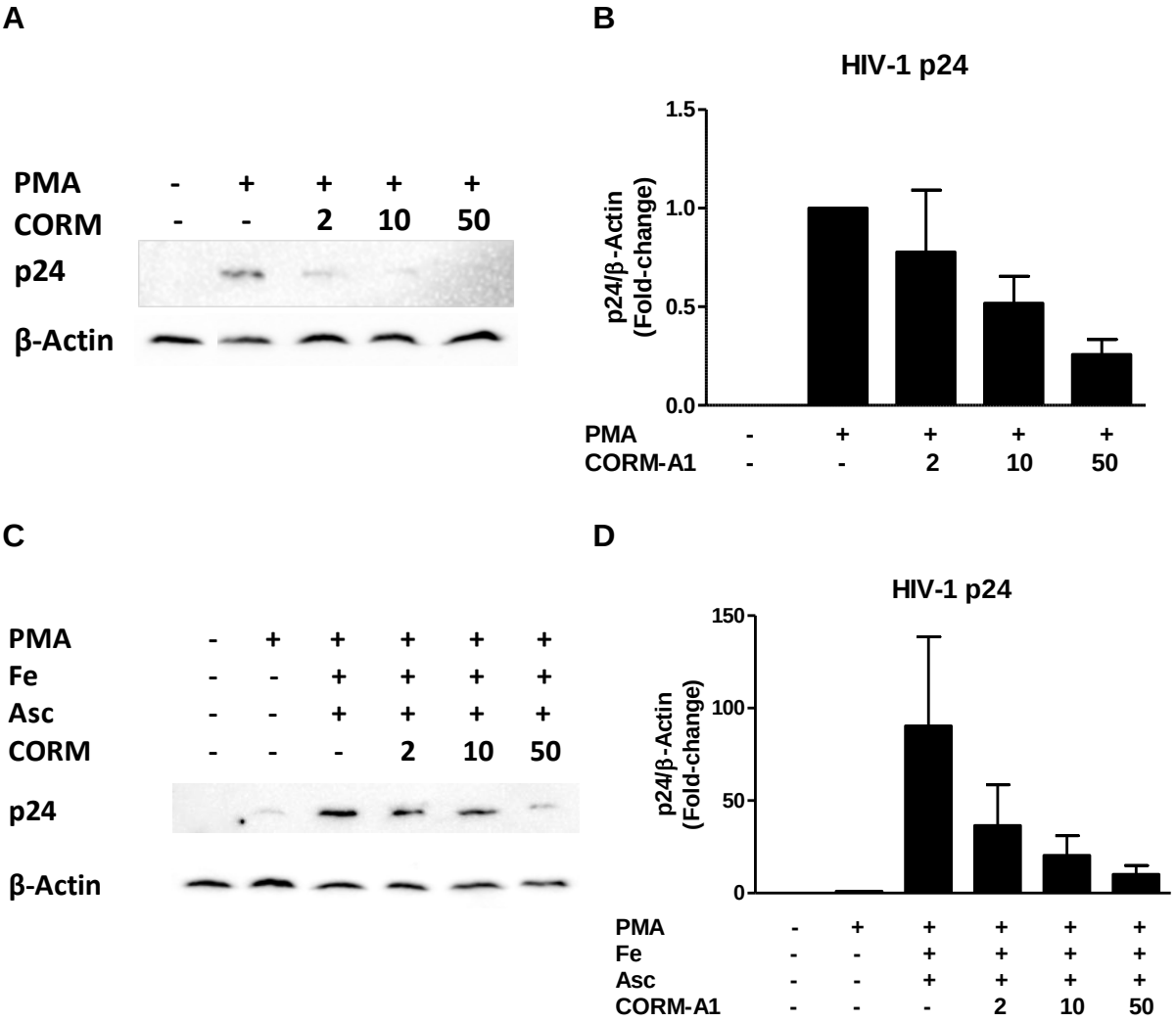


Fig. 21. Effect of iron and ascorbate on cell viability. A3.01 cells were treated with 10 μM ferric nitrate, 2, 1, 0.5 or 0.25 mM ascorbate with or without 0.5 ng/ml PMA for 48 h. Cell size and granulosity were analysed by flow cytometry. Graph presented as mean of three independent experiments performed in duplicates ± SEM. * Increase is statistically significant when compared to 0 in each treatment ($p<0.05$). The experiments were performed with a kind help of Michaela Madlenakova.

6.14. Effect of CO and bilirubin on PMA-stimulated reactivation of the latent HIV-1

Biliverdin formed as a result of heme degradation is unstable and is immediately reduced to bilirubin by biliverdin reductase. Both bilirubin and the carbon monoxide released from heme degradation are strong anti-oxidants. To elucidate the role of CO and bilirubin in latent HIV-1 reactivation stimulated by PMA, ACH-2 cells were treated with increasing concentrations of either a CO-donor CORM-A1 (2, 10 and 50 μM) for 5 minutes or with bilirubin (0.01, 0.1 and 1 μM) for 6 h, and treated with 0.5 ng/ml PMA for 24 h. Additionally, the effect of CORM-A1 was tested in PMA stimulated ACH-2 cells treated with ferric nitrate (Fe; 10 μM) and ascorbate (0.25 μM) for 24 h. HIV-1 p24 expression was analysed by western blotting and digital quantification was performed with ImageLab software (BioRad). The results revealed that CO (CORM-A1) dose dependently inhibited the reactivation of latent HIV-1 as indicated by the decreased levels of p24 in PMA-stimulated ACH-2 cells (Fig. 22A and B) as well as in PMA-stimulated ACH-2 cells treated with iron and ascorbate (Fig. 22C and D). Biliverdin also showed the same pattern of dose-dependent decrease in p24 levels in PMA-treated ACH-2 cells (Fig. 22E and F). The effects of

biliverdin in PMA-stimulated p24 expression in ACH-2 was tested, but it did not reveal any appreciable effects (data not included).



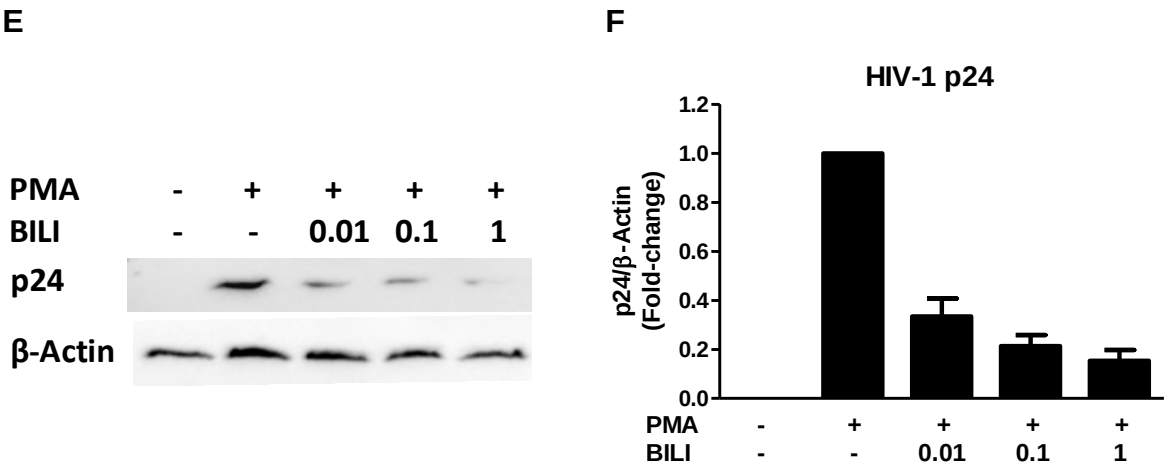


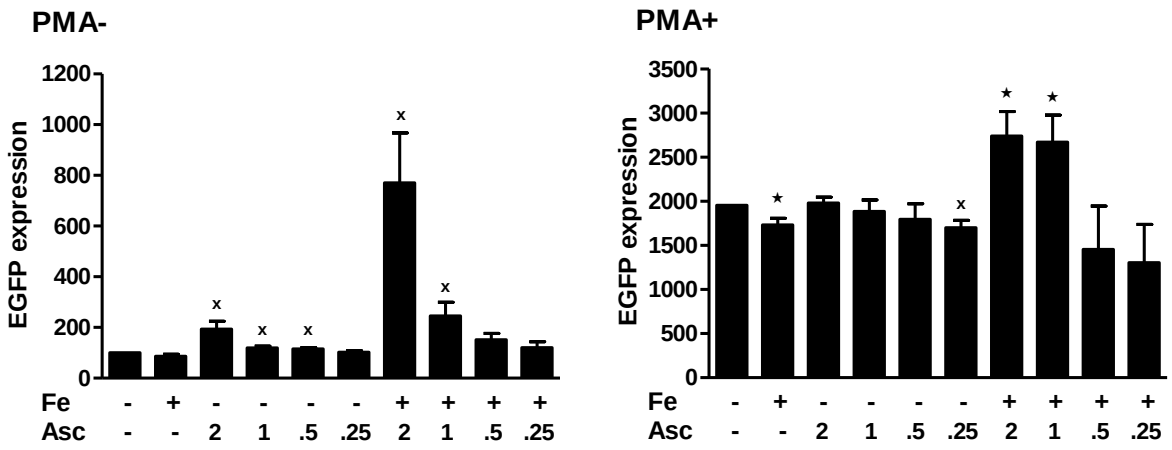
Fig. 22. Effect of CO and bilirubin on PMA-stimulated reactivation of the latent HIV-1. ACH-2 cells pre-treated with CORM-A1 (2, 10 and 50 µM) for 5 min or with bilirubin (0.01, 0.1 and 1 µM) for 6 h, and treated with 0.5 ng/ml PMA for 24 h. (ferric nitrate (Fe; 10 µM) and ascorbate (0.25 µM) were added extra, only to experiments with CORM-A1 treated cells) **(A, C, E)** Western blot detection of HIV-1 p24 expression. Representative results of two independent experiments performed in duplicates. **(B, D, F)** Quantification of the western blots presented in panel (A, C, E) respectively. The graphs represents densitometric analysis of 4 western blots ± S.E.M.

6.15. Effect of Iron and ascorbate on latent HIV-1 ‘‘mini-virus’’ reactivation and cell viability in H12 clone of Jurkat cells

In our previous experiments, the HA- and PMA-stimulated HIV-1 provirus reactivation in ACH-2 cells was reproducible in Jurkat clones. In addition, HA-alone treated for 48 h was also shown to induce the expression of EGFP in the Jurkat clones (Fig. 14). Therefore, we decided to determine the effect of iron and ascorbate on reactivation of HIV-1 “mini-virus” in H12 Jurkat cells. H12 cells were treated with ferric nitrate (Fe; 10 µM), ascorbate (2, 1, 0.5 and 0.25 mM) with or without PMA (0.5 ng/ml) either alone or in combinations for 48 h. The cells were collected and EGFP expression was analysed by flow cytometry. The combination of iron with different concentrations of ascorbate induced the expression of EGFP in H12 cells unstimulated or stimulated with PMA (Fig. 23A). Additionally, ascorbate 1 and 2 mM alone significantly increased the EGFP expression in unstimulated cells. Concentrations of ascorbate lower than 1 mM in combination with iron did not reveal any appreciable effect on EGFP expression.

In addition, apoptosis of the cells was determined by flow cytometry to assess the effect of iron and ascorbate on H12 cell viability (Fig. 23B). In general, the apoptosis induced by iron and ascorbate alone or in combination was proportional to EGFP expression and the pattern was similar in both PMA-treated and untreated

A



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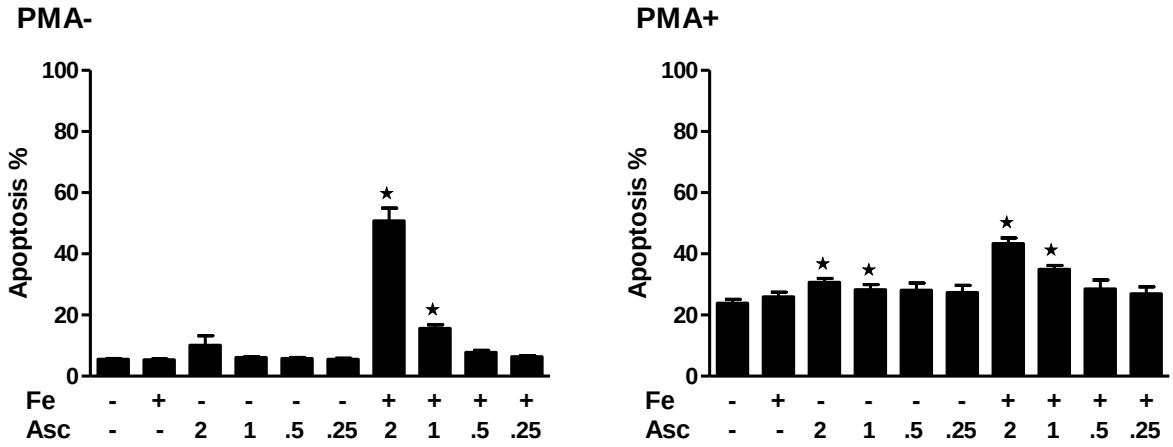


Fig. 23. Effect of Iron and ascorbate on reactivation of the latent HIV-1 ‘‘mini-virus’’ and cell viability in H12 clone of Jurkat cells. H12 cells treated with 10 μM ferric nitrate (Fe), 2, 1, 0.5 or 0.25 mM ascorbate (Asc), and 0.5 ng/ml PMA for 48 h. **(A)** Flow cytometric analysis of EGFP expression. The graphs show a quantification of EGFP expression calculated as the arithmetic mean of green fluorescence of green cell population X percentage of green cells. The graphs represent mean and standard error of mean (S.E.M.) of 3-8 experiments **(B)** Percentage of apoptosis -based on the size and granulosity as analysed by FACS (FSC-A x SSC-A). ^{x,} * Increase is statistically significant when compared to 0 in each treatment ($p<0.1$, 0.05 respectively). The experiments were performed with a kind help of Michaela Madlenakova.

cells. A 5-fold increase in apoptotic cell percentage was observed in PMA-alone treatment, when compared to unstimulated H12 cells.

6.16. Role of iron in HA-mediated and PMA-stimulated reactivation of the latent HIV-1

In order to confirm the role of iron also in the HA-mediated, PMA-induced reactivation of HIV-1 provirus, the ACH-2 cells were pretreated with DFO (125 and 62.5 μM) for 4 h and treated with HA (2.5 μl) for 24 h, followed by additional 24 h treatment with PMA (0.5 ng/ml). HIV-1 p24 expression in cell lysates was detected by western blot analysis and HIV-1 Gag mRNA expression was quantified by 1-step RT-ddPCR. Both p24 protein levels (Fig. 24A and B) and Gag mRNA expression levels (Fig. 24C) are dose dependently decreased by DFO in HA and PMA stimulated induction of HIV-1 provirus reactivation in ACH-2 cells. The results confirmed the possibility of role of iron in HA-mediated PMA stimulated latent HIV-1 reactivation. Of note, DFO decreased also the levels of control β-actin in the absence of HA in these experiments.

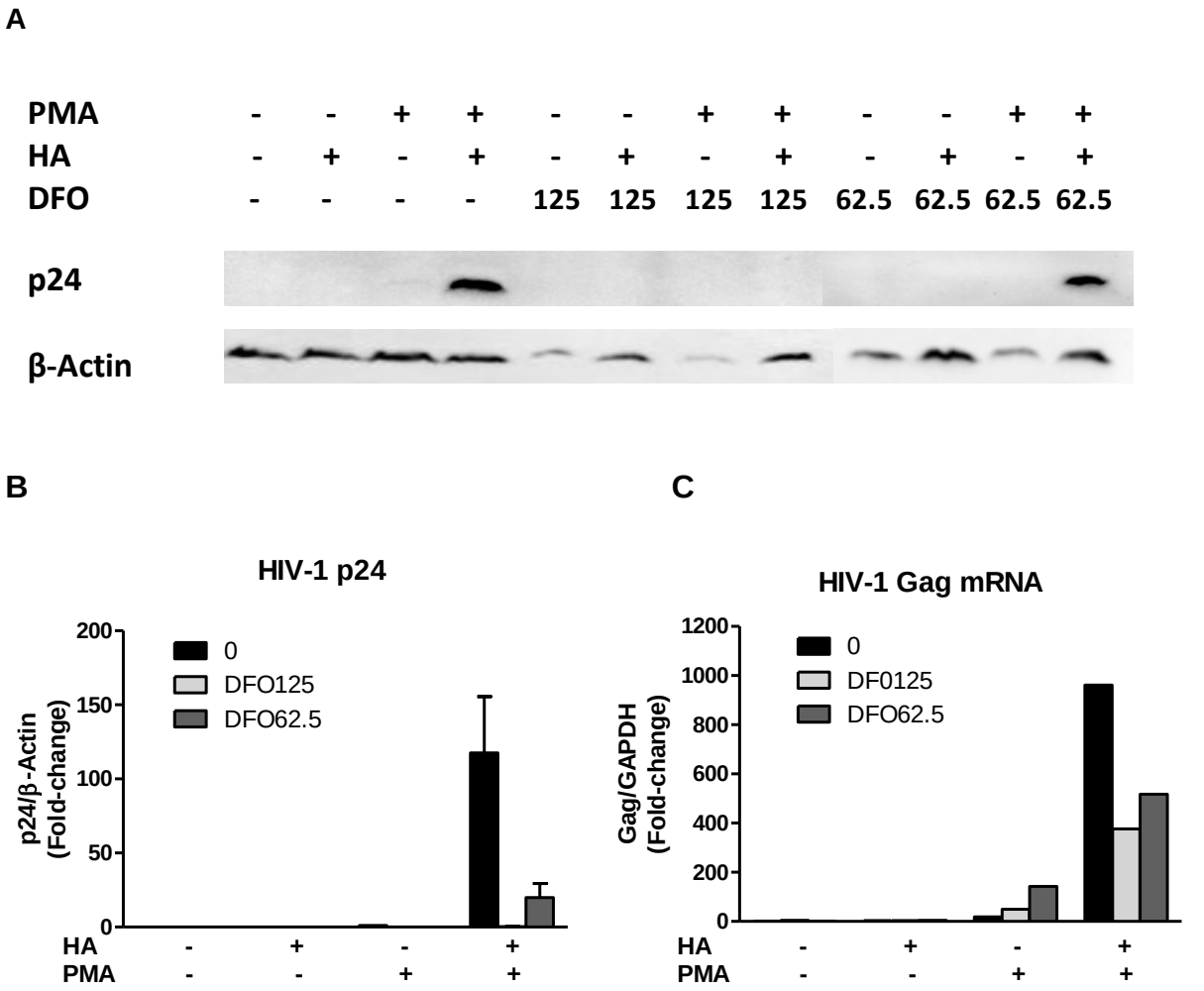


Fig. 24. Role of iron in HA-mediated and PMA-stimulated reactivation of the latent HIV-1. ACH-2 cells pre-treated with 125 and 62.5 µM DFO for 4 h, and treated with 2.5 ul of heme arginate/ml for 24 h and additional 24 h with PMA (0.5 ng/ml) **(A)** Western blot analysis of HIV-1 p24 antigen. Representative results of two independent experiments performed in duplicates. **(b)** Quantification of the western blots presented in panel (A). The graph represents densitometric analysis of 4 western blots ± S.E.M. **(C)** Analysis of HIV-1 Gag RNA using 1-step RT-ddPCR. The Gag mRNA quantified was standardised to human GAPDH mRNA. The graph represents mean of two independent experiments performed in duplicates.

6.17. Heme arginate stimulates HIV-1 expression in human HIV+ PBMC's ex vivo

Demonstrating the stimulatory effects of HA and PMA on latent HIV-1 reactivation directly in primary cell cultures would be more appropriate in reflecting the real situation *in vivo*, as the latency models used in previous experiments are known to reveal different mutations and clonal bias. For example, ACH-2 cells possess a mutation in TAR region, affecting Tat-mediated transactivation of latent

HIV-1 provirus transcription (Venkatachari et al., 2015). Also in the Jurkat clones, the HIV “mini-virus” genome integration site and differential methylation pattern influences the rate of transactivation (Jordan et al., 2001, Blazkova et al., 2009). Thus, in order to validate the effects of HA and PMA on reactivation of the latent HIV-1, PBMCs isolated from four HIV-1 infected patients on cART were treated with HA (2.5 ul) and PMA (0.5 or 2.5 ng/ml) for 18 h. In the first approach, we intentionally used PBMC’s instead of purified CD4+ T-cells because the heterogenic cell population is closer to the situation in vivo. The HIV-1 Gag mRNA was detected in the total RNA isolated from the PBMCs cultured ex vivo by two step semi-nested qPCR. Gag mRNA quantified was standardised to human GAPDH mRNA. As shown in Fig. 25, the cell-associated HIV-1 Gag RNA detected in all the samples at the time of isolation, went down during the 18 h-incubation ex vivo (based on relative quantification to GAPDH). PMA-alone and HA-alone increased the levels of HIV-1 RNA compared to untreated samples. On the other hand, HA synergized with PMA to induce a 2-12-fold increase in HIV-1 Gag RNA expression when compared to untreated samples. HIV-1 RNA levels were quantified relatively to human GAPDH. Additionally in a different series of experiments testing 10 house-keeping genes, we found GAPDH and β-2 microglobulin genes to be affected the least by individual treatments. Nevertheless, PMA treatment increased their expression also, affecting the relative changes in HIV-1 RNA levels.

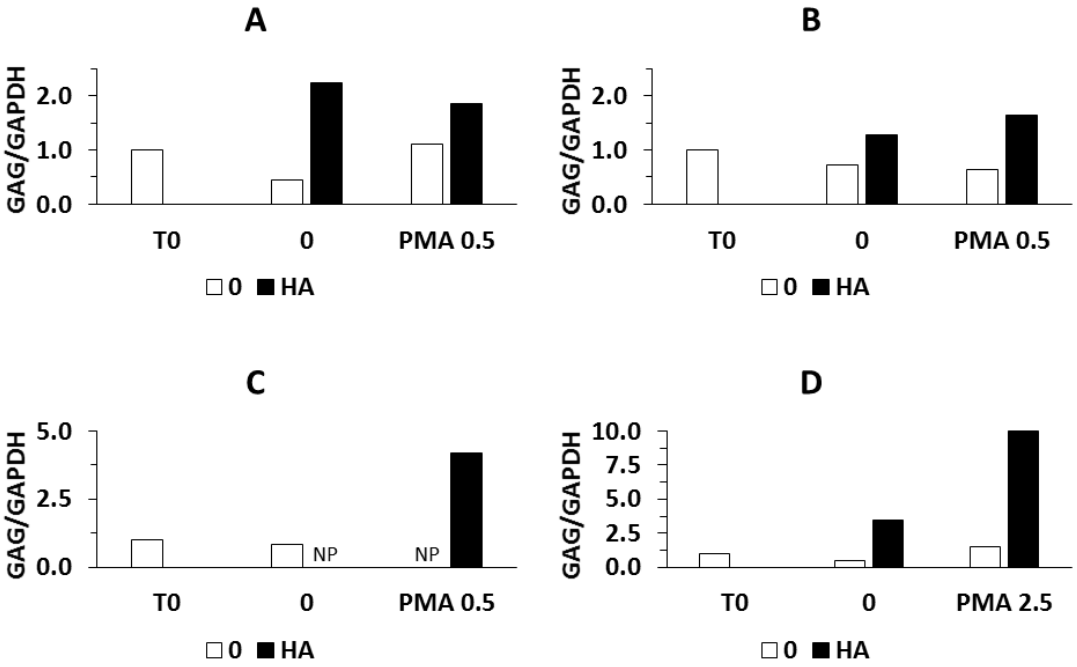


Fig. 25. Heme arginate stimulates HIV-1 expression in human HIV+ PBMC's *ex vivo.* 5 x 10^6/ml of human PBMC's were treated with HA 2.5 µl/ml with or without addition of 0.5 or 2.5 ng/ml PMA for 18 h. HIV-1 Gag RNA was analysed using semi-nested 2-step RT-qPCR and standardized to GAPDH mRNA. Results of 4 different HIV+ patients on cART are presented as fold-increase (A, B, C, D). T0 – time 0, 0 – no treatment, HA – heme arginate 2.5 µl/ml, PMA 0.5 or 2.5 ng/ml, NP – not performed. The experiments were performed with a kind help of Zora Melkova.

6.18. Comparison of HIV-1 reactivation by HA and other T cell activating agents

Tumor necrosis factor-α (TNF-α), phytohemagglutinin (PHA) and phorbol myristate acetate (PMA) are some of the well-known and routinely used T cell activators. The PMA concentration (0.5 ng/ml) used in our experiments was selected based on a preliminary screening as the lowest concentration of PMA required to detect p24 expression by western blot analysis. The weak stimulation and reactivation of HIV-1 provirus by this low concentration of PMA allows to detect a several hundred-fold increase in reactivation when potentiated by pre-treatment with HA (Fig. 13B). To assess the efficiency of HA-potentiated, PMA-stimulated HIV-1 provirus reactivation and to compare it with other cell stimulators, ACH-2 cells were treated with 5 µl/ml of HA, PMA (final concentration 0.5 ng/ml), HA and PMA, TNF-α

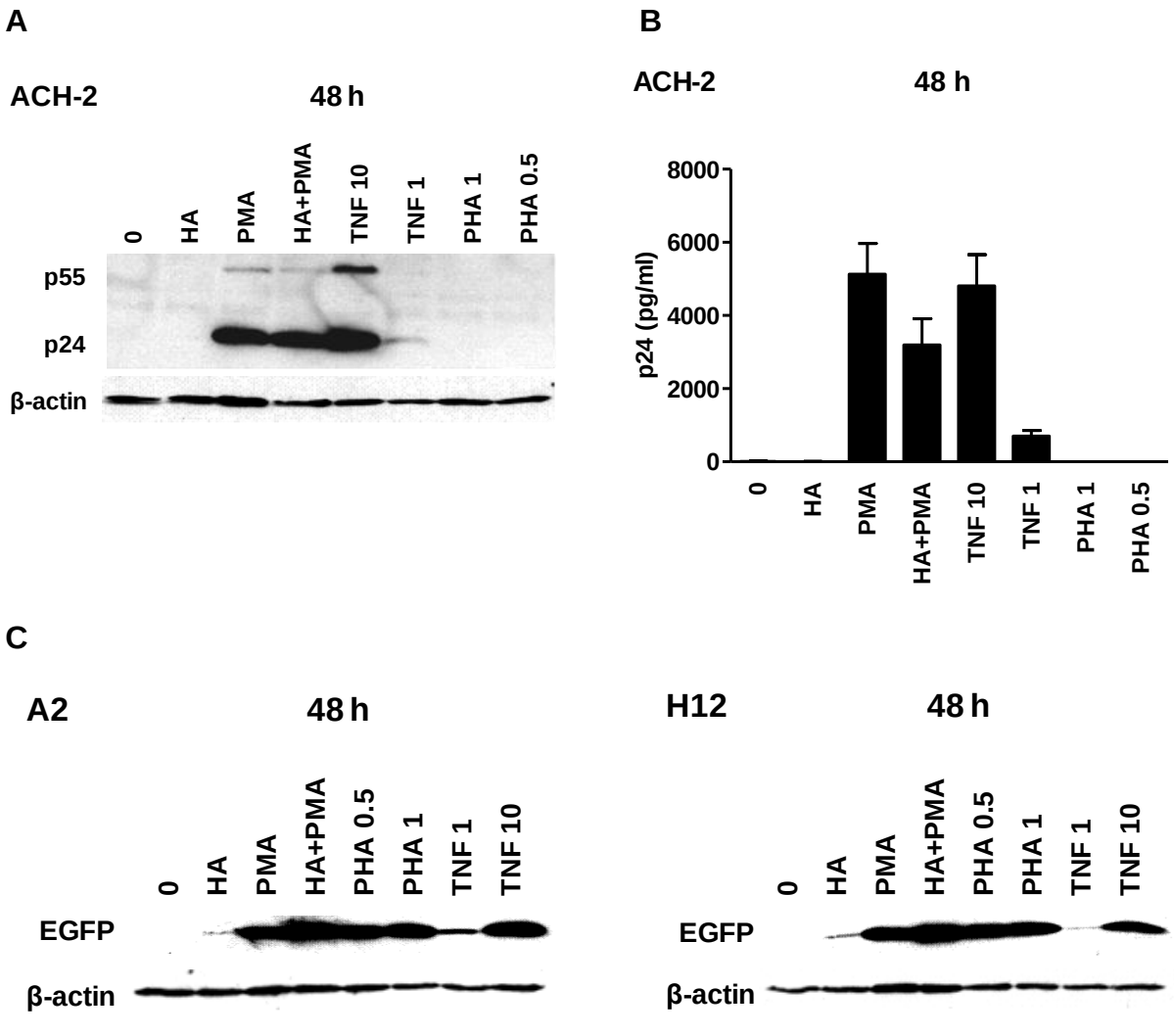
(final concentration 1 and 10 U/ml) or phytohemagglutinin (PHA; final concentration 0.5 and 1 µg/ml) for 48 h. HIV-1 provirus reactivation was characterized as p24 expression detected in cell lysate by western blot analysis and in culture supernatant by ELISA.

The results revealed that treatment with HA-alone as high as 5 µl/ml did not stimulate reactivation of HIV-1 in ACH-2 cells. p24 expression levels in PMA-alone treatment were increased comparably as in combination with HA for 48h. TNF-α 1 U/ml induced a relatively smaller expression of p24 Ag, whereas 10 U/ml of TNF-α led to maximum expression of p24 Ag of all the compounds. On the other hand, all the concentrations of phytohemagglutinin A tested (PHA; 0.5, 2.5; 5 µg/ml) alone or in combination with 1 µM ionomycin failed to yield a positive signal of p24 Ag in western blot analysis (Fig. 26A and data not shown).

The HIV-1 provirus reactivation and p24 expression in culture supernatants quantified by ELISA analysis revealed similar changes as the western blot analysis (Fig. 26B). It was clear that a 24 h pre-treatment with HA and followed by a stimulation with PMA for additional 24 hours increased p24 expression in ACH-2 cells several folds more than a 24 h stimulation with PMA alone (Fig. 13A and B). However, at 48 h, stimulation of ACH-2 cells by PMA-alone resulted in a stronger expression of p24 than by the combined action HA and PMA. This effect was possibly due to the death of the HA-and PMA-stimulated cells at 48 h - as indicated by the decreased β-actin levels (Fig. 26A) or due to the inhibitory effects of heme degradation end products CO and bilirubin (Fig. 22).

HA mediated reactivation of HIV-1 “mini-virus” in A2 and H12 cells was compared using the same stimulatory agents and experimental settings for 48 h. As observed previously (Fig. 14) the weak expression of EGFP in A2 and H12 cells by HA-alone treatment was increased strongly with PMA-alone and even more strongly with PMA and HA. The stimulation with 10 U/ml of TNF-α or 0.5–1 µg/ml PHA was comparable to the effect of PMA, while the stimulation with 1U/ml TNF-α induced a relatively weaker expression of EGFP. The effect of 1 U/ml TNF-α was comparable to the effect of HA (2.5 µl/ml) in H12 cells, while it was stronger in A2 cells.

EGFP expression by individual cell-stimulating agents was also determined using flow cytometry (Fig. 26D, Table 26E). As expected, the EGFP expression pattern was similar to the trend observed in western blot analysis. Also, as mentioned above, H12 cells reveal a higher background expression of EGFP in untreated cells than A2 cells, and in general respond with a smaller fold-increase than A2 cells. Based on various criteria used in this analysis, it can be concluded that A2 cells are more responsive to TNF-α than H12 cells. When analyzing the cell viability, neither PMA nor TNF-α alone or in combination with HA were found to decrease it. On the other hand, PHA reduced cell viability relatively strongly.


A
ACH-2
48 h
0
HA
PMA
HA+PMA
TNF 10
TNF 1
PHA 1
PHA 0.5
p55
p24
β-actin
B
ACH-2
48 h
p24 (pg/ml)
8000
6000
4000
2000
0
C
A2
48 h
H12
48 h
EGFP
β-actin
D
HA 0
HA 1.25
HA 2.5
A2
48 h
H12
48 h
EGFP expression
10000
1000
100
10
1

(E)

A2	EGFP+ Cells (%)				
	0	PMA	PHA 0.5	TNF 1	TNF 10
HA 0	2.74	8.10	13.57	14.68	72.55
HA 1.25	1.76	11.97	14.33	15.93	73.73
HA 2.5	2.81	17.75	15.33	20.90	75.40
			SD		
HA 0	0.57	2.78	2.09	1.92	3.20
HA 1.25	0.91	1.70	1.98	2.68	2.23
HA 2.5	0.26	1.01	1.43	1.74	1.91

H12	EGFP+ Cells (%)				
	0	PMA	PHA 0.5	TNF 1	TNF 10
HA 0	9.46	18.30	30.84	15.08	43.75
HA 1.25	9.29	16.49	29.25	14.83	43.20
HA 2.5	9.88	19.58	28.00	16.30	43.30
			SD		
HA 0	2.33	3.96	5.48	3.11	1.55
HA 1.25	1.93	2.58	2.06	2.57	0.98
HA 2.5	2.34	2.33	1.62	2.76	0.74

A2	Fluoresc. Intensity (EGFP+ Live Cells Median)				
	0	PMA	PHA 0.5	TNF 1	TNF 10
HA 0	979	1,897	5,342	1,367	4,556
HA 1.25	1,187	2,607	6,462	1,625	5,853
HA 2.5	1,597	4,189	7,702	2,074	7,891
			SD		
HA 0	251	242	615	148	441
HA 1.25	214	659	854	212	653
HA 2.5	212	338	796	165	661

H12	Fluoresc. Intensity (EGFP+ Live Cells Median)				
	0	PMA	PHA 0.5	TNF 1	TNF 10
HA 0	1,098	1,813	4,319	1,217	2,836
HA 1.25	1,167	1,951	5,388	1,335	3,325
HA 2.5	1,207	2,373	6,045	1,459	3,969
			SD		
HA 0	94	204	969	61	135
HA 1.25	101	241	154	84	152
HA 2.5	69	31	406	84	98

A2	Cell Viability (% of Live Cells)				
	0	PMA	PHA 0.5	TNF 1	TNF 10
HA 0	83.35	87.44	70.69	85.93	85.98
HA 1.25	80.77	83.74	68.20	82.85	82.50
HA 2.5	76.56	79.68	61.33	80.10	79.35
			SD		
HA 0	2.05	1.55	3.72	1.20	1.27
HA 1.25	1.68	2.35	1.64	1.54	1.22
HA 2.5	1.99	2.67	3.25	1.30	1.13

H12	Cell Viability (% of Live Cells)				
	0	PMA	PHA 0.5	TNF 1	TNF 10
HA 0	86.78	86.75	61.63	88.10	87.45
HA 1.25	84.45	85.33	69.85	85.63	85.03
HA 2.5	81.43	86.58	67.83	83.13	81.43
			SD		
HA 0	1.08	3.75	16.59	0.75	1.10
HA 1.25	1.58	5.20	5.20	0.85	0.40
HA 2.5	0.90	4.17	4.51	0.76	1.25

Fig. 26. Comparison of HIV-1 reactivation by HA and other T cell activating agents. ACH-2 cells were treated with 5 µl/ml of HA, PMA (final concentration 0.5 ng/ml), HA and PMA, TNF-α (final concentration 1 and 10 U/ml) or phytohemagglutinin (PHA; final concentration 0.5 and 1 µg/ml) for 48 h. **(A)** Levels of p24 and b-actin were characterized by western blot analysis and chemiluminescence. **(B)** p24 antigen in culture supernatants were determined by ELISA. Results of two independent experiments ±SD. **(C and D)** A2 and H12 cells were treated as above except the HA concentration used (HA 1.25 µl/ml or 2.5 µl/ml) for 48 h **(C)** Western blot analysis of EGFP in the cells. Representative results of two independent experiments. **(D)** Flow cytometric analysis of EGFP expression. The graphs show a quantification of EGFP expression calculated as the arithmetic mean of green fluorescence of green cell population X percentage of all EGFP-positive cells.
*Increase in EGFP expression is statistically significant when compared to HA 0 in each treatment ($p < 0.05$). x, Increase in EGFP expression in the presence of the stimulatory agent is statistically significant when compared to the same concentration of HA without the agent ($p < 0.05$). **(E)** Flow cytometric analysis of EGFP expression. Tables summarize percentage of all EGFP-positive cells, fluorescence intensity of EGFP+ live cells (median of green fluorescence), and cell viability (% of life cells) presented in Fig. 26D. Live and apoptotic cells were distinguished based on their size and granulosity (FSC-A x SSC-A; Fig. 8). The results represent means of two independent experiments performed in duplicates ± S.D. The experiments involving flow cytometry were performed with a kind help of Zora Melkova and Jana Liskova.

6.19. HA neither stimulated nor interfered with activation of T cell

CD69 is expressed on the activated T cells, and thus it is used as a surface marker for the identification of T cell activation. To determine whether HA-alone activates the T cells or whether it might interfere with PMA-stimulated T cell activation, the A3.01 cells were stimulated with PMA (0.5 ng/ml) with or without HA (1.25 and 2.5 µl/ml) for 24 h. Expression of CD69 on the cell surface was determined by using anti-CD69 antibody labelled with AlexaFluor 700 and flow cytometry. The results revealed that HA did not exhibit any effects on T-cell activation characterized by this activation marker at any concentration of PMA tested (1 and 10 ng/ml; data not shown), especially not even at the lowest concentration used throughout the experiments (0.5 ng/ml; Fig. 27). Importantly, HA alone did not activate the T cells when used alone.

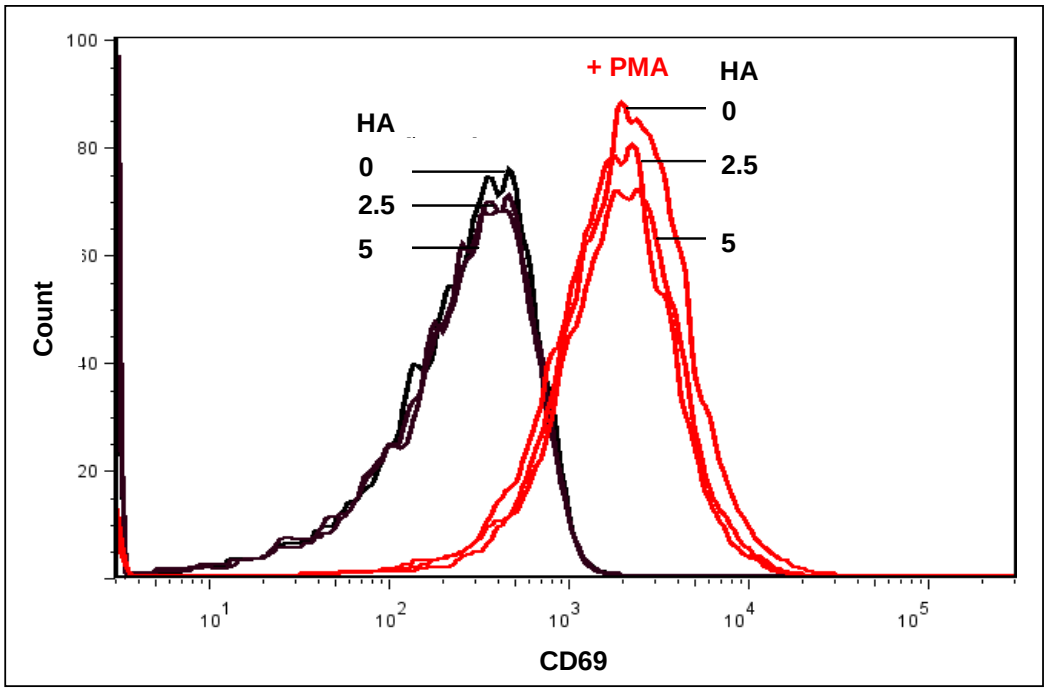
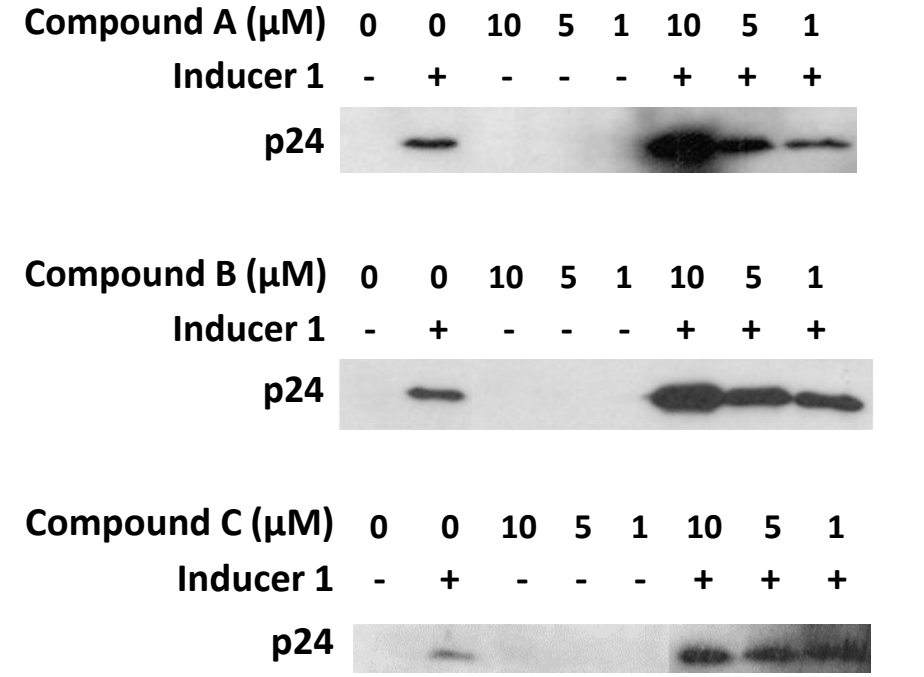


Fig. 27. HA neither stimulated nor interfered with activation of T cells. A3.01 cells were treated with increasing concentrations of HA in the presence or absence of PMA (final concentration 0.5 ng/ml) for 24 h. Flow cytometry detection of T cell activation marker CD69. Representative results of two independent experiments performed in duplicates.

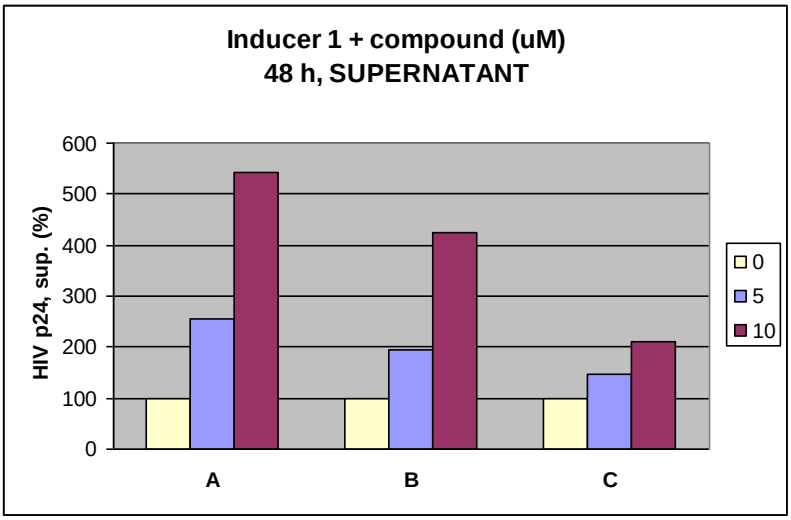
6.20. Selected compounds potentiate reactivation of the HIV-1 provirus in ACH-2 cells stimulated by different cell inducers.

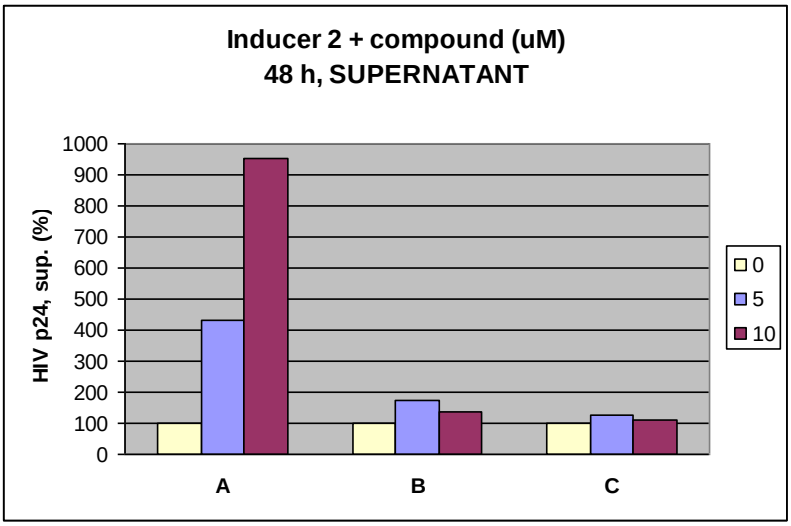
In order to identify new promising molecules that could possibly be developed and used as latency reversing agents, we screened several compounds and selected three among them for further evaluation of their effect on reactivation of latent HIV-1 in synergy with different activation inducers. The selected compounds (A, B and C) alone do not reveal any stimulatory effects, but they potentiate the reactivation of transcriptionally silent HIV-1 provirus induced by threshold levels of different inducers (1, 2 and 3). Therefore, they can be considered as sensitizers. The level of synergy is different for different inducer-compound combinations. In general, all three compounds increases the reactivation of HIV-1 by several folds when compared to the inducers alone (Fig. 28A and B), but compound A is the most efficient one among the compounds tested. More information about the compounds and inducers and their concentrations are included in the patent application.

A


Compound A (µM) 0 0 10 5 1 10 5 1
Inducer 1 - + - - - + + +
p24
Compound B (µM) 0 0 10 5 1 10 5 1
Inducer 1 - + - - - + + +
p24
Compound C (µM) 0 0 10 5 1 10 5 1
Inducer 1 - + - - - + + +
p24
Compounds A B C
(µM) 10 5 10 5 10 5
Inducer 2 - + + + + + + + + +
p24
Compounds A B C
(µM) 10 5 10 5 10 5
Inducer 3 - + + + + + + + + +
p24

B





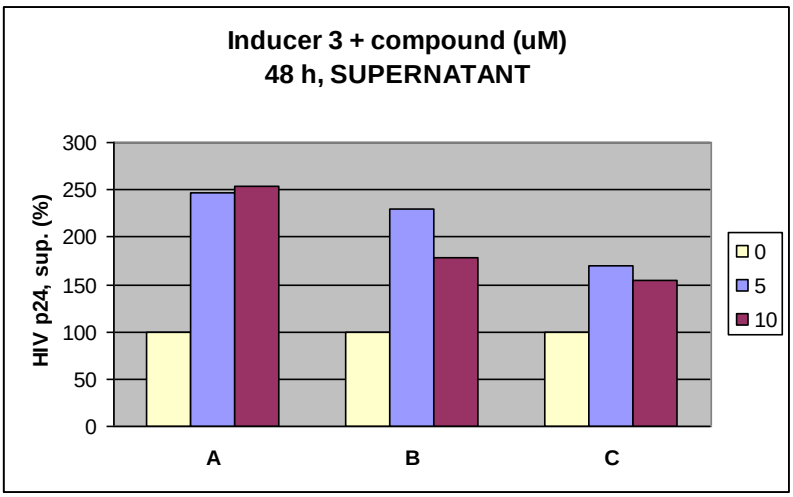


Fig. 28. Selected compounds potentiates reactivation of the HIV-1 provirus in ACH-2 cells stimulated by different cell inducers. 0.5 x 10^6/ml of ACH-2 cells were incubated without any inducer or with an inducer (inducer 1, 2, 3), additionally in combination with compounds A, B and C (5, 10 μM). **(A)** After 24 hours, the cells were collected and lysed, the

proteins were resolved by 14% SDS-PAGE, and HIV protein p24 was detected by western blot analysis using chemiluminescence. **(B)** The parallel samples were incubated for 48 hours, and then the levels of p24 Ag in the supernatant were determined by ELISA. The inducer alone = 100%. The experiments involving ELISA assay were performed with a kind help of Zora Melkova.

7. Discussion

Global HIV-AIDS epidemic reached the peak in 1997 when new infection rates started to be efficiently curbed down by highly active antiretroviral therapy (HAART). Out of 36.7 million people who are currently living with HIV (as of 2015) only 17 million people are having access to antiretroviral therapy yet. Despite this still unsufficient coverage, AIDS-related deaths have fallen by 45% since the peak in 2005 (http://www.unaids.org/en/resources/fact-sheet). The introduction of even more efficient combined antiretroviral therapy (cART) has significantly improved the clinical outcome and life expectancy of HIV infected patients. Despite the clinical benefits of the drug therapy, cART alone is not able to eliminate the virus from the organism, as the virus persists in reservoirs that are thought to be the source for viral re-emergence after treatment interruption (Chun et al., 2010 and Chun et al., 1997). Therefore, developing novel therapeutics targeting the latent reservoirs becomes crucial. Latently infected $CD4^+$ T cells constitute one reservoir of replication-competent HIV that needs to be eliminated to completely purge the virus from antiretroviral drug-treated patients. For the realization of a sterilizing cure, strategies to eradicate the latent virus reservoirs including the “shock and kill” method of therapeutic reactivation are being actively explored by researchers. Latency reversing agents often target the factors involved in establishing viral latency and reactivation and expression of transcriptionally silent latent virus. HDAC inhibitors, HMT inhibitors, DNMT inhibitors, protein kinase C activators and several unclassified molecules are examples of molecules being explored and developed as possible latency reversing agents (Ylisastigui et al., 2004, Archin et al., 2012, Bjerg Christensen, 2015, Kumar et al, 2015, Bernhard et al., 2011, Kauder et al., 2009, McKernan et al., 2012, Trushin et al., 2005, Gulakowski, 1997, Jose et al., 2014). Since increased redox stress plays an important role in HIV-1 replication (Pace and Leaf, 1995) our aim was to employ the effects of redox stress induced by redox modulators and to reactivate the latent HIV-1.

Heme arginate is consists of heme and L-arginine.One of its degradation products is ferrous iron (Fe^{2+}). Heme- and iron-mediated Fenton reaction generates reactive oxygen species (ROS) and creates an oxidative stress. Heme arginate is more stable and has less or no side effects on homeostasis when compared to its equivalent hematin (Volin et al., 1988). Heme has been previously shown to inhibit

replication of HIV-1 (Levere et al., 1991), specifically reverse transcriptase (Argyris et al., 2001). Further, heme derivative hemin has been demonstrated to inhibit HIV-1 growth in human PBMC-reconstituted NOD-SCID mice and to induce a dose-dependent inhibition of HIV-1 replication in tissue culture during a 7-day long infection (Devadas and Dhawan, 2006). Though the RT inhibition by heme or hematin has been demonstrated by different groups as stated above, there is a gap in the research exploring the effect of heme or hematin in HIV-1 latency reversal and there are no reports available in the literature about this. Hence, we sought to explore and evaluate the effect of heme arginate on HIV-1 replication and reactivation from latency.

In accordance with previous publications, our results revealed the inhibitory effects of HA on HIV-1 replication and reverse transcription in acutely infected cells, characterized by levels of p24 and reverse transcripts, respectively. Devadas and Dhawan (2006), also found hemin to induce expression of HO-1, and the inhibitory effects of hemin on HIV-1 replication could be reversed by certain concentrations of SnPP, the inhibitor of HO-1. Based on these results, they had concluded that the inhibition of HIV-1 growth was mediated by the action of HO-1. We also observed a HA-induced expression of HO-1 in ACH-2 cells, while its levels were already increased in untreated A2 and H12 cells. However simultaneously, we observed HA-induced stimulatory effects on HIV-1 provirus and ‘‘mini-virus’’ reactivation in ACH-2 and A2, H12 cells, respectively.

HA stimulated HIV-1 provirus reactivation in synergy with PMA or TNF-α, in ACH-2 cells while it acted alone and/or in synergy with the two agents in A2 and H12 cells. Further, the effects of HA in both ACH-2 cells and Jurkat clones A2 and H12 were increased by the addition of SnPP, the inhibitor of HO-1, and all the stimulatory effects could be inhibited by antioxidant NAC and iron chelator DFO. Thus based on our results, it can be suggested that in the experiments of Devadas and Dhawan (2006), the inhibitory effects of hemin on HIV-1 replication were in fact over-ridden by the increased redox stress due to inhibition of HO-1 by SnPP and the resulting increase in expression of the provirus.

Heme and hemin differ in the oxidation state of iron in the two compounds; they contain Fe2+ and Fe3+, respectively. In the organism, heme is mostly bound as a prosthetic group in various heme proteins. In the presence of various oxidizing agents, the heme moiety is oxidized to hemin, while the oxidized heme proteins as well as the free hemin readily undergo reduction driven by CO, both in biological systems and in vitro (Bickar et al., 1984). Changes in the oxidation state of iron in heme moiety are also mediated by another ubiquitously present gas signaling agent, nitric oxide (Ascenzi et al., 2010; Kilbourn et al., 1994). It is thus impossible to strictly separate the effects of heme and hemin as their mutual balance is dynamically regulated. On the other hand, only heme can serve as a substrate of HO-1. As a hydrophobic compound, hemin inserts into plasma membranes and translocates inside the cells. Inside the cells, the free iron is released namely by the action of heme oxygenases, hydrogen peroxide or other non-specific degradation (Belcher et al., 2010), leading to the generation of the hydroxyl radical (Kruszewski, 2003) and activation of the redox sensitive transcription factor NF-κB (Lander et al., 1993; Pantano et al., 2006). Heme also regulates levels of key enzymes involved in heme synthesis and degradation, non-specific synthase of 5-aminolevulinic acid (ALAS1), HO-1, and of oxidative stress response genes (Furuyama et al., 2007; Igarashi and Sun, 2006; Mense and Zhang, 2006).

In the time-course experiments (Fig. 9), HA inhibited HIV-1 replication characterized by levels of p24 Ag. In similar time-course experiments (Fig. 10), viability of the mock-infected and infected cells in the presence of HA was found comparable to the untreated mock-infected cells, while untreated infected cells succumbed to apoptosis. A long-term culture of the cells in the presence of HA in concentrations that inhibited HIV-1 replication did not therefore negatively affect cell growth and viability; on the contrary, HA protected the infected cells from dying. We cannot, though, exclude a possibility that a selection of HA-resistant cells could take place.

In contrast to the acutely infected cells, HA revealed stimulatory effects on HIV-1 provirus and ‘‘mini-virus’’ reactivation in ACH-2 and A2, H12 cells, respectively. In A2 and H12 cells, HA stimulated ‘‘mini-virus’’ reactivation even by itself, but its effects were much weaker than those of PMA, PHA, or TNF-α alone or

in combination with HA. The overall EGFP expression as well as percentage of EGFP-positive cells were dose-dependent for all agents. During a 48 h-incubation period, stimulatory effects of HA and TNF-α were more or less comparable to HA and PMA in H12 cells, while A2 cells appeared to be more responsive to TNF-α (Fig. 26D). Both cell lines seemed to respond similarly to PHA. H12 cells revealed a higher background fluorescence of untreated cells than A2 cells, similarly to the published data (Blazkova et al., 2009), but in general, they responded to the individual inducers with a smaller fold-increase than A2 cells. Perhaps, the lower responsiveness of H12 cells might be due to a somewhat higher CpG methylation of the 50 LTR region compared to A2 cells (Blazkova et al., 2009).

The observed effects of PMA on the HIV-1 provirus reactivation in ACH-2 cells were biphasic, possibly due to a low concentration of PMA used. During a 24 h-treatment, PMA stimulated the provirus reactivation only weakly, while a 48 h-treatment induced a 10-fold increase in the levels of p24 compared to a 24 h-treatment.

Apparently, PMA was inducing the provirus reactivation indirectly. It seems to induce expression and/or activity of certain factors that in turn mediate reactivation of the provirus. Phorbol esters mimic the action of diacyl glycerols (DAG), activators of protein kinase C family proteins (PKC) and of several non-PKC targets. In addition to DAG or phorbolester, the full activation of PKC's requires also Ca^{2+} and acidic phospholipids, leading to a synergistic activation of two different ligand binding domains and to the appropriate membrane targeting (Brose and Rosenmund, 2002; Goel et al., 2007). PKC was also found to mediate expression of HO-1 stimulated by PMA or LPS (Devadas et al., 2010; Naidu et al., 2008). Also, PMA is known to generate ROS (primarily superoxides) (Swindle et al., 2002).The effects of PMA in ACH-2 cells could be greatly potentiated with HA during a 24 h-treatment (Figs. 13 and 15). Possibly, HA could synergize with PMA by changing levels of cytoplasmic Ca^{2+}, membrane targeting of PKC's or by increasing the redox stress and changing the properties of zinc-finger-like repeats in C1 domain involved in PMA binding to its targets. Heme and PMA were independently shown to affect also other signal transduction pathways, e.g. Ras and MAPK, increasing chances for their synergistic action (Mense and Zhang, 2006; Sacks, 2006). The exact mechanism of stimulation

of HIV-1 reactivation by HA remains to be established, but a mechanism involving induction and/or activity of HO-1 along a with release of Fe^{2+}, increased redox stress and activation of the redox-sensitive transcription factor NF-κB can be suggested (Belcher et al., 2010; Devadas and Dhawan, 2006; Kruszewski, 2003; Lander et al., 1993; Morse et al., 2009; Pantano et al., 2006). Therefore, we propose a model in which heme arginate induces a redox stress leading to chromatin remodeling, binding of specific transcription factors to HIV-LTR and potentiation of HIV-1 expression induced by a PKC inducer.

Our results indicate a HA-induced expression of HO-1 in ACH-2 cells, while HO-1 was found present already in untreated A2 and H12 cells. In all cell lines, LTR-driven expression could be inhibited by pretreatment of the cells with antioxidant NAC, precursor of the potent anti-oxidant, GSH, and by iron chelator DFO, suggesting that the effect of HA involved an increased redox stress mediated by iron. In fact, we have also detected increased production of free radicals by A3.01 and Jurkat cells in the presence of HA or PMA (unpublished results). Additionally, we tested the effect of the inhibitor of HO-1, SnPP, in both ACH-2 and Jurkat clones A2 and H12. While SnPP was not found to affect basal expression of EGFP in either cell line, it strongly stimulated this expression in the presence of HA in both A2 and H12 cells. Most probably, EGFP expression could be stimulated by an increased redox stress imposed by HA that could not be counteracted by the anti-oxidative effects of HO-1 because of its inhibition by SnPP. Alternatively, electron transfer between the two porphyrin species and generation of ROS could take place. Again, the stimulatory effects of SnPP and HA on LTR-driven expression were inhibited by NAC.

The other two products of heme oxygenases, CO and biliverdin, further converted to bilirubin, reveal strong antioxidant and cytoprotective properties (Morse et al., 2009). The effect of HA addition thus can be shortly pro-oxidative and then anti-oxidative for a prolonged period of time. Thus, upon a massive induction of expression of HO-1 stimulated by HA and PMA in ACH-2 cells, the anti-oxidative effects could eventually prevail, and inhibit provirus reactivation during a longer incubation in the presence of HA. The situation seems to be different in A2 and H12 cells in which HO-1 was found expressed already in untreated cells and its levels

were not further increased by any treatment; HO-1 thus could start to effectively degrade HA immediately after its addition. Apparently, the kinetics and balance between the pro-oxidative and anti-oxidative effects of HO-1 products might be different in these cells.

We used A2 and H12 cells (Blazkova et al., 2009; Jordan et al., 2001) to characterize the effects of HA on LTR-driven expression, comparing western blot analysis detecting levels of EGFP and flow cytometry detecting fluorescence of EGFP. The flow cytometry results underestimate the numbers of EGFP-positive cells and/or levels of EGFP expressed, as high levels of EGFP are cytotoxic and dead cells loose EGFP fluorescence. Nevertheless, we assessed the overall expression of EGFP by the number of all EGFP-positive cells x arithmetic mean of green fluorescence of the green cell population. Using this approximation, the levels of EGFP expression were found increased even by treatment with 1.25 µl/ml of HA in most experiments, corresponding to the results of western blot analysis. The percentage of green (EGFP-positive) cells in samples treated with 1.25 µl/ml of HA used to be lower than in untreated cells, while the arithmetic mean and median of green fluorescence of all green and live green cells, respectively, were always higher. In higher concentrations of HA, as well as in other stimulatory treatments, all values were higher than in controls.

In general, in A2 and H12 cells, HA alone or in combination with other stimulatory agents increased LTR-driven EGFP expression as well as cell death. These tendencies seemed to be similar in ACH-2 cells. However, a long term incubation of A3.01 and Jurkat cells with HA did not significantly increase cell death. It is thus possible that the cytotoxicity of HA might be further increased due to expression of HIV or EGFP. In fact, it would be of advantage if latently infected cells were more prone to cell death induced by HA alone or in combinations. There might be several mechanisms involved in cell death induced by HA: first, a direct increase in ROS production due to a higher availability of heme and iron; second, an indirect cytotoxicity of HA that would further increase ROS production and HIV reactivation; third, the resulting increase in HIV reactivation would lead to the cell death. The excess of oxygen free radicals induces oxidation of proteins, lipids, lipoproteins, nucleic acids, carbohydrates and other cellular or viral targets. Thus, HA might not

only stimulate expression of the provirus, but also affect the viability and infectivity of the released virions. A similar inhibition of HIV-1 by reactive oxygen species was indeed shown in the case of bleomycin (Georgiou et al., 2004).

Heme oxygenase has been suggested to exert various immunoregulatory effects on innate and adaptive immune cells, and to inhibit pathogenesis of several immune-mediated inflammatory diseases (Soares et al., 2009). Further, analysis of HO-1 promoter polymorphism revealed that Caucasian HIV-1-infected patients who maintain low levels of immune activation and control HIV-1 viral loads to undetectable levels are more likely to possess a specific microsatellite (GT)n repeat and two single nucleotide polymorphisms in HO-1 promoter region that favour enhanced HO-1 gene expression (Seu et al., 2009).

With these findings of the effect of heme arginate on HIV-1 provirus reactivation, we then stepped further to decipher the role of individual heme degradation products- iron, carbon monoxide and bilirubin (reduced from biliverdin by biliverdin reductase) on HIV-1 latency reversal. Previously published results indicated that a longer pre-treatment with ascorbate suppressed the induction of HIV-1 reactivation in latently infected T-cell lines (Harakeh et al., 1990). However, in our experiments, ascorbate added shortly before PMA stimulated latent HIV-1 reactivation in both ACH-2 and H12 cells; these effects could be attributed to the double faced character of ascorbate that can act as an anti- or pro-oxidant, depending on its high or low concentrations (Atanassova and Tzatchev, 2008). Pro-oxidant character of ascorbate has been observed in a recent study where ascorbate induced necrosis of Hep2 cells via ROS generation and PKC induction, while the antioxidant NAC inhibited the effects of ascorbate (Beak et al., 2016). Methylation of DNA at the 5-position of cytosine (5mC) is an important epigenetic modification that repress gene transcription. In contrast, demethylation makes DNA accessible for active gene transcription. Interestingly, Fe(II) and ascorbate function as co-factors for enzymes that are involved in demethylation (Ponnaluri et al., 2013; Minor et al., 2013; Dickson et al., 2013). Ascorbate mediated increase in latent HIV-1 gene expression could also be explain in this light.

Further, the stimulatory effects of ascorbate but also those of PMA were inhibited by DFO, suggesting involvement of iron in these processes. In addition to direct effects of DFO on iron chelation, this inhibition might be explained also by previously described inhibitory effects of DFO on activation of NF-κB, the redox-sensitive transcription factor involved in both PMA signaling and HIV-1 reactivation (Sappey et al., 1995). On the other hand, addition of Fe^{3+}, similarly to NAC, was inhibitory to PMA- and ascorbate-stimulated latent HIV-1 reactivation; Fe^{3+} alone thus appears to reveal final antioxidant effects in this system, possibly due to its complex with EDTA. Evidently, the final outcome of chemical reactions and annihilations of individual compounds and free radicals, respectively, depends on many variables and can dynamically change in a biological system. Importantly, the concentrations of heme-degradation products used in our experiments were comparable or lower than effective concentrations of heme arginate. On the other hand, effective concentrations of heme arginate are achievable in vivo (Tokola et al., 1986).

In ACH-2 cells, the effect of ascorbate or iron with ascorbate on induction of latent HIV expression was relatively lower at both RNA and protein levels than the effect of HA. Indeed, HA serves as a source of both iron and hemin. Heme similarly to iron, generates ROS by Fenton reaction (Shibahara, 2003), while hemin is known to regulate expression of various genes (Furuyama et al., 2007; Mense and Zhang, 2006). On the other hand, pretreatment with a CO-donor or bilirubin dose-dependently decreased p24 levels in ACH-2 cells, while the CO donor increased EGFP expression and bilirubin was ineffective in H12 cells. These differences may be attributable to a different intracellular redox milieu, possibly affected by a constitutive expression of heme oxygenase-1 (Shankaran et al., 2011). In addition to its anti-oxidant property, bilirubin is also a PKC inhibitor (Sano et al., 1985), and thus can directly inhibit HIV-1 transcription stimulated by PMA (a PKC inducer). These results also suggest that the contribution of the individual heme-degradation products may vary in different cell types in vivo. In the first approach, we intentionally used PBMC's to verify the stimulatory effects of heme arginate as they are closer to the situation in vivo. Nevertheless, additional experiments on individual cell types,

namely on isolated total and resting CD4+ cells, will better assess the magnitude of HA-stimulatory effects and help to delineate the underlying mechanisms.

HIV-1 replication and spread is closely connected to the induction of cell death and can be modulated by its onset (Cummins and Badley, 2013; Wang et al., 2016; Wang et al., 2011). While the general apoptosis due to toxicity of latency reversing agents is not desirable, elimination of the infected cells is a goal of all approaches aiming at curing HIV-1. Increased redox stress characterized by decreased levels of reduced glutathione is associated with HIV-1 replication (Pace and Leaf, 1995), while it was suggested as an approach to reactivate and kill latently infected cells by several authors (Iordanskiy and Kashanchi, 2016; Lewis et al., 2011; Shankaran et al., 2011). Importantly, central memory and transition memory T-cells, the main reservoir cells containing the latent HIV-1, were shown to be more susceptible to the redox stress and apoptosis (Chirullo et al., 2013).

HIV/AIDS, similarly to other chronic infections, has been reported to lead to immune-mediated anemia of chronic disease, iron deficiency anemia or their combination (Kerkhoff and Lawn, 2015; Minchella et al., 2015), in which differential expression of hepcidin, hemojuvelin, ferroportin and other factors plays an important role (Drakesmith and Prentice, 2012; Krijt et al., 2004; Theurl et al., 2011; Xu et al., 2010). Consequently, iron supplements have often been administered. However, this can result in an increased labile iron pool (LIP) and reactivation, expression and dissemination of HIV-1. In fact, it has been recognized that iron plays a critical role in several steps of HIV-1 replication (Nekhai et al., 2013) and its levels are modulated by most nonpathogenic SIV (Koppensteiner et al., 2014). Several clinical studies also suggest that iron supplementation or hereditary defects leading to increased intracellular iron stores can fasten progression of HIV infection to AIDS in untreated patients (Gordeuk et al., 2001; McDermid et al., 2007; Rawat et al., 2009).

In vivo, HIV-1 infection can coincide with several conditions that lead to acute or chronic hemolysis that could cause a similar exposure to extracellular heme as the administration of HA. These conditions include genetically determined glucose-6-phosphate dehydrogenase deficiencies, sickle cell anemia, thalasemia or other hemoglobinopathies as well as various other diseases involving hemolytic episodes

or chronic hemolysis, especially malaria (Lopez et al., 2010; Pamplona et al., 2009). It would be worthwhile to determine a possible correlation of HIV-1/AIDS progression with these conditions. However, the situation is complex and therapeutic interventions, namely iron supplementation, could strongly affect the fine balance of pro-oxidative and anti-oxidative agents.

In clinics, HA is used to treat acute attacks of hepatic porphyrias. The mean maximum plasma levels of heme after a single dose of HA 3 mg/kg body weight was determined as 60 µg/ml (corresponds to 2.4 µl/ml of HA), with a plasma half-life of 10.8 h and a distribution volume of 3.4 L (Tokola et al., 1986). The concentrations of HA used throughout this research work are thus very close to the levels achieved in clinics. Additionally, 24–48 h after administration of the same dose of hemin, but in the form of Panhematin, plasma levels of HO-1 were increased 5-times, while its activity in venous leukocytes increased 15-times (Bharucha et al., 2010).

Heme mediates a feedback inhibition of the rate-limiting enzyme in the heme synthetic pathway, synthase of 5-aminolevulinic acid. It also reconstitutes heme stores and function of various hemoproteins, namely hemoglobin, cytochrome P450, guanylate synthase, nitric oxide synthases, tryptophan dioxygenase, catalase and peroxidase. However, neither the exact pathogenesis of the neurovisceral symptoms in acute porphyrias, nor the precise mechanism of action of heme arginate are understood (http://www.porphyria.uct.ac.za/professional/prof-haem-therapy.htm; Herrick and McColl, 2005; Siegesmund et al., 2010). Nevertheless since HA has been approved for human use, it can be suggested that HA could be tested as a supplement of HAART in selected cases. For example its administration could be suggested as an additional measure in early stages of HIV/AIDS disease to release the virus from the existing latent pool, while inhibiting its dissemination to the new viral reservoirs. Since the levels of TNF-α and other cytokines are increased and/or dysregulated in HIV/AIDS, HA might synergize with these cytokines in provirus reactivation also in vivo. The suggestion of HA use in HIV/AIDS is further supported by a case of an HIV-positive individual that was administered one infusion of Normosang because of anemia. This patient then remained p24 negative for several months (Pavel Martasek, General Faculty Hospital in Prague, personal communication). Obviously, the use of HA should be tested first in animal models of

retrovirus infection to assess its therapeutic potential against retroviruses more closely.

Also, the administration of HA can be complicated by its adverse side effects. Vascular side effects of HA, especially on hemostasis, can occur, but they are reported to be much weaker than after administration of hematin (Panhaematin). Additionally, since hemin decreased HIV growth in humanized mice even when administered intraperitoneally (Devadas and Dhawan, 2006), it is possible that the i.p. or some other way of administration of HA would be also effective against HIV in humans. Repeated administrations of HA could lead to an iron overload. However, HIV/AIDS disease is often accompanied by the anaemia due to a chronic immune activation, altered porphyrin metabolism caused by iron deficiency (Adetifa and Okomo, 2009; Fuchs et al., 1990) as well as by treatment with antiretrovirals (Bozzi et al., 2004; Fox et al., 1999). All these conditions would be improved by the administration of heme, while iron overload might not develop.

The ability of cells to become activated remained unaffected by HA as demonstrated by expression of the early activation marker CD69, characterized by flow cytometry. Since the activation of T cells constitutes an essential component of immune responses to the virus itself as well as to other infections, we consider the finding that HA does not seem to generally decrease the activation of T cells as important. Moreover, HA did not induce any global activation of T-cells either; this finding is significant as well, since a nonspecific T-cell activation and release of pro-inflammatory cytokines should be avoided. The effect of HA thus could be compared to the effect of 5-hydroxynaphthalene-1,4-dione, a compound described to reactivate the latent provirus without cellular activation (Yang et al., 2009).

In addition to the experiments studying a role of HA and heme degradation products in HIV-1 latency reversal, we selected additional three compounds and assessed their potency to reactivate HIV-1 provirus in ACH-2 cells either alone or in combination with three different inducers. None of the selected compounds alone could induce the latent HIV-1 expression, while they all stimulated the reactivation of HIV-1 provirus induced by the three inducers and increased p24 expression several folds. Thus, they could be rather called sensitizers. The final effect of the

combination of the two agents is higher than the sum of the effects of the two agents used individually (“1+1 > 2”); therefore, it can be classified as a true synergy. Understandably, different concentrations of compounds and their combination with different inducers resulted in varying levels of reactivation of latent HIV-1. The best working compound-inducer combinations we found in our experiments were filed for Czech and International patent right protection. In fact very recently, an approach towards using a combination of compounds to reactivate HIV-1 provirus and reservoir clearance has been eagerly explored. For example, the combination of romidepsin (HDAC inhibitor) and bryostatin-1 (PKC agonist) has been shown to be very effective in latency reversal in primary CD4+ T cells. Our HA+PMA-mediated stimulation and compounds combinations also follow this new approach of HIV-1 therapeutic reactivation and reservoir clearance.

To our knowledge, this is the first work demonstrating the stimulatory effect of heme on reactivation of the latent provirus. HA seems to possess a combination of two unique properties: it can help to reactivate the provirus from latent pools, and simultaneously prevent HIV-1 dissemination into new cells and/or expansion of the latent pool. On the whole, these results suggest a possibility of an alternative approach to the management of HIV/AIDS disease.

8. Conclusions

Despite the successful suppression of HIV-1 viraemia by cART, the transcriptionally silent provirus and the inaccessible reservoirs of latent virus are the main hurdles to eradicate the HIV-1 infection. “Shock and kill” strategy aiming to reactivate the latent virus and wipe out the latent reservoirs, is currently a hot topic in HIV-1 therapy research. Our original work focussed at exploring the effects of heme arginate on HIV-1 acute infection and reactivation of the latent provirus. The key findings and contributions of our research could be summarised as follows:

1. Heme arginate (HA) inhibited HIV-1 replication in acute infection.
2. The effective concentrations of HA (2.5 µl/ml and 1.25 µl/ml) that inhibited the HIV-1 acute infection did not reveal any significant cytotoxicity.
3. HA synergized with PMA in reactivating latent HIV-1 in ACH-2 cells and HIV-1 “mini-virus” in Jurkat clones A2 and H12.
4. HA alone reactivated HIV-1 “mini-virus” in Jurkat clones A2 and H12.
5. HO-1 enzyme expression was induced by HA in PMA-stimulated ACH-2 cells, but not in A2 and H12 clones, in which the enzyme was found expressed constitutively even in untreated cells.
6. Ascorbate added to generate Fe^{2+}, one of heme degradation products, increased the reactivation of HIV-1 provirus in PMA-stimulated ACH-2 and H12 cells. In contrast, Fe^{3+} revealed a final anti-oxidant effect and decreased HIV-1 reactivation stimulated by PMA alone and in combination with ascorbate.
7. Carbon monooxide and bilirubin, other two heme degradation products, inhibited HIV-1 reactivation stimulated by PMA alone and in combination with ascorbate.
8. Anti-oxidant N-acetyl cysteine inhibited the reactivation of latent HIV-1 by PMA alone and in combination with HA or ascorbate, suggesting that the stimulatory effects ware mediated by reactive oxygen species.

9. Iron chelator desferrioxamine (DFO) prevented latent HIV-1 reactivation by PMA alone or in combination with HA or ascorbate, indicating a causal role of iron.

10. HA and PMA stimulated HIV-1 expression also in primary PBMCs of HIV-1 infected cART-treated patients during *ex vivo* culture.

11. HA did not activate nor inhibit activation of T cells.

12. Our results suggest that HA could be used as a safe HIV-1 latency reversing agent *in vivo*.

9. References

Abraham, N.G., M.L. Friedland, and R.D. Levere. 1983. Heme metabolism in hepatic and erythroid cells. Grune and Stratton, New York.

Abraham, N.G., and A. Kappas. 2008. Pharmacological and clinical aspects of heme oxygenase. *Pharmacol Rev*. 60:79-127.

Abreu, C.M., S.L. Price, E.N. Shirk, R.D. Cunha, L.F. Pianowski, J.E. Clements, A. Tanuri, and L. Gama. 2014. Dual role of novel ingenol derivatives from Euphorbia tirucalli in HIV replication: inhibition of de novo infection and activation of viral LTR. *PloS one*. 9:e97257.

Adetifa, I., and U. Okomo. 2009. Iron supplementation for reducing morbidity and mortality in children with HIV. *Cochrane Database Syst Rev*:CD006736.

Ahluwalia, J.K., S.Z. Khan, K. Soni, P. Rawat, A. Gupta, M. Hariharan, V. Scaria, M. Lalwani, B. Pillai, D. Mitra, and S.K. Brahmachari. 2008. Human cellular microRNA hsa-miR-29a interferes with viral nef protein expression and HIV-1 replication. *Retrovirology*. 5:117.

Aires, S.P., B. Schaaf, C. Muller, K. Dalhoff, and R.H. Dennin. 1995. Fas (CD95) expression on CD4+ T cells from HIV-infected patients increases with disease progression. *J Mol Med*. 73:591-593.

Alonso, J.R., F. Cardellach, S. Lopez, J. Casademont, and O. Miro. 2003. Carbon monoxide specifically inhibits cytochrome c oxidase of human mitochondrial respiratory chain. *Pharmacol Toxicol*. 93:142-146.

Andreas, M., A.I. Schmid, D. Doberer, K. Schewzow, S. Weisshaar, G. Heinze, M. Bilban, E. Moser, and M. Wolzt. 2012. Heme arginate improves reperfusion patterns after ischemia: a randomized, placebo-controlled trial in healthy male subjects. *J Cardiovasc Magn Reson*. 14:55.

Archin, N.M., J.J. Eron, S. Palmer, A. Hartmann-Duff, J.A. Martinson, A. Wiegand, N. Bandarenko, J.L. Schmitz, R.J. Bosch, A.L. Landay, J.M. Coffin, and D.M. Margolis. 2008. Valproic acid without intensified antiviral therapy has limited impact on persistent HIV infection of resting CD4+ T cells. *Aids*. 22:1131-1135.

Archin, N.M., A.L. Liberty, A.D. Kashuba, S.K. Choudhary, J.D. Kuruc, A.M. Crooks, D.C. Parker, E.M. Anderson, M.F. Kearney, M.C. Strain, D.D. Richman, M.G. Hudgens, R.J. Bosch, J.M. Coffin, J.J. Eron, D.J. Hazuda, and D.M. Margolis. 2012. Administration of vorinostat disrupts HIV-1 latency in patients on antiretroviral therapy. *Nature*. 487:482-485.

Archin, N.M., J.M. Sung, C. Garrido, N. Soriano-Sarabia, and D.M. Margolis. 2014. Eradicating HIV-1 infection: seeking to clear a persistent pathogen. *Nat Rev Microbiol*. 12:750-764.

Argyris, E.G., J.M. Vanderkooi, and Y. Paterson. 2001. Mutagenesis of key residues identifies the connection subdomain of HIV-1 reverse transcriptase as the site of inhibition by heme. *Eur J Biochem*. 268:925-931.

Armitage, A.E., A.R. Stacey, E. Giannoulatou, E. Marshall, P. Sturges, K. Chatha, N.M. Smith, X. Huang, X. Xu, S.R. Pasricha, N. Li, H. Wu, C. Webster, A.M. Prentice, P. Pellegrino, I. Williams, P.J. Norris, H. Drakesmith, and P. Borrow. 2014. Distinct patterns of hepcidin and iron regulation during HIV-1, HBV, and HCV infections. *Proceedings of the National Academy of Sciences of the United States of America*. 111:12187-12192.

Arney, K.L., S. Bao, A.J. Bannister, T. Kouzarides, and M.A. Surani. 2002. Histone methylation defines epigenetic asymmetry in the mouse zygote. *The International journal of developmental biology*. 46:317-320.

Ascenzi, P., Y. Cao, A. di Masi, F. Gullotta, G. De Sanctis, G. Fanali, M. Fasano, and M. Coletta. 2010. Reductive nitrosylation of ferric human serum heme-albumin. *FEBS J*. 277:2474-2485.

Atanassova, B.D., and K.N. Tzatchev. 2008. Ascorbic acid--important for iron metabolism. *Folia Med (Plovdiv)*. 50:11-16.

Badia, R., J. Grau, E. Riveira-Munoz, E. Ballana, G. Giannini, and J.A. Este. 2015. The

thioacetate-omega(gamma-lactam carboxamide) HDAC inhibitor ST7612AA1 as HIV-1 latency reactivation agent. *Antiviral research*. 123:62-69.

Baek, M.W., H.S. Cho, S.H. Kim, W.J. Kim, and J.Y. Jung. 2016. Ascorbic acid induces necrosis in human laryngeal squamous cell carcinoma via ROS, PKC, and calcium signaling. *J Cell Physiol*.

Bannister, A.J., R. Schneider, and T. Kouzarides. 2002. Histone methylation: dynamic or static? *Cell*. 109:801-806.

Baranano, D.E., M. Rao, C.D. Ferris, and S.H. Snyder. 2002. Biliverdin reductase: a major physiologic cytoprotectant. *Proceedings of the National Academy of Sciences of the United States of America*. 99:16093-16098.

Barre-Sinoussi, F., J.C. Chermann, F. Rey, M.T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum, and L. Montagnier. 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science*. 220:868-871.

Bauer, U.M., S. Daujat, S.J. Nielsen, K. Nightingale, and T. Kouzarides. 2002. Methylation at arginine 17 of histone H3 is linked to gene activation. *EMBO reports*. 3:39-44.

Bednarik, D.P., J.A. Cook, and P.M. Pitha. 1990. Inactivation of the HIV LTR by DNA CpG methylation: evidence for a role in latency. *The EMBO journal*. 9:1157-1164.

Belcher, J.D., J.D. Beckman, G. Balla, J. Balla, and G. Vercellotti. 2010. Heme degradation and vascular injury. *Antioxid Redox Signal*. 12:233-248.

Bergamaschi, A., and G. Pancino. 2010. Host hindrance to HIV-1 replication in monocytes and macrophages. *Retrovirology*. 7:31.

Berkhout, B., R.H. Silverman, and K.T. Jeang. 1989. Tat trans-activates the human immunodeficiency virus through a nascent RNA target. *Cell*. 59:273-282.

Berne, J.P., B. Lauzier, L. Rochette, and C. Vergely. 2012. Carbon monoxide protects against ischemia-reperfusion injury in vitro via antioxidant properties. *Cell Physiol Biochem*. 29:475-484.

Bernhard, W., K. Barreto, A. Saunders, M.S. Dahabieh, P. Johnson, and I. Sadowski. 2011. The Suv39H1 methyltransferase inhibitor chaetocin causes induction of integrated HIV-1 without producing a T cell response. *FEBS letters*. 585:3549-3554.

Bernstein, B.E., E.L. Humphrey, R.L. Erlich, R. Schneider, P. Bouman, J.S. Liu, T. Kouzarides, and S.L. Schreiber. 2002. Methylation of histone H3 Lys 4 in coding regions of active genes. *Proceedings of the National Academy of Sciences of the United States of America*. 99:8695-8700.

Betteridge, D.J. 2000. What is oxidative stress? *Metabolism - Clinical and Experimental* 49:3 - 8.

Bharucha, A.E., A. Kulkarni, K.M. Choi, M. Camilleri, M. Lempke, G.J. Brunn, S.J. Gibbons, A.R. Zinsmeister, and G. Farrugia. 2010. First-in-human study demonstrating pharmacological activation of heme oxygenase-1 in humans. *Clin Pharmacol Ther*. 87:187-190.

Bickar, D., C. Bonaventura, and J. Bonaventura. 1984. Carbon monoxide-driven reduction of ferric heme and heme proteins. *The Journal of biological chemistry*. 259:10777-10783.

Bjerg Christensen, A., A. Dige, J. Vad-Nielsen, C.R. Brinkmann, M. Bendix, L. Ostergaard, M. Tolstrup, O.S. Sogaard, T.A. Rasmussen, J. Randel Nyengaard, J. Agnholt, and P.W. Denton. 2015. Administration of Panobinostat Is Associated with Increased IL-17A mRNA in the Intestinal Epithelium of HIV-1 Patients. *Mediators of inflammation*. 2015:120605.

Blazkova, J., D. Murray, J.S. Justement, E.K. Funk, A.K. Nelson, S. Moir, T.W. Chun, and A.S. Fauci. 2012. Paucity of HIV DNA methylation in latently infected, resting CD4+ T cells from infected individuals receiving antiretroviral therapy. *Journal of virology*. 86:5390-5392.

Blazkova, J., K. Trejbalova, F. Gondois-Rey, P. Halfon, P. Philibert, A. Guiguen, E. Verdin, D. Olive, C. Van Lint, J. Hejnar, and I. Hirsch. 2009. CpG methylation controls

reactivation of HIV from latency. *PLoS pathogens*. 5:e1000554.
Bouchat, S., J.S. Gatot, K. Kabeya, C. Cardona, L. Colin, G. Herbein, S. De Wit, N. Clumeck, O. Lambotte, C. Rouzioux, O. Rohr, and C. Van Lint. 2012. Histone methyltransferase inhibitors induce HIV-1 recovery in resting CD4(+) T cells from HIV-1-infected HAART-treated patients. *Aids*. 26:1473-1482.
Bozzi, A., F. Brisdelli, A.M. D'Alessandro, G. D'Andrea, A.R. Lizzi, A.C. Rinaldi, and A. Oratore. 2004. Effects of AZT on cellular iron homeostasis. *Biometals*. 17:443-450.
Bres, V., R.E. Kiernan, L.K. Linares, C. Chable-Bessia, O. Plechakova, C. Treand, S. Emiliani, J.M. Peloponese, K.T. Jeang, O. Coux, M. Scheffner, and M. Benkirane. 2003. A non-proteolytic role for ubiquitin in Tat-mediated transactivation of the HIV-1 promoter. *Nature cell biology*. 5:754-761.
Brooks, D.G., S.G. Kitchen, C.M. Kitchen, D.D. Scripture-Adams, and J.A. Zack. 2001. Generation of HIV latency during thymopoiesis. *Nature medicine*. 7:459-464.
Brose, N., and C. Rosenmund. 2002. Move over protein kinase C, you've got company: alternative cellular effectors of diacylglycerol and phorbol esters. *J Cell Sci*. 115:4399-4411.
Buccigrossi, V., G. Laudiero, E. Nicastro, E. Miele, F. Esposito, and A. Guarino. 2011. The HIV-1 transactivator factor (Tat) induces enterocyte apoptosis through a redox-mediated mechanism. *PloS one*. 6:e29436.
Calis, J.C., K.S. Phiri, R.J. Vet, R.J. de Haan, F. Munthali, R.J. Kraaijenhagen, P.J. Hulshof, M.E. Molyneux, B.J. Brabin, M. Boele van Hensbroek, and I. Bates. 2010. Erythropoiesis in HIV-infected and uninfected Malawian children with severe anemia. *Aids*. 24:2883-2887.
Carter, C.C., A. Onafuwa-Nuga, L.A. McNamara, J.t. Riddell, D. Bixby, M.R. Savona, and K.L. Collins. 2010. HIV-1 infects multipotent progenitor cells causing cell death and establishing latent cellular reservoirs. *Nature medicine*. 16:446-451.
Cedar, H., and Y. Bergman. 2009. Linking DNA methylation and histone modification: patterns and paradigms. *Nature reviews. Genetics*. 10:295-304.
Centers for Disease, C. 1981. Kaposi's sarcoma and Pneumocystis pneumonia among homosexual men--New York City and California. *MMWR Morb Mortal Wkly Rep*. 30:305-308.
Centers for Disease, C. 1982. Update on acquired immune deficiency syndrome (AIDS)--United States. *MMWR Morb Mortal Wkly Rep*. 31:507-508, 513-504.
Chang, H.C., M. Bayeva, B. Taiwo, F.J. Palella, Jr., T.J. Hope, and H. Ardehali. 2015. Short communication: high cellular iron levels are associated with increased HIV infection and replication. *AIDS research and human retroviruses*. 31:305-312.
Chau, L.Y. 2015. Heme oxygenase-1: emerging target of cancer therapy. *J Biomed Sci*. 22:22.
Chavez, L., V. Calvanese, and E. Verdin. 2015. HIV Latency Is Established Directly and Early in Both Resting and Activated Primary CD4 T Cells. *PLoS pathogens*. 11:e1004955.
Chen, H.M., C.M. Yang, J.F. Chang, C.S. Wu, K.C. Sia, and W.N. Lin. 2016. AdipoR-increased intracellular ROS promoted cPLA2 and COX-2 expression via activation of PKC and p300 in adiponectin-stimulated human alveolar type II cells. *Am J Physiol Lung Cell Mol Physiol*:ajplung 00218 02015.
Chirullo, B., R. Sgarbanti, D. Limongi, I.L. Shytaj, D. Alvarez, B. Das, A. Boe, S. DaFonseca, N. Chomont, L. Liotta, E.I. Petricoin, S. Norelli, E. Pelosi, E. Garaci, A. Savarino, and A.T. Palamara. 2013. A candidate anti-HIV reservoir compound, auranofin, exerts a selective 'anti-memory' effect by exploiting the baseline oxidative status of lymphocytes. *Cell Death Dis*. 4:e944.
Chiu, Y.L., V.B. Soros, J.F. Kreisberg, K. Stopak, W. Yonemoto, and W.C. Greene. 2005. Cellular APOBEC3G restricts HIV-1 infection in resting CD4+ T cells. *Nature*. 435:108-114.
Chou, S., H. Upton, K. Bao, U. Schulze-Gahmen, A.J. Samelson, N. He, A. Nowak, H. Lu,

N.J. Krogan, Q. Zhou, and T. Alber. 2013. HIV-1 Tat recruits transcription elongation factors dispersed along a flexible AFF4 scaffold. *Proceedings of the National Academy of Sciences of the United States of America*. 110:E123-131.

Choudhary, A.K., J. Rennie, C. Cairns, G. Borthwick, J. Hughes, N.M. Morton, D. Kluth, and B.R. Conway. 2013. Administration of heme arginate ameliorates murine type 2 diabetes independently of heme oxygenase activity. *PloS one*. 8:e78209.

Chun, T.W., R.T. Davey, Jr., D. Engel, H.C. Lane, and A.S. Fauci. 1999. Re-emergence of HIV after stopping therapy. *Nature*. 401:874-875.

Chun, T.W., D. Engel, S.B. Mizell, L.A. Ehler, and A.S. Fauci. 1998. Induction of HIV-1 replication in latently infected CD4+ T cells using a combination of cytokines. *The Journal of experimental medicine*. 188:83-91.

Chun, T.W., D. Engel, S.B. Mizell, C.W. Hallahan, M. Fischette, S. Park, R.T. Davey, Jr., M. Dybul, J.A. Kovacs, J.A. Metcalf, J.M. Mican, M.M. Berrey, L. Corey, H.C. Lane, and A.S. Fauci. 1999. Effect of interleukin-2 on the pool of latently infected, resting CD4+ T cells in HIV-1-infected patients receiving highly active anti-retroviral therapy. *Nature medicine*. 5:651-655.

Chun, T.W., D. Finzi, J. Margolick, K. Chadwick, D. Schwartz, and R.F. Siliciano. 1995. In vivo fate of HIV-1-infected T cells: quantitative analysis of the transition to stable latency. *Nature medicine*. 1:1284-1290.

Chun, T.W., J.S. Justement, D. Murray, C.W. Hallahan, J. Maenza, A.C. Collier, P.M. Sheth, R. Kaul, M. Ostrowski, S. Moir, C. Kovacs, and A.S. Fauci. 2010. Rebound of plasma viremia following cessation of antiretroviral therapy despite profoundly low levels of HIV reservoir: implications for eradication. *Aids*. 24:2803-2808.

Chun, T.W., L. Stuyver, S.B. Mizell, L.A. Ehler, J.A. Mican, M. Baseler, A.L. Lloyd, M.A. Nowak, and A.S. Fauci. 1997. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. *Proceedings of the National Academy of Sciences of the United States of America*. 94:13193-13197.

Churchill, M.J., S.L. Wesselingh, D. Cowley, C.A. Pardo, J.C. McArthur, B.J. Brew, and P.R. Gorry. 2009. Extensive astrocyte infection is prominent in human immunodeficiency virus-associated dementia. *Ann Neurol*. 66:253-258.

Cillo, A.R., M.D. Sobolewski, R.J. Bosch, E. Fyne, M. Piatak, Jr., J.M. Coffin, and J.W. Mellors. 2014. Quantification of HIV-1 latency reversal in resting CD4+ T cells from patients on suppressive antiretroviral therapy. *Proceedings of the National Academy of Sciences of the United States of America*. 111:7078-7083.

Clavel, F., D. Guetard, F. Brun-Vezinet, S. Chamaret, M.A. Rey, M.O. Santos-Ferreira, A.G. Laurent, C. Dauguet, C. Katlama, C. Rouzioux, and et al. 1986. Isolation of a new human retrovirus from West African patients with AIDS. *Science*. 233:343-346.

Clouse, K.A., D. Powell, I. Washington, G. Poli, K. Strebel, W. Farrar, P. Barstad, J. Kovacs, A.S. Fauci, and T.M. Folks. 1989. Monokine regulation of human immunodeficiency virus-1 expression in a chronically infected human T cell clone. *Journal of immunology*. 142:431-438.

Cochrane, A. 2014. HIV-1 Rev function and RNA nuclear-cytoplasmic export. *Methods Mol Biol*. 1087:103-114.

Colin, L., and C. Van Lint. 2009. Molecular control of HIV-1 postintegration latency: implications for the development of new therapeutic strategies. *Retrovirology*. 6:111.

Costantini, A., S. Giuliodoro, L. Butini, G. Silvestri, P. Leoni, and M. Montroni. 2009. Abnormalities of erythropoiesis during HIV-1 disease: a longitudinal analysis. *J Acquir Immune Defic Syndr*. 52:70-74.

Craig, J.M. 2005. Heterochromatin--many flavours, common themes. *BioEssays : news and reviews in molecular, cellular and developmental biology*. 27:17-28.

Craigie, R., and F.D. Bushman. 2012. HIV DNA integration. *Cold Spring Harbor perspectives in medicine*. 2:a006890.

Cuadrado, A., and A.I. Rojo. 2008. Heme oxygenase-1 as a therapeutic target in neurodegenerative diseases and brain infections. *Curr Pharm Des*. 14:429-442.

Cummins, N.W., and A.D. Badley. 2013. Anti-apoptotic mechanisms of HIV: lessons and novel approaches to curing HIV. *Cell Mol Life Sci*. 70:3355-3363.

Daujat, S., U.M. Bauer, V. Shah, B. Turner, S. Berger, and T. Kouzarides. 2002. Crosstalk between CARM1 methylation and CBP acetylation on histone H3. *Current biology : CB*. 12:2090-2097.

Davey, R.T., Jr., N. Bhat, C. Yoder, T.W. Chun, J.A. Metcalf, R. Dewar, V. Natarajan, R.A. Lempicki, J.W. Adelsberger, K.D. Miller, J.A. Kovacs, M.A. Polis, R.E. Walker, J. Falloon, H. Masur, D. Gee, M. Baseler, D.S. Dimitrov, A.S. Fauci, and H.C. Lane. 1999. HIV-1 and T cell dynamics after interruption of highly active antiretroviral therapy (HAART) in patients with a history of sustained viral suppression. *Proceedings of the National Academy of Sciences of the United States of America*. 96:15109-15114.

de Gaetano Donati, K., R. Cauda, and L. Iacoviello. 2010. HIV Infection, Antiretroviral Therapy and Cardiovascular Risk. *Mediterranean journal of hematology and infectious diseases*. 2:e2010034.

De Maria, A., G. Pantaleo, S.M. Schnittman, J.J. Greenhouse, M. Baseler, J.M. Orenstein, and A.S. Fauci. 1991. Infection of CD8+ T lymphocytes with HIV. Requirement for interaction with infected CD4+ cells and induction of infectious virus from chronically infected CD8+ cells. *Journal of immunology*. 146:2220-2226.

Devadas, K., and S. Dhawan. 2006. Hemin activation ameliorates HIV-1 infection via heme oxygenase-1 induction. *Journal of immunology*. 176:4252-4257.

Devadas, K., I.K. Hewlett, and S. Dhawan. 2010. Lipopolysaccharide suppresses HIV-1 replication in human monocytes by protein kinase C-dependent heme oxygenase-1 induction. *Journal of leukocyte biology*. 87:915-924.

Dickson, K.M., C.B. Gustafson, J.I. Young, S. Zuchner, and G. Wang. 2013. Ascorbate-induced generation of 5-hydroxymethylcytosine is unaffected by varying levels of iron and 2-oxoglutarate. *Biochemical and biophysical research communications*. 439:522-527.

Dieffenbach, C.W., and A.S. Fauci. 2011. Thirty years of HIV and AIDS: future challenges and opportunities. *Ann Intern Med*. 154:766-771.

Dixon, S.J., and B.R. Stockwell. 2014. The role of iron and reactive oxygen species in cell death. *Nature chemical biology*. 10:9-17.

Doyon, G., J. Zerbato, J.W. Mellors, and N. Sluis-Cremer. 2013. Disulfiram reactivates latent HIV-1 expression through depletion of the phosphatase and tensin homolog. *Aids*. 27:F7-F11.

Drakesmith, H., and A. Prentice. 2008. Viral infection and iron metabolism. *Nat Rev Microbiol*. 6:541-552.

du Chene, I., E. Basyuk, Y.L. Lin, R. Triboulet, A. Knezevich, C. Chable-Bessia, C. Mettling, V. Baillat, J. Reynes, P. Corbeau, E. Bertrand, A. Marcello, S. Emiliani, R. Kiernan, and M. Benkirane. 2007. Suv39H1 and HP1gamma are responsible for chromatin-mediated HIV-1 transcriptional silencing and post-integration latency. *The EMBO journal*. 26:424-435.

Dulak, J., and A. Jozkowicz. 2003. Carbon monoxide -- a "new" gaseous modulator of gene expression. *Acta Biochim Pol*. 50:31-47.

Duverger, A., F. Wolschendorf, M. Zhang, F. Wagner, B. Hatcher, J. Jones, R.Q. Cron, R.M. van der Sluis, R.E. Jeeninga, B. Berkhout, and O. Kutsch. 2013. An AP-1 binding site in the enhancer/core element of the HIV-1 promoter controls the ability of HIV-1 to establish latent infection. *Journal of virology*. 87:2264-2277.

Dybul, M., B. Hidalgo, T.W. Chun, M. Belson, S.A. Migueles, J.S. Justement, B. Herpin, C. Perry, C.W. Hallahan, R.T. Davey, J.A. Metcalf, M. Connors, and A.S. Fauci. 2002. Pilot study of the effects of intermittent interleukin-2 on human immunodeficiency virus (HIV)-specific immune responses in patients treated during recently acquired HIV infection. *The Journal of infectious diseases*. 185:61-68.

Elliott, J.H., J.H. McMahon, C.C. Chang, S.A. Lee, W. Hartogensis, N. Bumpus, R. Savic, J.

Roney, R. Hoh, A. Solomon, M. Piatak, R.J. Gorelick, J. Lifson, P. Bacchetti, S.G. Deeks, and S.R. Lewin. 2015. Short-term administration of disulfiram for reversal of latent HIV infection: a phase 2 dose-escalation study. *The lancet. HIV*. 2:e520-529.

Faller, M., D. Toso, M. Matsunaga, I. Atanasov, R. Senturia, Y. Chen, Z.H. Zhou, and F. Guo. 2010. DGCR8 recognizes primary transcripts of microRNAs through highly cooperative binding and formation of higher-order structures. *RNA*. 16:1570-1583.

Feinberg, M.B., D. Baltimore, and A.D. Frankel. 1991. The role of Tat in the human immunodeficiency virus life cycle indicates a primary effect on transcriptional elongation. *Proceedings of the National Academy of Sciences of the United States of America*. 88:4045-4049.

Fernandez, G., and S.L. Zeichner. 2010. Cell line-dependent variability in HIV activation employing DNMT inhibitors. *Virology journal*. 7:266.

Finzi, D., J. Blankson, J.D. Siliciano, J.B. Margolick, K. Chadwick, T. Pierson, K. Smith, J. Lisziewicz, F. Lori, C. Flexner, T.C. Quinn, R.E. Chaisson, E. Rosenberg, B. Walker, S. Gange, J. Gallant, and R.F. Siliciano. 1999. Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. *Nature medicine*. 5:512-517.

Finzi, D., M. Hermankova, T. Pierson, L.M. Carruth, C. Buck, R.E. Chaisson, T.C. Quinn, K. Chadwick, J. Margolick, R. Brookmeyer, J. Gallant, M. Markowitz, D.D. Ho, D.D. Richman, and R.F. Siliciano. 1997. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science*. 278:1295-1300.

Folks, T.M., K.A. Clouse, J. Justement, A. Rabson, E. Duh, J.H. Kehrl, and A.S. Fauci. 1989. Tumor necrosis factor alpha induces expression of human immunodeficiency virus in a chronically infected T-cell clone. *Proceedings of the National Academy of Sciences of the United States of America*. 86:2365-2368.

Folks, T.M., J. Justement, A. Kinter, C.A. Dinarello, and A.S. Fauci. 1987. Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. *Science*. 238:800-802.

Fortuny, C., A. Deya-Martinez, E. Chiappini, L. Galli, M. de Martino, and A. Noguera-Julian. 2015. Metabolic and renal adverse effects of antiretroviral therapy in HIV-infected children and adolescents. *The Pediatric infectious disease journal*. 34:S36-43.

Freed, E.O. 2015. HIV-1 assembly, release and maturation. *Nat Rev Microbiol*. 13:484-496.

Friedman, J., W.K. Cho, C.K. Chu, K.S. Keedy, N.M. Archin, D.M. Margolis, and J. Karn. 2011. Epigenetic silencing of HIV-1 by the histone H3 lysine 27 methyltransferase enhancer of Zeste 2. *Journal of virology*. 85:9078-9089.

Fuchs, D., E. Artner-Dworzak, A. Hausen, G. Reibnegger, E.R. Werner, G. Werner-Felmayer, M.P. Dierich, and H. Wachter. 1990. Urinary excretion of porphyrins is increased in patients with HIV-1 infection. *Aids*. 4:341-344.

Fuks, F., P.J. Hurd, D. Wolf, X. Nan, A.P. Bird, and T. Kouzarides. 2003. The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. *The Journal of biological chemistry*. 278:4035-4040.

Furuyama, K., Kaneko, K., Vargas, P. D. 2007. Heme as a magnificent molecule with multiple missions: heme determines its own fate and governs cellular homeostasis. *Tohoku J. Exp. Med.* . 213:1-16.

Galaris, D., and K. Pantopoulos. 2008. Oxidative stress and iron homeostasis: mechanistic and health aspects. *Crit Rev Clin Lab Sci*. 45:1-23.

Gallo, R.C., S.Z. Salahuddin, M. Popovic, G.M. Shearer, M. Kaplan, B.F. Haynes, T.J. Palker, R. Redfield, J. Oleske, B. Safai, and et al. 1984. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science*. 224:500-503.

Gao, F., E. Bailes, D.L. Robertson, Y. Chen, C.M. Rodenburg, S.F. Michael, L.B. Cummins, L.O. Arthur, M. Peeters, G.M. Shaw, P.M. Sharp, and B.H. Hahn. 1999. Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. *Nature*. 397:436-441.

Garcia, J.V., and A.D. Miller. 1992. Downregulation of cell surface CD4 by nef. *Res Virol*.

143:52-55.
Georgiou, N.A., T. van der Bruggen, M. Oudshoorn, P. de Bie, C.A. Jansen, H.S. Nottet, J.J. Marx, and B.S. van Asbeck. 2004. Mechanism of inhibition of the human immunodeficiency virus type 1 by the oxygen radical generating agent bleomycin. *Antiviral research*. 63:97-106.
Gibbs, P.E., and M.D. Maines. 2007. Biliverdin inhibits activation of NF-kappaB: reversal of inhibition by human biliverdin reductase. *Int J Cancer*. 121:2567-2574.
Gil del Valle, L. 2011. Pathophysiological implications of altered redox balance in HIV/AIDS infection: diagnosis and counteract intervention in Oxidative stress diagnosis, prevention and therapy. *ACS Symposium Series*:37-70.
Goel, G., H.P. Makkar, G. Francis, and K. Becker. 2007. Phorbol esters: structure, biological activity, and toxicity in animals. *Int J Toxicol*. 26:279-288.
Goldberg, A.D., C.D. Allis, and E. Bernstein. 2007. Epigenetics: a landscape takes shape. *Cell*. 128:635-638.
Gordeuk, V.R., J.R. Delanghe, M.R. Langlois, and J.R. Boelaert. 2001. Iron status and the outcome of HIV infection: an overview. *J Clin Virol*. 20:111-115.
Gottlinger, H.G., J.G. Sodroski, and W.A. Haseltine. 1989. Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. *Proceedings of the National Academy of Sciences of the United States of America*. 86:5781-5785.
Greiner, D., T. Bonaldi, R. Eskeland, E. Roemer, and A. Imhof. 2005. Identification of a specific inhibitor of the histone methyltransferase SU(VAR)3-9. *Nature chemical biology*. 1:143-145.
Guenzel, C.A., C. Herate, and S. Benichou. 2014. HIV-1 Vpr-a still "enigmatic multitasker". *Frontiers in microbiology*. 5:127.
Gulakowski, R.J., J.B. McMahon, R.W. Buckheit, Jr., K.R. Gustafson, and M.R. Boyd. 1997. Antireplicative and anticytopathic activities of prostratin, a non-tumor-promoting phorbol ester, against human immunodeficiency virus (HIV). *Antiviral research*. 33:87-97.
Gustafson, K.R., J.H. Cardellina, 2nd, J.B. McMahon, R.J. Gulakowski, J. Ishitoya, Z. Szallasi, N.E. Lewin, P.M. Blumberg, O.S. Weislow, J.A. Beutler, and et al. 1992. A nonpromoting phorbol from the samoan medicinal plant Homalanthus nutans inhibits cell killing by HIV-1. *Journal of medicinal chemistry*. 35:1978-1986.
Gutierrez, C., S. Serrano-Villar, N. Madrid-Elena, M.J. Perez-Elias, M.E. Martin, C. Barbas, J. Ruiperez, E. Munoz, M.A. Munoz-Fernandez, T. Castor, and S. Moreno. 2016. Bryostatin-1 for latent virus reactivation in HIV-infected patients on antiretroviral therapy: a phase i, double-blind clinical trial. *Aids*.
Haberland, M., R.L. Montgomery, and E.N. Olson. 2009. The many roles of histone deacetylases in development and physiology: implications for disease and therapy. *Nature reviews. Genetics*. 10:32-42.
Hammond, S.M. 2005. Dicing and slicing: the core machinery of the RNA interference pathway. *FEBS letters*. 579:5822-5829.
Han, Y., K. Lassen, D. Monie, A.R. Sedaghat, S. Shimoji, X. Liu, T.C. Pierson, J.B. Margolick, R.F. Siliciano, and J.D. Siliciano. 2004. Resting CD4+ T cells from human immunodeficiency virus type 1 (HIV-1)-infected individuals carry integrated HIV-1 genomes within actively transcribed host genes. *Journal of virology*. 78:6122-6133.
Handel, A.E., G.C. Ebers, and S.V. Ramagopalan. 2010. Epigenetics: molecular mechanisms and implications for disease. *Trends in molecular medicine*. 16:7-16.
Harakeh, S., R.J. Jariwalla, and L. Pauling. 1990. Suppression of human immunodeficiency virus replication by ascorbate in chronically and acutely infected cells. *Proceedings of the National Academy of Sciences of the United States of America*. 87:7245-7249.
He, N., M. Liu, J. Hsu, Y. Xue, S. Chou, A. Burlingame, N.J. Krogan, T. Alber, and Q. Zhou. 2010. HIV-1 Tat and host AFF4 recruit two transcription elongation factors into a bifunctional complex for coordinated activation of HIV-1 transcription. *Molecular cell*.

38:428-438.
Henderson, A.J., X. Zou, and K.L. Calame. 1995. C/EBP proteins activate transcription from the human immunodeficiency virus type 1 long terminal repeat in macrophages/monocytes. *Journal of virology*. 69:5337-5344.
Hermes-Lima, M., P. Ponka, and H.M. Schulman. 2000. The iron chelator pyridoxal isonicotinoyl hydrazone (PIH) and its analogues prevent damage to 2-deoxyribose mediated by ferric iron plus ascorbate. *Biochim Biophys Acta*. 1523:154-160.
Herrick, A.L., and K.E. McColl. 2005. Acute intermittent porphyria. *Best Pract Res Clin Gastroenterol*. 19:235-249.
Ho, Y.C., L. Shan, N.N. Hosmane, J. Wang, S.B. Laskey, D.I. Rosenbloom, J. Lai, J.N. Blankson, J.D. Siliciano, and R.F. Siliciano. 2013. Replication-competent noninduced proviruses in the latent reservoir increase barrier to HIV-1 cure. *Cell*. 155:540-551.
Honeycutt, J.B., A. Wahl, N. Archin, S. Choudhary, D. Margolis, and J.V. Garcia. 2013. HIV-1 infection, response to treatment and establishment of viral latency in a novel humanized T cell-only mouse (TOM) model. *Retrovirology*. 10:121.
Hong, K.J., H.S. Lee, Y.S. Kim, and S.S. Kim. 2011. Ingenol Protects Human T Cells From HIV-1 Infection. *Osong public health and research perspectives*. 2:109-114.
Hu, W.S., and S.H. Hughes. 2012. HIV-1 reverse transcription. *Cold Spring Harbor perspectives in medicine*. 2.
Huang, J., F. Wang, E. Argyris, K. Chen, Z. Liang, H. Tian, W. Huang, K. Squires, G. Verlinghieri, and H. Zhang. 2007. Cellular microRNAs contribute to HIV-1 latency in resting primary CD4+ T lymphocytes. *Nature medicine*. 13:1241-1247.
Huber, K., G. Doyon, J. Plaks, E. Fyne, J.W. Mellors, and N. Sluis-Cremer. 2011. Inhibitors of histone deacetylases: correlation between isoform specificity and reactivation of HIV type 1 (HIV-1) from latently infected cells. *The Journal of biological chemistry*. 286:22211-22218.
Igarashi, K., and J. Sun. 2006. The heme-Bach1 pathway in the regulation of oxidative stress response and erythroid differentiation. *Antioxid Redox Signal*. 8:107-118.
Imai, K., and T. Okamoto. 2006. Transcriptional repression of human immunodeficiency virus type 1 by AP-4. *The Journal of biological chemistry*. 281:12495-12505.
Imai, K., H. Togami, and T. Okamoto. 2010. Involvement of histone H3 lysine 9 (H3K9) methyltransferase G9a in the maintenance of HIV-1 latency and its reactivation by BIX01294. *The Journal of biological chemistry*. 285:16538-16545.
Immenschuh, S., and H. Schroder. 2006. Heme oxygenase-1 and cardiovascular disease. *Histol Histopathol*. 21:679-685.
International, A.S.S.W.G.o.H.I.V.C., S.G. Deeks, B. Autran, B. Berkhout, M. Benkirane, S. Cairns, N. Chomont, T.W. Chun, M. Churchill, M. Di Mascio, C. Katlama, A. Lafeuillade, A. Landay, M. Lederman, S.R. Lewin, F. Maldarelli, D. Margolis, M. Markowitz, J. Martinez-Picado, J.I. Mullins, J. Mellors, S. Moreno, U. O'Doherty, S. Palmer, M.C. Penicaud, M. Peterlin, G. Poli, J.P. Routy, C. Rouzioux, G. Silvestri, M. Stevenson, A. Telenti, C. Van Lint, E. Verdin, A. Woolfrey, J. Zaia, and F. Barre-Sinoussi. 2012. Towards an HIV cure: a global scientific strategy. *Nat Rev Immunol*. 12:607-614.
Iordanskiy, S., and F. Kashanchi. 2016. Potential of Radiation-Induced Cellular Stress for Reactivation of Latent HIV-1 and Killing of Infected Cells. *AIDS research and human retroviruses*. 32:120-124.
Itzen, F., A.K. Greifenberg, C.A. Bosken, and M. Geyer. 2014. Brd4 activates P-TEFb for RNA polymerase II CTD phosphorylation. *Nucleic acids research*. 42:7577-7590.
Jarmi, T., and A. Agarwal. 2009. Heme oxygenase and renal disease. *Curr Hypertens Rep*. 11:56-62.
Jenuwein, T., and C.D. Allis. 2001. Translating the histone code. *Science*. 293:1074-1080.
Ji, H., Z. Jiang, P. Lu, L. Ma, C. Li, H. Pan, Z. Fu, X. Qu, P. Wang, J. Deng, X. Yang, J. Wang, and H. Zhu. 2016. Specific Reactivation of Latent HIV-1 by dCas9-SunTag-VP64-mediated Guide RNA Targeting the HIV-1 Promoter. *Molecular therapy : the*

journal of the American Society of Gene Therapy.
Jin, B., Y. Li, and K.D. Robertson. 2011. DNA methylation: superior or subordinate in the epigenetic hierarchy? *Genes & cancer*. 2:607-617.
Jones, K.A., and B.M. Peterlin. 1994. Control of RNA initiation and elongation at the HIV-1 promoter. *Annual review of biochemistry*. 63:717-743.
Jordan, A., D. Bisgrove, and E. Verdin. 2003. HIV reproducibly establishes a latent infection after acute infection of T cells in vitro. *The EMBO journal*. 22:1868-1877.
Jordan, A., P. Defechereux, and E. Verdin. 2001. The site of HIV-1 integration in the human genome determines basal transcriptional activity and response to Tat transactivation. *The EMBO journal*. 20:1726-1738.
Juarez, J.C., M. Manuia, M.E. Burnett, O. Betancourt, B. Boivin, D.E. Shaw, N.K. Tonks, A.P. Mazar, and F. Donate. 2008. Superoxide dismutase 1 (SOD1) is essential for H2O2-mediated oxidation and inactivation of phosphatases in growth factor signaling. *Proceedings of the National Academy of Sciences of the United States of America*. 105:7147-7152.
Kalbacova, M., M. Vrbacky, Z. Humlova, and Z. Melkova. 2002. Protooncogene Bcl-2 induces apoptosis in several cell lines. *Folia Biol (Praha)*. 48:15-27.
Kapitulnik, J., and M.D. Maines. 2009. Pleiotropic functions of biliverdin reductase: cellular signaling and generation of cytoprotective and cytotoxic bilirubin. *Trends Pharmacol Sci*. 30:129-137.
Karn, J., and C.M. Stoltzfus. 2012. Transcriptional and posttranscriptional regulation of HIV-1 gene expression. *Cold Spring Harbor perspectives in medicine*. 2:a006916.
Kauder, S.E., A. Bosque, A. Lindqvist, V. Planelles, and E. Verdin. 2009. Epigenetic regulation of HIV-1 latency by cytosine methylation. *PLoS pathogens*. 5:e1000495.
Kerkhoff, A.D., and S.D. Lawn. 2015. Iron replacement therapy and anemia associated with chronic infectious diseases in sub-Saharan Africa. *Clin Infect Dis*. 60:1438-1439.
Kilbourn, R.G., G. Joly, B. Cashon, J. DeAngelo, and J. Bonaventura. 1994. Cell-free hemoglobin reverses the endotoxin-mediated hyporesponsivity of rat aortic rings to alpha-adrenergic agents. *Biochemical and biophysical research communications*. 199:155-162.
Kiselinova, M., A.O. Pasternak, W. De Spiegelaere, D. Vogelaers, B. Berkhout, and L. Vandekerckhove. 2014. Comparison of droplet digital PCR and seminested real-time PCR for quantification of cell-associated HIV-1 RNA. *PloS one*. 9:e85999.
Koppensteiner, H., K. Hohne, M.V. Gondim, F.X. Gobert, M. Widder, S. Gundlach, A. Heigele, F. Kirchhoff, M. Winkler, P. Benaroch, and M. Schindler. 2014. Lentiviral Nef suppresses iron uptake in a strain specific manner through inhibition of Transferrin endocytosis. *Retrovirology*. 11:1.
Kouzarides, T. 2002. Histone methylation in transcriptional control. *Current opinion in genetics & development*. 12:198-209.
Kouzarides, T. 2007. Chromatin modifications and their function. *Cell*. 128:693-705.
Kravets, A., Z. Hu, T. Miralem, M.D. Torno, and M.D. Maines. 2004. Biliverdin reductase, a novel regulator for induction of activating transcription factor-2 and heme oxygenase-1. *The Journal of biological chemistry*. 279:19916-19923.
Krebs, F.C., M.M. Goodenow, and B. Wigdahl. 1997. Neuroglial ATF/CREB factors interact with the human immunodeficiency virus type 1 long terminal repeat. *Journal of neurovirology*. 3 Suppl 1:S28-32.
Krijt, J., M. Vokurka, K.T. Chang, and E. Necas. 2004. Expression of Rgmc, the murine ortholog of hemojuvelin gene, is modulated by development and inflammation, but not by iron status or erythropoietin. *Blood*. 104:4308-4310.
Kruszewski, M. 2003. Labile iron pool: the main determinant of cellular response to oxidative stress. *Mutat Res*. 531:81-92.
Kubicek, S., R.J. O'Sullivan, E.M. August, E.R. Hickey, Q. Zhang, M.L. Teodoro, S. Rea, K. Mechtler, J.A. Kowalski, C.A. Homon, T.A. Kelly, and T. Jenuwein. 2007. Reversal of H3K9me2 by a small-molecule inhibitor for the G9a histone methyltransferase.

Molecular cell. 25:473-481.
Kulkosky, J., D.M. Culnan, J. Roman, G. Dornadula, M. Schnell, M.R. Boyd, and R.J. Pomerantz. 2001. Prostratin: activation of latent HIV-1 expression suggests a potential inductive adjuvant therapy for HAART. *Blood*. 98:3006-3015.
Kumar, A., G. Darcis, C. Van Lint, and G. Herbein. 2015. Epigenetic control of HIV-1 post integration latency: implications for therapy. *Clinical epigenetics*. 7:103.
Laird, G.M., C.K. Bullen, D.I. Rosenbloom, A.R. Martin, A.L. Hill, C.M. Durand, J.D. Siliciano, and R.F. Siliciano. 2015. Ex vivo analysis identifies effective HIV-1 latency-reversing drug combinations. *The Journal of clinical investigation*. 125:1901-1912.
Laird, G.M., E.E. Eisele, S.A. Rabi, J. Lai, S. Chioma, J.N. Blankson, J.D. Siliciano, and R.F. Siliciano. 2013. Rapid quantification of the latent reservoir for HIV-1 using a viral outgrowth assay. *PLoS pathogens*. 9:e1003398.
Landau, N.R., M. Warton, and D.R. Littman. 1988. The envelope glycoprotein of the human immunodeficiency virus binds to the immunoglobulin-like domain of CD4. *Nature*. 334:159-162.
Lander, H.M., P. Sehajpal, D.M. Levine, and A. Novogrodsky. 1993. Activation of human peripheral blood mononuclear cells by nitric oxide-generating compounds. *Journal of immunology*. 150:1509-1516.
Langhoff, E., E.F. Terwilliger, H.J. Bos, K.H. Kalland, M.C. Poznansky, O.M. Bacon, and W.A. Haseltine. 1991. Replication of human immunodeficiency virus type 1 in primary dendritic cell cultures. *Proceedings of the National Academy of Sciences of the United States of America*. 88:7998-8002.
Lehrman, G., I.B. Hogue, S. Palmer, C. Jennings, C.A. Spina, A. Wiegand, A.L. Landay, R.W. Coombs, D.D. Richman, J.W. Mellors, J.M. Coffin, R.J. Bosch, and D.M. Margolis. 2005. Depletion of latent HIV-1 infection in vivo: a proof-of-concept study. *Lancet*. 366:549-555.
Levere, R.D., Y.F. Gong, A. Kappas, D.J. Bucher, G.P. Wormser, and N.G. Abraham. 1991. Heme inhibits human immunodeficiency virus 1 replication in cell cultures and enhances the antiviral effect of zidovudine. *Proceedings of the National Academy of Sciences of the United States of America*. 88:1756-1759.
Levere, R.D., P. Martasek, B. Escalante, M.L. Schwartzman, and N.G. Abraham. 1990. Effect of heme arginate administration on blood pressure in spontaneously hypertensive rats. *The Journal of clinical investigation*. 86:213-219.
Levy, J.A., A.D. Hoffman, S.M. Kramer, J.A. Landis, J.M. Shimabukuro, and L.S. Oshiro. 1984. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. *Science*. 225:840-842.
Lewinski, M.K., D. Bisgrove, P. Shinn, H. Chen, C. Hoffmann, S. Hannenhalli, E. Verdin, C.C. Berry, J.R. Ecker, and F.D. Bushman. 2005. Genome-wide analysis of chromosomal features repressing human immunodeficiency virus transcription. *Journal of virology*. 79:6610-6619.
Lewis, M.G., S. DaFonseca, N. Chomont, A.T. Palamara, M. Tardugno, A. Mai, M. Collins, W.L. Wagner, J. Yalley-Ogunro, J. Greenhouse, B. Chirullo, S. Norelli, E. Garaci, and A. Savarino. 2011. Gold drug auranofin restricts the viral reservoir in the monkey AIDS model and induces containment of viral load following ART suspension. *Aids*. 25:1347-1356.
Lister, R., M. Pelizzola, R.H. Dowen, R.D. Hawkins, G. Hon, J. Tonti-Filippini, J.R. Nery, L. Lee, Z. Ye, Q.M. Ngo, L. Edsall, J. Antosiewicz-Bourget, R. Stewart, V. Ruotti, A.H. Millar, J.A. Thomson, B. Ren, and J.R. Ecker. 2009. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature*. 462:315-322.
Lizard, G., V. Deckert, L. Dubrez, M. Moisant, P. Gambert, and L. Lagrost. 1996. Induction of apoptosis in endothelial cells treated with cholesterol oxides. *Am J Pathol*. 148:1625-1638.
Lopez, C., C. Saravia, A. Gomez, J. Hoebeke, and M.A. Patarroyo. 2010. Mechanisms of genetically-based resistance to malaria. *Gene*. 467:1-12.

Lucic, B., and M. Lusic. 2016. Connecting HIV-1 Integration and Transcription: a step forward towards new treatments. *FEBS letters*.

Ma, Q. 2013. Role of nrf2 in oxidative stress and toxicity. *Annu Rev Pharmacol Toxicol*. 53:401-426.

MacLeod, M.K., J.W. Kappler, and P. Marrack. 2010. Memory CD4 T cells: generation, reactivation and re-assignment. *Immunology*. 130:10-15.

Maghzal, G.J., M.C. Leck, E. Collinson, C. Li, and R. Stocker. 2009. Limited role for the bilirubin-biliverdin redox amplification cycle in the cellular antioxidant protection by biliverdin reductase. *The Journal of biological chemistry*. 284:29251-29259.

Maines, M.D., G.M. Trakshel, and R.K. Kutty. 1986. Characterization of two constitutive forms of rat liver microsomal heme oxygenase. Only one molecular species of the enzyme is inducible. *The Journal of biological chemistry*. 261:411-419.

Marcello, A. 2006. Latency: the hidden HIV-1 challenge. *Retrovirology*. 3:7.

Marsden, M.D., M. Kovochich, N. Suree, S. Shimizu, R. Mehta, R. Cortado, G. Bristol, D.S. An, and J.A. Zack. 2012. HIV latency in the humanized BLT mouse. *Journal of virology*. 86:339-347.

Masanetz, S., and M.H. Lehmann. 2011. HIV-1 Nef increases astrocyte sensitivity towards exogenous hydrogen peroxide. *Virology journal*. 8:35.

Mauclere, P., I. Loussert-Ajaka, F. Damond, P. Fagot, S. Souquieres, M. Monny Lobe, F.X. Mbopi Keou, F. Barre-Sinoussi, S. Saragosti, F. Brun-Vezinet, and F. Simon. 1997. Serological and virological characterization of HIV-1 group O infection in Cameroon. *Aids*. 11:445-453.

McDermid, J.M., A. Jaye, M.F. Schim van der Loeff, J. Todd, C. Bates, S. Austin, D. Jeffries, A.A. Awasana, A.A. Whittlex, and A. Prentice. 2007. Elevated iron status strongly predicts mortality in West African adults with HIV infection. *J Acquir Immune Defic Syndr*. 46:498-507.

McDonald, D., M.A. Vodicka, G. Lucero, T.M. Svitkina, G.G. Borisy, M. Emerman, and T.J. Hope. 2002. Visualization of the intracellular behavior of HIV in living cells. *J Cell Biol*. 159:441-452.

McElrath, M.J., R.M. Steinman, and Z.A. Cohn. 1991. Latent HIV-1 infection in enriched populations of blood monocytes and T cells from seropositive patients. *The Journal of clinical investigation*. 87:27-30.

McKernan, L.N., D. Momjian, and J. Kulkosky. 2012. Protein Kinase C: One Pathway towards the Eradication of Latent HIV-1 Reservoirs. *Advances in virology*. 2012:805347.

McPhee, F., P.S. Caldera, G.W. Bemis, A.F. McDonagh, I.D. Kuntz, and C.S. Craik. 1996 Bile pigments as HIV-1 protease inhibitors and their effects on HIV-1 viral maturation and infectivity in vitro. *Biochem J.* 1:681-686.

Mehla, R., S. Bivalkar-Mehla, R. Zhang, I. Handy, H. Albrecht, S. Giri, P. Nagarkatti, M. Nagarkatti, and A. Chauhan. 2010. Bryostatin modulates latent HIV-1 infection via PKC and AMPK signaling but inhibits acute infection in a receptor independent manner. *PloS one*. 5:e11160.

Mense, S.M., and L. Zhang. 2006. Heme: a versatile signaling molecule controlling the activities of diverse regulators ranging from transcription factors to MAP kinases. *Cell Res*. 16:681-692.

Merson, M.H., J. O'Malley, D. Serwadda, and C. Apisuk. 2008. The history and challenge of HIV prevention. *Lancet*. 372:475-488.

Miller, J.C., S. Tan, G. Qiao, K.A. Barlow, J. Wang, D.F. Xia, X. Meng, D.E. Paschon, E. Leung, S.J. Hinkley, G.P. Dulay, K.L. Hua, I. Ankoudinova, G.J. Cost, F.D. Urnov, H.S. Zhang, M.C. Holmes, L. Zhang, P.D. Gregory, and E.J. Rebar. 2011. A TALE nuclease architecture for efficient genome editing. *Nature biotechnology*. 29:143-148.

Miller, M.D., M.T. Warmerdam, I. Gaston, W.C. Greene, and M.B. Feinberg. 1994. The human immunodeficiency virus-1 nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages. *The Journal of*

experimental medicine. 179:101-113.
Minchella, P.A., A.E. Armitage, B. Darboe, M.W. Jallow, H. Drakesmith, A. Jaye, A.M. Prentice, and J.M. McDermid. 2015. Elevated Hepcidin Is Part of a Complex Relation That Links Mortality with Iron Homeostasis and Anemia in Men and Women with HIV Infection. *J Nutr*. 145:1194-1201.
Minor, E.A., B.L. Court, J.I. Young, and G. Wang. 2013. Ascorbate induces ten-eleven translocation (Tet) methylcytosine dioxygenase-mediated generation of 5-hydroxymethylcytosine. *The Journal of biological chemistry*. 288:13669-13674.
Morgan, M.J., and Z.G. Liu. 2011. Crosstalk of reactive oxygen species and NF-kappaB signaling. *Cell Res*. 21:103-115.
Morse, D., L. Lin, A.M. Choi, and S.W. Ryter. 2009. Heme oxygenase-1, a critical arbitrator of cell death pathways in lung injury and disease. *Free Radic Biol Med*. 47:1-12.
Motterlini, R. 2007. Carbon monoxide-releasing molecules (CO-RMs): vasodilatory, anti-ischaemic and anti-inflammatory activities. *Biochem Soc Trans*. 35:1142-1146.
Motterlini, R., and L.E. Otterbein. 2010. The therapeutic potential of carbon monoxide. *Nat Rev Drug Discov*. 9:728-743.
Muesing, M.A., D.H. Smith, C.D. Cabradilla, C.V. Benton, L.A. Lasky, and D.J. Capon. 1985. Nucleic acid structure and expression of the human AIDS/lymphadenopathy retrovirus. *Nature*. 313:450-458.
Nabel, G., and D. Baltimore. 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature*. 326:711-713.
Naidu, S., N. Wijayanti, S. Santoso, T. Kietzmann, and S. Immenschuh. 2008. An atypical NF-kappa B-regulated pathway mediates phorbol ester-dependent heme oxygenase-1 gene activation in monocytes. *Journal of immunology*. 181:4113-4123.
Nekhai, S., N. Kumari, and S. Dhawan. 2013. Role of cellular iron and oxygen in the regulation of HIV-1 infection. *Future Virol*. 8:301-311.
North, T.W., J. Higgins, J.D. Deere, T.L. Hayes, A. Villalobos, L. Adamson, B.L. Shacklett, R.F. Schinazi, and P.A. Luciw. 2010. Viral sanctuaries during highly active antiretroviral therapy in a nonhuman primate model for AIDS. *Journal of virology*. 84:2913-2922.
Olerup, O., and H. Zetterquist. 1992. HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. *Tissue Antigens*. 39:225-235.
Pace, G.W., and C.D. Leaf. 1995. The role of oxidative stress in HIV disease. *Free Radic Biol Med*. 19:523-528.
Pace, M.J., L. Agosto, E.H. Graf, and U. O'Doherty. 2011. HIV reservoirs and latency models. *Virology*. 411:344-354.
Palacios, J.A., T. Perez-Pinar, C. Toro, B. Sanz-Minguela, V. Moreno, E. Valencia, C. Gomez-Hernando, and B. Rodes. 2012. Long-term nonprogressor and elite controller patients who control viremia have a higher percentage of methylation in their HIV-1 proviral promoters than aviremic patients receiving highly active antiretroviral therapy. *Journal of virology*. 86:13081-13084.
Paller, M.S., and H.S. Jacob. 1994. Cytochrome P-450 mediates tissue-damaging hydroxyl radical formation during reoxygenation of the kidney. *Proceedings of the National Academy of Sciences of the United States of America*. 91:7002-7006.
Pamplona, A., T. Hanscheid, S. Epiphanio, M.M. Mota, and A.M. Vigario. 2009. Cerebral malaria and the hemolysis/methemoglobin/heme hypothesis: shedding new light on an old disease. *Int J Biochem Cell Biol*. 41:711-716.
Pandelo Jose, D., K. Bartholomeeusen, R.D. da Cunha, C.M. Abreu, J. Glinski, T.B. da Costa, A.F. Bacchi Rabay, L.F. Pianowski Filho, L.W. Dudycz, U. Ranga, B.M. Peterlin, L.F. Pianowski, A. Tanuri, and R.S. Aguiar. 2014. Reactivation of latent HIV-1 by new semi-synthetic ingenol esters. *Virology*. 462-463:328-339.
Pantano, C., N.L. Reynaert, A. van der Vliet, and Y.M. Janssen-Heininger. 2006. Redox-

sensitive kinases of the nuclear factor-kappaB signaling pathway. *Antioxid Redox Signal*. 8:1791-1806.

Parfenova, H., S. Basuroy, S. Bhattacharya, D. Tcheranova, Y. Qu, R.F. Regan, and C.W. Leffler. 2006. Glutamate induces oxidative stress and apoptosis in cerebral vascular endothelial cells: contributions of HO-1 and HO-2 to cytoprotection. *Am J Physiol Cell Physiol*. 290:C1399-1410.

Partridge, J.F., K.S. Scott, A.J. Bannister, T. Kouzarides, and R.C. Allshire. 2002. cis-acting DNA from fission yeast centromeres mediates histone H3 methylation and recruitment of silencing factors and cohesin to an ectopic site. *Current biology : CB*. 12:1652-1660.

Pasternak, A.O., K.W. Adema, M. Bakker, S. Jurriaans, B. Berkhout, M. Cornelissen, and V.V. Lukashov. 2008. Highly sensitive methods based on seminested real-time reverse transcription-PCR for quantitation of human immunodeficiency virus type 1 unspliced and multiply spliced RNA and proviral DNA. *J Clin Microbiol*. 46:2206-2211.

Pasternak, A.O., V.V. Lukashov, and B. Berkhout. 2013. Cell-associated HIV RNA: a dynamic biomarker of viral persistence. *Retrovirology*. 10:41.

Pasupathi, P., T. Ramchandran, P.J. Sindhu, G. Saranavan, and G. Bakthavathsalam. 2009. Enhanced oxidative stress markers and antioxidant imbalance in HIV infection and AIDS patients. *J Sci Res*. 1:370-380.

Patel, P., M.Y. Ansari, S. Bapat, M. Thakar, R. Gangakhedkar, and S. Jameel. 2014. The microRNA miR-29a is associated with human immunodeficiency virus latency. *Retrovirology*. 11:108.

Patki, A.H., D.L. Georges, and M.M. Lederman. 1997. CD4+-T-cell counts, spontaneous apoptosis, and Fas expression in peripheral blood mononuclear cells obtained from human immunodeficiency virus type 1-infected subjects. *Clin Diagn Lab Immunol*. 4:736-741.

Perdigao, P., T. Gaj, M. Santa-Marta, C.F. Barbas, 3rd, and J. Goncalves. 2016. Reactivation of Latent HIV-1 Expression by Engineered TALE Transcription Factors. *PloS one*. 11:e0150037.

Pereira, E.A., and L.L. daSilva. 2016. HIV-1 Nef: taking control of protein trafficking. *Traffic*.

Peterlin, B.M., and D.H. Price. 2006. Controlling the elongation phase of transcription with P-TEFb. *Molecular cell*. 23:297-305.

Plantier, J.C., M. Leoz, J.E. Dickerson, F. De Oliveira, F. Cordonnier, V. Lemee, F. Damond, D.L. Robertson, and F. Simon. 2009. A new human immunodeficiency virus derived from gorillas. *Nature medicine*. 15:871-872.

Pollicita, M., C. Muscoli, A. Sgura, A. Biasin, T. Granato, L. Masuelli, V. Mollace, C. Tanzarella, C. Del Duca, P. Rodino, C.F. Perno, and S. Aquaro. 2009. Apoptosis and telomeres shortening related to HIV-1 induced oxidative stress in an astrocytoma cell line. *BMC Neurosci*. 10:51.

Ponnaluri, V.K., J.P. Maciejewski, and M. Mukherji. 2013. A mechanistic overview of TET-mediated 5-methylcytosine oxidation. *Biochemical and biophysical research communications*. 436:115-120.

Price, D.H. 2000. P-TEFb, a cyclin-dependent kinase controlling elongation by RNA polymerase II. *Molecular and cellular biology*. 20:2629-2634.

Pumfery, A., L. Deng, A. Maddukuri, C. de la Fuente, H. Li, J.D. Wade, P. Lambert, A. Kumar, and F. Kashanchi. 2003. Chromatin remodeling and modification during HIV-1 Tat-activated transcription. *Current HIV research*. 1:343-362.

Pyo, C.W., Y.L. Yang, N.K. Yoo, and S.Y. Choi. 2008. Reactive oxygen species activate HIV long terminal repeat via post-translational control of NF-kappaB. *Biochemical and biophysical research communications*. 376:180-185.

Qi, M., R. Yang, and C. Aiken. 2008. Cyclophilin A-dependent restriction of human immunodeficiency virus type 1 capsid mutants for infection of nondividing cells. *Journal of virology*. 82:12001-12008.

Raha, T., S.W. Cheng, and M.R. Green. 2005. HIV-1 Tat stimulates transcription complex assembly through recruitment of TBP in the absence of TAFs. *PLoS biology*. 3:e44.
Rambaut, A., D. Posada, K.A. Crandall, and E.C. Holmes. 2004. The causes and consequences of HIV evolution. *Nature reviews. Genetics*. 5:52-61.
Rana, T.M., and K.T. Jeang. 1999. Biochemical and functional interactions between HIV-1 Tat protein and TAR RNA. *Archives of biochemistry and biophysics*. 365:175-185.
Rasmussen, T.A., O. Schmeltz Sogaard, C. Brinkmann, F. Wightman, S.R. Lewin, J. Melchjorsen, C. Dinarello, L. Ostergaard, and M. Tolstrup. 2013. Comparison of HDAC inhibitors in clinical development: effect on HIV production in latently infected cells and T-cell activation. *Human vaccines & immunotherapeutics*. 9:993-1001.
Raval, C.M., and P.J. Lee. 2010. Heme oxygenase-1 in lung disease. *Curr Drug Targets*. 11:1532-1540.
Rawat, R., J.H. Humphrey, R. Ntozini, K. Mutasa, P.J. Iliff, and R.J. Stoltzfus. 2009. Elevated iron stores are associated with HIV disease severity and mortality among postpartum women in Zimbabwe. *Public Health Nutr*. 12:1321-1329.
Redig, A.J., and N. Berliner. 2013. Pathogenesis and clinical implications of HIV-related anemia in 2013. *Hematology Am Soc Hematol Educ Program*. 2013:377-381.
Richman, D.D., D.M. Margolis, M. Delaney, W.C. Greene, D. Hazuda, and R.J. Pomerantz. 2009. The challenge of finding a cure for HIV infection. *Science*. 323:1304-1307.
Richter, S., Y.H. Ping, and T.M. Rana. 2002. TAR RNA loop: a scaffold for the assembly of a regulatory switch in HIV replication. *Proceedings of the National Academy of Sciences of the United States of America*. 99:7928-7933.
Rohr, O., C. Marban, D. Aunis, and E. Schaeffer. 2003. Regulation of HIV-1 gene transcription: from lymphocytes to microglial cells. *Journal of leukocyte biology*. 74:736-749.
Romerio, F., M.N. Gabriel, and D.M. Margolis. 1997. Repression of human immunodeficiency virus type 1 through the novel cooperation of human factors YY1 and LSF. *Journal of virology*. 71:9375-9382.
Romerio, F., and J.C. Zapata. 2015. Detection and enrichment to near purity of rare HIV-1 infected cells by PrimeFlow RNA. *Journal of Virus Eradication* 1:5.
Rossi, A., R. Mukerjee, P. Ferrante, K. Khalili, S. Amini, and B.E. Sawaya. 2006. Human immunodeficiency virus type 1 Tat prevents dephosphorylation of Sp1 by TCF-4 in astrocytes. *The Journal of general virology*. 87:1613-1623.
Saayman, S.M., D.C. Lazar, T.A. Scott, J.R. Hart, M. Takahashi, J.C. Burnett, V. Planelles, K.V. Morris, and M.S. Weinberg. 2015. Potent and Targeted Activation of Latent HIV-1 Using the CRISPR/dCas9 Activator Complex. *Molecular therapy : the journal of the American Society of Gene Therapy*.
Sabharwal, S.S., and P.T. Schumacker. 2014. Mitochondrial ROS in cancer: initiators, amplifiers or an Achilles' heel? *Nat Rev Cancer*. 14:709-721.
Sacks, D.B. 2006. The role of scaffold proteins in MEK/ERK signalling. *Biochem. Soc. Trans.* 34:833-836.
Salmen, S., and L. Berrueta. 2012. Immune Modulators of HIV Infection: The Role of Reactive Oxygen Species. . *J Clin Cell Immunol* 3:doi:10.4172/2155-9899.1000121.
Salter, J.D., G.A. Morales, and H.C. Smith. 2014. Structural insights for HIV-1 therapeutic strategies targeting Vif. *Trends Biochem Sci*. 39:373-380.
Sanchez-Del Cojo, M., M.R. Lopez-Huertas, E. Mateos, J. Alcami, and M. Coiras. 2011. Mechanisms of RNA interference in the HIV-1-host cell interplay. *AIDS reviews*. 13:149-160.
Sandalio, L.M., M. Rodriguez-Serrano, M.C. Romero-Puertas, and L.A. del Rio. 2013. Role of peroxisomes as a source of reactive oxygen species (ROS) signaling molecules. *Subcell Biochem*. 69:231-255.
Sano, K., H. Nakamura, and T. Matsuo. 1985. Mode of inhibitory action of bilirubin on protein kinase C. *Pediatr Res*. 19:587-590.
Sappey, C., J.R. Boelaert, S. Legrand-Poels, C. Forceille, A. Favier, and J. Piette. 1995. Iron

chelation decreases NF-kappa B and HIV type 1 activation due to oxidative stress. *AIDS research and human retroviruses*. 11:1049-1061.

Schiralli Lester, G.M., and A.J. Henderson. 2012. Mechanisms of HIV Transcriptional Regulation and Their Contribution to Latency. *Molecular biology international*. 2012:614120.

Schmidtmayerova, H., M. Alfano, G. Nuovo, and M. Bukrinsky. 1998. Human immunodeficiency virus type 1 T-lymphotropic strains enter macrophages via a CD4- and CXCR4-mediated pathway: replication is restricted at a postentry level. *Journal of virology*. 72:4633-4642.

Schubert, U., S. Bour, A.V. Ferrer-Montiel, M. Montal, F. Maldarell, and K. Strebel. 1996. The two biological activities of human immunodeficiency virus type 1 Vpu protein involve two separable structural domains. *Journal of virology*. 70:809-819.

Schulze-Forster, K., F. Gotz, H. Wagner, H. Kroger, and D. Simon. 1990. Transcription of HIV1 is inhibited by DNA methylation. *Biochemical and biophysical research communications*. 168:141-147.

Serras, F. 2016. The benefits of oxidative stress for tissue repair and regeneration. *Fly (Austin)*:1-6.

Seu, L., T.D. Burt, D.W. Williamson, J.N. Martin, S.G. Deeks, and J.M. McCune. 2009. Heme oxygenase-1 promoter polymorphisms correlate with favorable virologic and immunological parameters in HIV-1 infection. *Retrovirology*. 6:109.

Shan, L., K. Deng, N.S. Shroff, C.M. Durand, S.A. Rabi, H.C. Yang, H. Zhang, J.B. Margolick, J.N. Blankson, and R.F. Siliciano. 2012. Stimulation of HIV-1-specific cytolytic T lymphocytes facilitates elimination of latent viral reservoir after virus reactivation. *Immunity*. 36:491-501.

Shang, H.T., J.W. Ding, S.Y. Yu, T. Wu, Q.L. Zhang, and F.J. Liang. 2015. Progress and challenges in the use of latent HIV-1 reactivating agents. *Acta pharmacologica Sinica*. 36:908-916.

Shankaran, P., L. Vlkova, J. Liskova, and Z. Melkova. 2011. Heme arginate potentiates latent HIV-1 reactivation while inhibiting the acute infection. *Antiviral research*. 92:434-446.

Sharp, P.M., and B.H. Hahn. 2011. Origins of HIV and the AIDS pandemic. *Cold Spring Harbor perspectives in medicine*. 1:a006841.

Shaw, J.P., P.J. Utz, D.B. Durand, J.J. Toole, E.A. Emmel, and G.R. Crabtree. 1988. Identification of a putative regulator of early T cell activation genes. *Science*. 241:202-205.

Shibahara, S. 2003. The heme oxygenase dilemma in cellular homeostasis: new insights for the feedback regulation of heme catabolism. *Tohoku J Exp Med*. 200:167-186.

Siegesmund, M., A.M. van Tuyll van Serooskerken, P. Poblete-Gutierrez, and J. Frank. 2010. The acute hepatic porphyrias: current status and future challenges. *Best Pract Res Clin Gastroenterol*. 24:593-605.

Siliciano, J.D., J. Kajdas, D. Finzi, T.C. Quinn, K. Chadwick, J.B. Margolick, C. Kovacs, S.J. Gange, and R.F. Siliciano. 2003. Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4+ T cells. *Nature medicine*. 9:727-728.

Siliciano, J.D., and R.F. Siliciano. 2005. Enhanced culture assay for detection and quantitation of latently infected, resting CD4+ T-cells carrying replication-competent virus in HIV-1-infected individuals. *Methods Mol Biol*. 304:3-15.

Siliciano, R.F., and W.C. Greene. 2011. HIV latency. *Cold Spring Harbor perspectives in medicine*. 1:a007096.

Simon, F., P. Mauclere, P. Roques, I. Loussert-Ajaka, M.C. Muller-Trutwin, S. Saragosti, M.C. Georges-Courbot, F. Barre-Sinoussi, and F. Brun-Vezinet. 1998. Identification of a new human immunodeficiency virus type 1 distinct from group M and group O. *Nature medicine*. 4:1032-1037.

Soares, M.P., I. Marguti, A. Cunha, and R. Larsen. 2009. Immunoregulatory effects of HO-1: how does it work? *Curr Opin Pharmacol*. 9:482-489.

Sobhian, B., N. Laguette, A. Yatim, M. Nakamura, Y. Levy, R. Kiernan, and M. Benkirane. 2010. HIV-1 Tat assembles a multifunctional transcription elongation complex and stably associates with the 7SK snRNP. *Molecular cell*. 38:439-451.

Son, Y., Y.K. Cheong, N.H. Kim, H.T. Chung, D.G. Kang, and H.O. Pae. 2011. Mitogen-Activated Protein Kinases and Reactive Oxygen Species: How Can ROS Activate MAPK Pathways? *J Signal Transduct*. 2011:792639.

Son, Y., J.H. Lee, H.T. Chung, and H.O. Pae. 2013. Therapeutic roles of heme oxygenase-1 in metabolic diseases: curcumin and resveratrol analogues as possible inducers of heme oxygenase-1. *Oxid Med Cell Longev*. 2013:639541.

Spina, C.A., J. Anderson, N.M. Archin, A. Bosque, J. Chan, M. Famiglietti, W.C. Greene, A. Kashuba, S.R. Lewin, D.M. Margolis, M. Mau, D. Ruelas, S. Saleh, K. Shirakawa, R.F. Siliciano, A. Singhania, P.C. Soto, V.H. Terry, E. Verdin, C. Woelk, S. Wooden, S. Xing, and V. Planelles. 2013. An in-depth comparison of latent HIV-1 reactivation in multiple cell model systems and resting CD4+ T cells from aviremic patients. *PLoS pathogens*. 9:e1003834.

Spitaler, M., and D.A. Cantrell. 2004. Protein kinase C and beyond. *Nature immunology*. 5:785-790.

Staudinger, R., N.G. Abraham, R.D. Levere, and A. Kappas. 1996. Inhibition of human immunodeficiency virus-1 reverse transcriptase by heme and synthetic heme analogs. *Proc Assoc Am Physicians*. 108:47-54.

Steffan, A.M., M.E. Lafon, J.L. Gendrault, C. Schweitzer, C. Royer, D. Jaeck, J.P. Arnaud, M.P. Schmitt, A.M. Aubertin, and A. Kirn. 1992. Primary cultures of endothelial cells from the human liver sinusoid are permissive for human immunodeficiency virus type 1. *Proceedings of the National Academy of Sciences of the United States of America*. 89:1582-1586.

Stoszko, M., E. De Crignis, C. Rokx, M.M. Khalid, C. Lungu, R.J. Palstra, T.W. Kan, C. Boucher, A. Verbon, E.C. Dykhuizen, and T. Mahmoudi. 2016. Small Molecule Inhibitors of BAF; A Promising Family of Compounds in HIV-1 Latency Reversal. *EBioMedicine*. 3:108-121.

Sun, G., H. Li, X. Wu, M. Covarrubias, L. Scherer, K. Meinking, B. Luk, P. Chomchan, J. Alluin, A.F. Gombart, and J.J. Rossi. 2012. Interplay between HIV-1 infection and host microRNAs. *Nucleic acids research*. 40:2181-2196.

Sundquist, W.I., and H.G. Krausslich. 2012. HIV-1 assembly, budding, and maturation. *Cold Spring Harbor perspectives in medicine*. 2:a006924.

Sung, T.L., and A.P. Rice. 2009. miR-198 inhibits HIV-1 gene expression and replication in monocytes and its mechanism of action appears to involve repression of cyclin T1. *PLoS pathogens*. 5:e1000263.

Swaminathan, G., S. Navas-Martin, and J. Martin-Garcia. 2014. MicroRNAs and HIV-1 infection: antiviral activities and beyond. *Journal of molecular biology*. 426:1178-1197.

Swiggard, W.J., C. Baytop, J.J. Yu, J. Dai, C. Li, R. Schretzenmair, T. Theodosopoulos, and U. O'Doherty. 2005. Human immunodeficiency virus type 1 can establish latent infection in resting CD4+ T cells in the absence of activating stimuli. *Journal of virology*. 79:14179-14188.

Swindle, E.J., J.A. Hunt, and J.W. Coleman. 2002. A comparison of reactive oxygen species generation by rat peritoneal macrophages and mast cells using the highly sensitive real-time chemiluminescent probe pholasin: inhibition of antigen-induced mast cell degranulation by macrophage-derived hydrogen peroxide. *Journal of immunology*. 169:5866-5873.

Tan Gana, N.H., T. Onuki, A.F. Victoriano, and T. Okamoto. 2012. MicroRNAs in HIV-1 infection: an integration of viral and cellular interaction at the genomic level. *Frontiers in microbiology*. 3:306.

Tenhunen, R., H.S. Marver, and R. Schmid. 1968. The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proceedings of the National Academy of*

Sciences of the United States of America. 61:748-755.
Tenhunen, R., O. Tokola, and I.B. Linden. 1987. Haem arginate: a new stable haem compound. *J Pharm Pharmacol*. 39:780-786.
Terwilliger, E.F., E. Langhoff, D. Gabuzda, E. Zazopoulos, and W.A. Haseltine. 1991. Allelic variation in the effects of the nef gene on replication of human immunodeficiency virus type 1. *Proceedings of the National Academy of Sciences of the United States of America*. 88:10971-10975.
Theurl, I., A. Schroll, M. Nairz, M. Seifert, M. Theurl, T. Sonnweber, H. Kulaksiz, and G. Weiss. 2011. Pathways for the regulation of hepcidin expression in anemia of chronic disease and iron deficiency anemia in vivo. *Haematologica*. 96:1761-1769.
Tiwari, S., and J.F. Ndisang. 2014. The heme oxygenase system and type-1 diabetes. *Curr Pharm Des*. 20:1328-1337.
Tokola, O., R. Tenhunen, L. Volin, and P. Mustajoki. 1986. Pharmacokinetics of intravenously administered haem arginate. *Br J Clin Pharmacol*. 22:331-335.
Trejbalova, K., D. Kovarova, J. Blazkova, L. Machala, D. Jilich, J. Weber, D. Kucerova, O. Vencalek, I. Hirsch, and J. Hejnar. 2016. Development of 5' LTR DNA methylation of latent HIV-1 provirus in cell line models and in long-term-infected individuals. *Clinical epigenetics*. 8:19.
Triboulet, R., B. Mari, Y.L. Lin, C. Chable-Bessia, Y. Bennasser, K. Lebrigand, B. Cardinaud, T. Maurin, P. Barbry, V. Baillat, J. Reynes, P. Corbeau, K.T. Jeang, and M. Benkirane. 2007. Suppression of microRNA-silencing pathway by HIV-1 during virus replication. *Science*. 315:1579-1582.
Tripathy, M.K., M.E. McManamy, B.D. Burch, N.M. Archin, and D.M. Margolis. 2015. H3K27 Demethylation at the Proviral Promoter Sensitizes Latent HIV to the Effects of Vorinostat in Ex Vivo Cultures of Resting CD4+ T Cells. *Journal of virology*. 89:8392-8405.
Trushin, S.A., G.D. Bren, S. Asin, K.N. Pennington, C.V. Paya, and A.D. Badley. 2005. Human immunodeficiency virus reactivation by phorbol esters or T-cell receptor ligation requires both PKCalpha and PKCtheta. *Journal of virology*. 79:9821-9830.
Tyagi, M., and J. Karn. 2007. CBF-1 promotes transcriptional silencing during the establishment of HIV-1 latency. *The EMBO journal*. 26:4985-4995.
Vallari, A., V. Holzmayer, B. Harris, J. Yamaguchi, C. Ngansop, F. Makamche, D. Mbanya, L. Kaptue, N. Ndembi, L. Gurtler, S. Devare, and C.A. Brennan. 2011. Confirmation of putative HIV-1 group P in Cameroon. *Journal of virology*. 85:1403-1407.
Van Damme, N., D. Goff, C. Katsura, R.L. Jorgenson, R. Mitchell, M.C. Johnson, E.B. Stephens, and J. Guatelli. 2008. The interferon-induced protein BST-2 restricts HIV-1 release and is downregulated from the cell surface by the viral Vpu protein. *Cell host & microbe*. 3:245-252.
Van Heuverswyn, F., Y. Li, C. Neel, E. Bailes, B.F. Keele, W. Liu, S. Loul, C. Butel, F. Liegeois, Y. Bienvenue, E.M. Ngolle, P.M. Sharp, G.M. Shaw, E. Delaporte, B.H. Hahn, and M. Peeters. 2006. Human immunodeficiency viruses: SIV infection in wild gorillas. *Nature*. 444:164.
Van Lint, C., S. Emiliani, M. Ott, and E. Verdin. 1996. Transcriptional activation and chromatin remodeling of the HIV-1 promoter in response to histone acetylation. *The EMBO journal*. 15:1112-1120.
Venkatachari, N.J., J.M. Zerbato, S. Jain, A.E. Mancini, A. Chattopadhyay, N. Sluis-Cremer, Z. Bar-Joseph, and V. Ayyavoo. 2015. Temporal transcriptional response to latency reversing agents identifies specific factors regulating HIV-1 viral transcriptional switch. *Retrovirology*. 12:85.
Verdin, E., P. Paras, Jr., and C. Van Lint. 1993. Chromatin disruption in the promoter of human immunodeficiency virus type 1 during transcriptional activation. *The EMBO journal*. 12:3249-3259.
Volin, L., V. Rasi, E. Vahtera, and R. Tenhunen. 1988. Heme arginate: effects on hemostasis. *Blood*. 71:625-628.

Wang, P., X. Qu, X. Wang, X. Zhu, H. Zeng, H. Chen, and H. Zhu. 2014. Specific reactivation of latent HIV-1 with designer zinc-finger transcription factors targeting the HIV-1 5'-LTR promoter. *Gene therapy*. 21:490-495.

Wang, P., X. Qu, X. Zhou, Y. Shen, H. Ji, Z. Fu, J. Deng, P. Lu, W. Yu, H. Lu, and H. Zhu. 2015. Two cellular microRNAs, miR-196b and miR-1290, contribute to HIV-1 latency. *Virology*. 486:228-238.

Wang, R. 1998. Resurgence of carbon monoxide: an endogenous gaseous vasorelaxing factor. *Can J Physiol Pharmacol*. 76:1-15.

Wang, X., C. Mbondji-Wonje, J. Zhao, and I. Hewlett. 2016. IL-1beta and IL-18 inhibition of HIV-1 replication in Jurkat cells and PBMCs. *Biochemical and biophysical research communications*. 473:926-930.

Wang, X., V. Ragupathy, J. Zhao, and I. Hewlett. 2011. Molecules from apoptotic pathways modulate HIV-1 replication in Jurkat cells. *Biochemical and biophysical research communications*. 414:20-24.

Wang, X., P. Wang, Z. Fu, H. Ji, X. Qu, H. Zeng, X. Zhu, J. Deng, P. Lu, S. Zha, Z. Song, and H. Zhu. 2015. Designed transcription activator-like effector proteins efficiently induced the expression of latent HIV-1 in latently infected cells. *AIDS research and human retroviruses*. 31:98-106.

Wang, X., L. Ye, W. Hou, Y. Zhou, Y.J. Wang, D.S. Metzger, and W.Z. Ho. 2009. Cellular microRNA expression correlates with susceptibility of monocytes/macrophages to HIV-1 infection. *Blood*. 113:671-674.

Wei, D.G., V. Chiang, E. Fyne, M. Balakrishnan, T. Barnes, M. Graupe, J. Hesselgesser, A. Irrinki, J.P. Murry, G. Stepan, K.M. Stray, A. Tsai, H. Yu, J. Spindler, M. Kearney, C.A. Spina, D. McMahon, J. Lalezari, D. Sloan, J. Mellors, R. Geleziunas, and T. Cihlar. 2014. Histone deacetylase inhibitor romidepsin induces HIV expression in CD4 T cells from patients on suppressive antiretroviral therapy at concentrations achieved by clinical dosing. *PLoS pathogens*. 10:e1004071.

Wei, P., M.E. Garber, S.M. Fang, W.H. Fischer, and K.A. Jones. 1998. A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. *Cell*. 92:451-462.

Wilen, C.B., J.C. Tilton, and R.W. Doms. 2012. HIV: cell binding and entry. *Cold Spring Harbor perspectives in medicine*. 2.

Willey, R.L., F. Maldarelli, M.A. Martin, and K. Strebel. 1992. Human immunodeficiency virus type 1 Vpu protein induces rapid degradation of CD4. *Journal of virology*. 66:7193-7200.

Williams, S.A., L.F. Chen, H. Kwon, C.M. Ruiz-Jarabo, E. Verdin, and W.C. Greene. 2006. NF-kappaB p50 promotes HIV latency through HDAC recruitment and repression of transcriptional initiation. *The EMBO journal*. 25:139-149.

WM, A. 2006. Iron and heam metabolism. *In* Textbook of biochemistry with clinical correlations. D. TM, editor. Wiley – Liss, New Jersey. 824– 830.

Wong, J.K., M. Hezareh, H.F. Gunthard, D.V. Havlir, C.C. Ignacio, C.A. Spina, and D.D. Richman. 1997. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. *Science*. 278:1291-1295.

Wu, L., and R. Wang. 2005. Carbon monoxide: endogenous production, physiological functions, and pharmacological applications. *Pharmacol Rev*. 57:585-630.

Xing, S., C.K. Bullen, N.S. Shroff, L. Shan, H.C. Yang, J.L. Manucci, S. Bhat, H. Zhang, J.B. Margolick, T.C. Quinn, D.M. Margolis, J.D. Siliciano, and R.F. Siliciano. 2011. Disulfiram reactivates latent HIV-1 in a Bcl-2-transduced primary CD4+ T cell model without inducing global T cell activation. *Journal of virology*. 85:6060-6064.

Xu, M., F. Kashanchi, A. Foster, J. Rotimi, W. Turner, V.R. Gordeuk, and S. Nekhai. 2010. Hepcidin induces HIV-1 transcription inhibited by ferroportin. *Retrovirology*. 7:104.

Yamaguchi, Y., N. Inukai, T. Narita, T. Wada, and H. Handa. 2002. Evidence that negative elongation factor represses transcription elongation through binding to a DRB sensitivity-inducing factor/RNA polymerase II complex and RNA. *Molecular and*

cellular biology. 22:2918-2927.
Yamashita, K., J. McDaid, R. Ollinger, T.Y. Tsui, P.O. Berberat, A. Usheva, E. Csizmadia, R.N. Smith, M.P. Soares, and F.H. Bach. 2004. Biliverdin, a natural product of heme catabolism, induces tolerance to cardiac allografts. *FASEB J*. 18:765-767.
Yang, H.C., S. Xing, L. Shan, K. O'Connell, J. Dinoso, A. Shen, Y. Zhou, C.K. Shrum, Y. Han, J.O. Liu, H. Zhang, J.B. Margolick, and R.F. Siliciano. 2009. Small-molecule screening using a human primary cell model of HIV latency identifies compounds that reverse latency without cellular activation. *The Journal of clinical investigation*. 119:3473-3486.
Yang, Z., N. He, and Q. Zhou. 2008. Brd4 recruits P-TEFb to chromosomes at late mitosis to promote G1 gene expression and cell cycle progression. *Molecular and cellular biology*. 28:967-976.
Yang, Z., Q. Zhu, K. Luo, and Q. Zhou. 2001. The 7SK small nuclear RNA inhibits the CDK9/cyclin T1 kinase to control transcription. *Nature*. 414:317-322.
Yap, M.W., M.P. Dodding, and J.P. Stoye. 2006. Trim-cyclophilin A fusion proteins can restrict human immunodeficiency virus type 1 infection at two distinct phases in the viral life cycle. *Journal of virology*. 80:4061-4067.
Yeung, M.L., Y. Bennasser, K. Watashi, S.Y. Le, L. Houzet, and K.T. Jeang. 2009. Pyrosequencing of small non-coding RNAs in HIV-1 infected cells: evidence for the processing of a viral-cellular double-stranded RNA hybrid. *Nucleic acids research*. 37:6575-6586.
Yin, H., Y. Zhang, X. Zhou, and H. Zhu. 2011. Histonedeacetylase inhibitor Oxamflatin increase HIV-1 transcription by inducing histone modification in latently infected cells. *Molecular biology reports*. 38:5071-5078.
Ylisastigui, L., N.M. Archin, G. Lehrman, R.J. Bosch, and D.M. Margolis. 2004. Coaxing HIV-1 from resting CD4 T cells: histone deacetylase inhibition allows latent viral expression. *Aids*. 18:1101-1108.
Yoshikawa, T., and Y. Naito. 2002. What is oxidative stress? *JMAJ*. 45:271–276.
Zegerman, P., B. Canas, D. Pappin, and T. Kouzarides. 2002. Histone H3 lysine 4 methylation disrupts binding of nucleosome remodeling and deacetylase (NuRD) repressor complex. *The Journal of biological chemistry*. 277:11621-11624.
Zhang, A.S., F. Canonne-Hergaux, S. Gruenheid, P. Gros, and P. Ponka. 2008. Use of Nramp2-transfected Chinese hamster ovary cells and reticulocytes from mk/mk mice to study iron transport mechanisms. *Exp Hematol* 36:1227-1235.
Zhang, Z., T. Schuler, M. Zupancic, S. Wietgrefe, K.A. Staskus, K.A. Reimann, T.A. Reinhart, M. Rogan, W. Cavert, C.J. Miller, R.S. Veazey, D. Notermans, S. Little, S.A. Danner, D.D. Richman, D. Havlir, J. Wong, H.L. Jordan, T.W. Schacker, P. Racz, K. Tenner-Racz, N.L. Letvin, S. Wolinsky, and A.T. Haase. 1999. Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. *Science*. 286:1353-1357.

10. Publications

The results obtained during the work on this doctoral dissertation were summarized in two research and one review articles in peer-reviewed international journals with impact factor and resulted in submission of a Czech and international patent applications.

10.1. Research articles

Shankaran P., Vlkova L., Liskova J., and Melkova Z. 2011. Heme arginate potentiates latent HIV-1 reactivation while inhibiting the acute infection. Antiviral Research. 92(3):434-446. IF (2011): 4.301

Shankaran P., Madlenakova M., Hajkova V., Jilich D., Svobodova I., Horinek A., Fujikura Y., and Melkova Z. Effects of heme degradation products on reactivation of latent HIV-1. Acta Virologica. Submitted.

10.2. Review article

Melkova Z, **Shankaran P,** Madlenakova M and Bodor J. Current views on HIV-1 latency, persistence, and cure. Folia Microbiologica. Accepted. IF (2015): 1.335

10.3. Patent applications

Shankaran P and Melkova Z. 2013. Kombinovany přípravek a jeho použití pro reaktivaci latentniho HIV. **Národní Přihláška Vynálezu**, PV 2013 - 474. (*Application retracted and under revision*)

Melkova Z and **Shankaran P**. 2014. Combination preparation and use thereof for Latent HIV reactivation**. International patent application**, PCT/CZ2014/000070 *(Application retracted and under revision)*

10.4. Conferences

Madlenakova M, **Shankaran P**, Hajkova V, Fujikura Y, Jilich D, Machala L, Belacek J, Melkova Z.Characterization of plasma and cell-associated viremia and its correlation with redox state and heme metabolism in HIV-1-infected patients. 27th Congress of the Czechoslovak Society for Microbiology. September, 2016, Prague, Czech Republic,

Melkova Z, **Shankaran P**, Hajkova V, Jilich D, Fujikura Z. Effects of Heme Degradation Products on Reactivation of Latent HIV-1. 7th International Workshop on HIV Persistence During Therapy. December, 2015. Miami, FL, USA.

Melkova Z, **Shankaran P**, Hajkova V and Jilich D. Reactivation of latent HIV-1 by iron-mediated redox stress. "CYTOKINES" 3rd Annual meeting of ICIS. October, 2015. Bamberg, Germany.

Melkova Z, **Shankaran P**, Fujikura Z and Hajkova V. Reactivation of HIV from the latent stage using Normosang. 9th International Conference on Modern Drug Delivery Systems and Recombinant Vaccines. June, 2015. Telč, Czech Republic.

Melkova Z and **Shankaran P.** The Effect of Heme Arginate on Reactivation of the Latent HIV-1. 17th Annual Meeting of ESCV. September, 2014. Prague, Czech Republic.

Shankaran P. Reactivation of the latent HIV-1. Seminar at Institute of Immunology and Microbiology, 1st faculty of medicine, Charles university. October, 2013. Prague, Czech Republic.

Melkova Z and **Shankaran P**. The Effect of Heme Arginate on Reactivation of the Latent HIV-1 at 6th International Workshop on HIV Persistence During Therapy. December, 2013. Miami, FL, USA.

Melkova Z and **Shankaran P**. Role of redox stress and use of redox-modulating agents in the reactivation of latent HIV-1. On seminar at McGill University AIDS centre, Lady Davis Institute for Medical Research. December, 2013. Montreal, Canada.

Melkova Z, **Shankaran P** and Liskova J. Heme arginate potentiates latent HIV-1 reactivation while inhibiting the acute infection. 9th joint meeting of the international cytokine society and international society for interferon and cytokine research. October, 2011. Florence, Italy.

Shankaran P, Liskova J and Melkova Z. Double-barreled action of heme arginate on HIV-1: inhibition of acute infection and reactivation of latent provirus. Poster no. 15. VI Analytical cytometry conference. October, 2011. Prague, Czech Republic.

Shankaran P and Melkova Z. New ways to treat HIV/AIDS? 12th Students' science conference. First Medical Faculty, Charles University. May, 2011. Prague, Czech Republic. **(Awarded best presentation)**

Shankaran P and Melokova Z. Inhibitory effects of Heme Arginate on HIV-1 growth and replication. Antivirals Congress. November, 2010. Amsterdam, The Netherlands.

Shankaran P and Melkova Z. Effect of heme arginate in the reactivation and inhibition of latent HIV-1 infection. Centennial Retrovirus Meeting (CRM2010). May, 2010. Prague, Czech Republic.

Shankaran P, Knitlova J, Cizek Z and Melkova Z. Effect of the ethacrynic acid on immune responses towards vaccinia virus. FEBS Congress. July, 2009. Prague, Czech Republic.

Shankaran P. Effect of redox potential on the growth and replication of HIV-1. Joint scientific-research seminar of the Institute of Immunology and microbiology. June, 2009. Prague, Czech Republic.

Shankaran P. Effect of ethacrynic acid derivatives on cell growth in vitro. Postgraduate students project conference, GAČR. 7. 11. 2008, Prague, Czech Republic.

11. Appendix

11.1. Research article 1

Shankaran P., Vlkova L., Liskova J., and Melkova Z. 2011. Heme arginate potentiates latent HIV-1 reactivation while inhibiting the acute infection. Antiviral Research. 92(3):434-446. IF (2011): 4.301

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Heme arginate potentiates latent HIV-1 reactivation while inhibiting the acute infection

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ABSTRACT

Human immunodeficiency virus-1 (HIV-1) successfully escapes from host immune surveillance, vaccines and antiretroviral agents. The available antiretroviral compounds can only control viremia, but it is impossible to eliminate the virus from the organism, namely because HIV-1 provirus persists in the reservoir cells from which the virus repeatedly disseminates into new cells. Current therapeutic approaches, however, do not specifically address the stage of virus reactivation.

Heme has been demonstrated as very efficient in inhibiting HIV-1 reverse transcription, while its derivative hemin ameliorated HIV-1 infection via induction of heme oxygenase-1. Normosang (heme arginate; HA) is a human hemin-containing compound used to treat acute porphyria. In this work, we studied the effects of HA in HIV-1-acutely infected T-cell lines, and in cell lines harboring either a complete HIV-1 provirus (ACH-2 cells) or an HIV-1 "mini-virus" (Jurkat clones expressing EGFP under control of HIV LTR). We demonstrate that HA inhibited HIV-1 replication during the acute infection, which was accompanied by the inhibition of reverse transcription. On the other hand, HA alone stimulated the reactivation of HIV-1 "mini-virus" and synergized with phorbol ester or TNF-α in the reactivation of HIV-1 provirus. The stimulatory effects of HA were inhibited by *N*-acetyl cysteine, suggesting an increased redox stress and activation of NF-κB. Further, HA induced expression of heme oxygenase-1 (HO-1) in ACH-2 cells, while HO-1 was found expressed in untreated Jurkat clones. Inhibitor of HO-1 activity, tin protoporphyrin IX, further increased HA-mediated reactivation of HIV-1 "mini-virus" in Jurkat clones, and this effect was also inhibited by *N*-acetyl cysteine. The stimulatory effects of HA on HIV-1 reactivation thus seem to involve HO-1 and generation of free radicals. Additionally, the effective concentrations of HA did neither affect normal T-cell activation with PMA nor induce activation of the unstimulated cells.

In conclusion, HA appears to possess a combination of unique properties that could help to decrease the pool of latently infected reservoir cells, while simultaneously inhibiting HIV-1 replication in newly infected cells. Our results thus suggest a new direction to explore in treatment of HIV/AIDS disease.

1. Introduction

Overall, 2 million people die of AIDS every year. The causative agent of this deadly disease, Human immunodeficiency virus-1 (HIV-1), is one of the most variable viruses. The high evolution rate helps the virus to escape from host immune surveillance, vaccines and antiretroviral agents. The available antiretroviral compounds can only control viremia, and it is currently impossible to eliminate the virus from the organism, namely because HIV-1 provirus persists in the reservoir cells. During intercurrent infections, the provirus is repeatedly reactivated and disseminated into new cells, thus enlarging the pool of reservoir cells. Current therapeutic approaches consist of combinations of several drugs inhibiting various steps in HIV-1 growth cycle, but these drugs reveal serious side effects, and the virus often gains resistance to them (Mehellou and De Clercq, 2010; Walmsley and Loutfy, 2002). Therefore, more potent and/or less toxic therapeutic approaches effective against HIV are intensively sought.

Pathogenesis of HIV/AIDS infection is known to include an increased redox stress that is characterized by the increased production of reactive oxygen and nitrogen species, decreased levels of reduced glutathione (GSH) and GSH-dependent antioxidant mechanisms, as well as depletion of the main antioxidant enzymes, such as glutathione peroxidase, thioredoxin or catalase (Pace and Leaf, 1995). The increased redox stress leads not only to the reactivation of the latent HIV-1 provirus, but also to an increased apoptosis and depletion of uninfected CD4+ cells (Pace and Leaf, 1995). The activation of the host cell is accompanied by the activation of the redox-sensitive transcription factor NF-κB (Lander et al., 1993; Pantano et al., 2006) and its translocation to the nucleus (Greene,

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1991), where it binds to the Long Terminal Repeat (LTR) of the integrated HIV-1 provirus and induces its replication (Nabel and Baltimore, 1987; Pyo et al., 2008; Williams et al., 2007). The redox state of the cell thus simultaneously affects both activation of NF-κB and reactivation of the latent provirus.

Current therapeutic approaches focus primarily on the inhibition of HIV-encoded enzymes reverse transcriptase and protease; fusion inhibitors and inhibitors of co-receptors or integrase are also available (Mehellou and De Clercq, 2010). Due to a high mutation rate of HIV-1 and development of resistance, adherence to strict regimes consisting of administration of several drugs a day that keep the replication rates of HIV-1 in check, and thus decrease the possibility of the outgrow of the resistant clones, is an essential part of the therapy. Non-adherence to the regime as well as other factors then support an increased mutation rate and development of resistance. Evidently, it is desirable to pharmacologically target host cell factors that cannot mutate and gain resistance as fast as the virus. One such a target would be NF-κB and/or the process of reactivation of HIV-1 provirus. However, a focused approach trying to affect the redox stress and reactivation of the provirus (outside of the use of vitamins and the effort to avoid common diseases in general) is not generally included in the therapeutic approaches.

In vitro, heme (Fe^{2+}, ferroprotoporphyrin IX) has been demonstrated as very efficient in inhibiting HIV-1 reverse transcription (Argyris et al., 2001; Levere et al., 1991). Further, hemin (Fe^{3+}, ferriprotoporphyrin IX) ameliorated HIV-1 infection in humanized mice, and heme oxygenase-1 (HO-1) was suggested to be responsible for the inhibitory effect (HO-1; Devadas and Dhawan, 2006). Normosang (heme arginate, HA; Orphan Europe) is a human hemin-containing compound used to treat acute porphyria. It is composed of hemin and L-arginine as an additive to increase solubility and stability of the product (Siegesmund et al., 2010), and it shows fewer side effects in hemostasis compared to Panhaematin (Ovation Pharmaceuticals; Volin et al., 1988). However, there are no reports on the effect of HA on HIV-1 growth and reactivation. Hence, we attempted to study the effect of HA on HIV-1 replication in acutely infected T-cell lines A3.01 and Jurkat, as well as its effects on the latent provirus reactivation in PMA-stimulated ACH-2 cells harboring HIV-1 provirus and in A2 and H12 clones of Jurkat cells latently infected with an HIV-1 "mini-virus" containing EGFP under control of HIV-1 LTR. Here we demonstrate that HA inhibited HIV-1 replication during the acute infection of T-cell lines, which was accompanied by the inhibition of reverse transcription. On the other hand, HA alone stimulated the reactivation of HIV-1 "mini-virus" and in combination with PMA or other stimulatory agents the reactivation of HIV-1 provirus, with the stimulatory effects involving reactive oxygen species and activity of HO-1. Additionally, heme arginate did not activate T-cells nor inhibit the activation of T cells by PMA.

2. Materials and methods

2.1. Chemicals

All the media and growth supplements were purchased from Invitrogen Corporation (Carlsbad, CA) or PAA Laboratories GmbH (Pasching, Austria). Heme arginate (Normosang) was purchased from Orphan Europe (Paris, France), tin protoporphyrin IX (SnPP) from Frontier Scientific (Logan, UT), TNF-α from Peprotech (London, United Kingdom), and RETRO-TEK HIV-1 p24 Antigen ELISA from ZeptoMetrix Corp. (Buffalo, NY). Other chemicals used were purchased from Sigma unless otherwise specified.

2.2. Cell lines

Human T-cell lines A3.01 and Jurkat (a clone with high expression of CD4), ACH-2 cells harboring an integrated HIV-1 provirus (clone #4; Clouse et al., 1989), and A2 and H12 clones of Jurkat cells latently infected with a "mini-virus" containing the HIV-1 LTR-Tat-IRES-EGFP-LTR (Blazkova et al., 2009; Jordan et al., 2003) were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 12.5 mM Hepes, and antibiotics (penicillin 1×10^5 U/l, streptomycin 100 mg/l; 10% FBS-RPMI). The cells were treated with increasing concentrations of HA (1.25 and 2.5 μl/ml of HA correspond to 31.25 and 62.5 μg/ml of hemin or 48 and 96 μM hemin, respectively). ACH-2, A2 and H12 cells were stimulated with phorbol myristate acetate (PMA; final concentration 0.5 ng/ml was used throughout the experiments) to express HIV-1 or EGFP, respectively. The cells were also treated with *N*-acetyl cysteine (final concentration 5 and 10 mM), SnPP (final concentration 6.25 μM), TNF-α (final concentration 1 and 10 U/ml), PHA (final concentration 0.5 and 1 μg/ml).

2.3. Viruses and infection

The stock of HIV-1 was prepared using a transient transfection of Jurkat cells with pNL4–3 (Adachi et al., 1986). The culture supernatant was collected at day 7 after transfection and virus titer was estimated as 4.8×10^{10} TU/ml (transducing units/ml) based on levels of p24 antigen determined by RETRO-TEK HIV-1 p24 antigen ELISA according to the manufacturer's protocol. For time course experiments, 0.2×10^6 cells in 0.2 ml of 10% FBS-RPMI were infected with 2 μl of the stock; after 4 h of adsorption of inoculum, 0.8 ml of 10% FBS-RPMI was added and supplemented with HA (final concentration 1.25 and 2.5 μl/ml). The cells were split 1:4 at the indicated times after infection and the media was supplemented with HA to keep the final concentrations as indicated. The growth of HIV-1 was characterized by levels of p24 antigen in culture supernatants. For detection of HIV-1 reverse transcripts, virus stock was treated with RNAse-free DNase I (Sigma, Germany; final concentration 300 U/100 μl of virus stock) and incubated at room temperature for 45 min to remove plasmid and cellular DNA present in the inoculum. 0.5×10^6 A3.01 and Jurkat cells in 0.2 ml of 10% FBS-RPMI were infected with 100 μl of the DNase I-treated virus stock, and after 4 h of adsorption of inoculum, 0.8 ml of 10% FBS-RPMI was added and supplemented with HA (final concentration 2.5 μl/ml) or Azidothymidine (AZT; final concentration 10 μM) as a control. Forty eight hours after infection, the cells were collected in PBS, trypsinized and used for DNA isolation.

2.4. PCR detection of HIV-1 reverse transcripts

Total cellular DNA was isolated using a modified method of Miller's salting-out procedure, without proteinase K and with addition of a chloroform extraction phase (Olerup and Zetterquist, 1992). Ethanol-precipitated DNA was dissolved in TE buffer and quantified by measuring the absorbance at 260 nm using UV spectrophotometer BioPhotometer (Eppendorff AG, Germany). HIV-1 reverse transcripts were determined by PCR using primers specific for LTR/gag (Schmidtmayerova et al., 1998) and for GAPDH (sense 5′-TTC TGT CTT CCA CTC ACT CC-3′, antisense 5′-GTA TTC CCC CAG GTT TAC ATG-3′) in a 50 μl reaction volume containing 1 U of Taq DNA polymerase (Top-Bio, Czech Republic), 1x PCR buffer (10 mM Tris–HCl, pH 8.8; 50 mM KCl; 0.1% Triton X-100), 200 nM each primer, 200 μM dNTPs, $MgCl_2$ (1 mM for LTR/gag; 0.75 mM for GAPDH) and sample DNA (1000 ng for LTR/gag; 200 ng for GAPDH. PCR conditions: initial denaturation 94 °C/4 min and 35 cycles of

94 °C/30 s, 52 °C/30 s for LTR/gag or 57 °C/30 s for GAPDH, 72 °C/60 s, with final extension 72 °C/10 min. The PCR products were resolved using a 1.5% agarose gel electrophoresis in 1× TBE buffer and 0.5 μg/ml ethidium bromide, and visualized under UV transilluminator.

2.5. Western blot analysis

Cells were collected and lysed in Laemmli reducing sample buffer, boiled and analyzed by SDS–PAGE and western blotting as previously described (Harlow and Lane, 1988; Laemmli, 1970), using chemiluminescence (West Femto, Thermo Fisher Scientific – Pierce, Rockford, IL). For p24 antigen, the cell lysates were resolved on a 14% SDS–PAGE and detected using a monoclonal antibody ND-1 (dilution 1:500; Exbio, Prague, Czech Republic) and a peroxidase-conjugated goat anti-mouse IgG (dilution 1:20,000; Sigma Co., St. Louis, MO). EGFP was detected using a 12% SDS–PAGE, a rabbit polyclonal antibody (dilution 1:1000; Exbio, Prague, Czech Republic) and a peroxidase-conjugated goat anti-rabbit IgG (dilution 1:20,000; MP Biomedicals – Cappel, Solon, OH), HO-1 was detected using a 10% SDS–PAGE, a rabbit polyclonal antibody (dilution 1:20,000; Abcam, Cambridge, United Kingdom) and a peroxidase-conjugated goat anti-rabbit IgG (dilution 1:20,000). β-Actin was detected on a 10% gel, using either a goat polyclonal antibody (dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and a peroxidase-conjugated donkey anti-goat IgG (dilution 1:20,000; Jackson ImmunoResearch Laboratories, West Grove, PA) or using a rabbit polyclonal antibody (dilution 1:7500; Abcam, Cambridge, United Kingdom) and a peroxidase-conjugated goat anti-rabbit IgG (dilution 1:20,000).

2.6. Flow cytometry analysis

Flow cytometer Canto II (Becton Dickinson) equipped with 3 lasers emitting at 488, 405 and 633 nm, and with 8 detectors was used. Flow cytometry measurements were performed using the Diva 6 software (Becton Dickinson, Franklin Lakes, NJ). Subsequent analyses of the flow cytometric data were performed using Diva 6 and/or FlowJo (Tree Star, Inc., Ashland, OR).

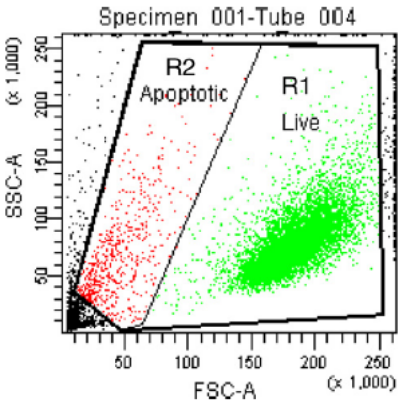


Fig. 1. Example of a flow cytometric analysis. Gating of the cell populations in dependence on their size and granularity. Cell populations in Regions 1 and 2 were used for further analysis; the two regions contained live and apoptotic cells, respectively. The samples were collected, incubated with individual fluorescent indicators as indicated, and analyzed using flow cytometry and Diva 6 or FlowJo software.

At each time point, cells were collected, stained with a fluorochrome, and used for further analysis in the appropriate detecting channel. Ten thousand cells were collected upon gating on a FSC-A × SSC-A dot plot. The region used for further analysis contained live cells, as well as their apoptotic counterparts (Fig. 1). Discrimination of apoptotic cells was performed on a FSC-A × SSC-A dot plot and/or using staining with Hoechst 33342 (Intergen; final concentration 0.1 μg/ml; Kalbacova et al., 2002; Lizard et al., 1996) and 7-AAD (final concentration (1 μg/ml) followed by flow cytometry analysis in FL5 (detecting at 474–496 nm) and FL4 (detecting at 750–810 nm), respectively. Percentage of apoptotic cells determined on a FSC-A × SSC-A dot plot correlated with the percentage of apoptotic cells determined on a Hoechst 33342 × 7-AAD dot plot (not shown). For assessment of cell viability of the infected cells during the time course experiment, the cells were first fixed with 1% paraformaldehyde, and then analyzed as described above.

EGFP fluorescence was characterized by a flow cytometry analysis in FL1 (detecting at 515–545 nm). EGFP expression was assessed as the arithmetic mean of green fluorescence of green cell population × percentage of all EGFP-positive cells. EGFP fluorescence intensity was characterized by the median fluorescence of live green cells. Detection of CD69 expression was performed using a mouse monoclonal antibody against human CD69 labeled with Alexa Fluor-700 (dilution 1:50; Exbio, Prague, Czech Republic) followed by flow cytometry analysis in FL7 (detecting at 700–720 nm).

2.7. Cytotoxicity assays and determination of CC_{50}

Cytotoxicity of heme arginate was characterized by determination of induction of apoptosis using flow cytometry (see above) and by the effects on cell viability and growth using a protocol adapted according to TOX-1 kit (Sigma Co., St. Louis, MO). Briefly, A3.01 and Jurkat cells were diluted with fresh culture medium and 24 h later, they were plated in 24-well plates at a density of 0.06×10^6/ml/well in culture medium containing increasing concentrations of HA. In parallel, wells with culture medium and HA were incubated to be used as individual blanks for each particular concentration of HA. After 2 days of incubation, cell growth and viability were characterized by activity of mitochondrial dehydrogenases using the MTT assay. The conversion of MTT to formazan was determined photometrically at 540 nm after dissolving the product in the acidified isopropanol. The cytotoxic concentration was expressed as CC50, the concentration of the tested compound that reduced cell growth to 50% compared to vehiculum-treated controls.

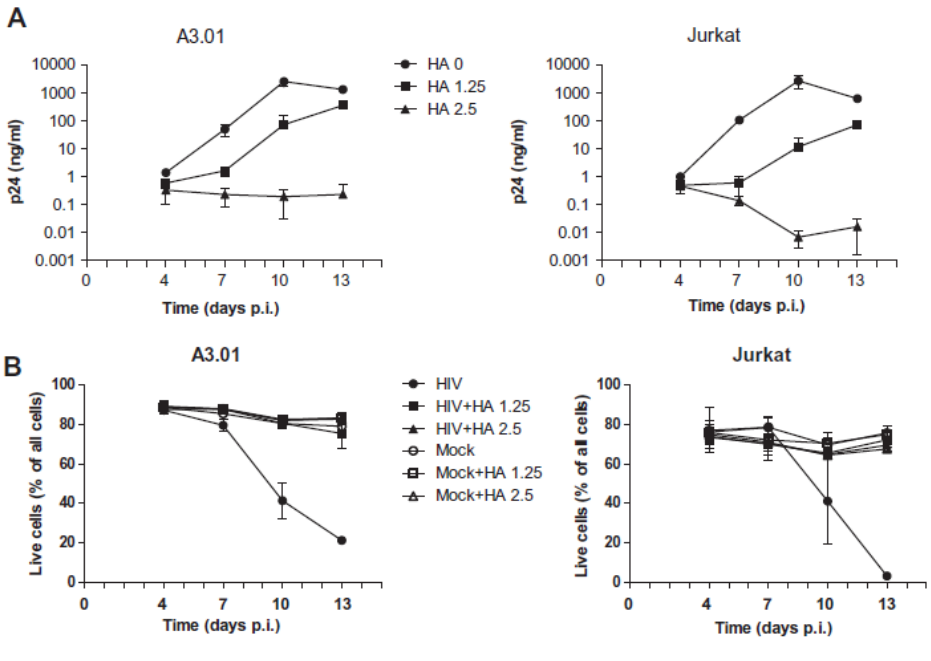
2.8. Statistical analysis

Results are presented as means ± SD (standard deviation). Statistical differences between each group and control or between two groups were determined using a two-sample two-tailed Student's *t*-test for either equal or unequal variances. Equality of variances was tested with *F*-test.

3. Results

3.1. Heme arginate inhibits HIV-1 growth during the acute infection of cells with HIV-1

The overall effect of heme arginate (HA) was assessed during a time course experiment characterizing the acute infection of T-cell lines A3.01 and Jurkat with HIV-1. As demonstrated in Fig. 2A, addition of HA strongly inhibited growth of HIV-1 characterized by levels of p24 in culture supernatants in both cell lines. The



C

A3.01

HA (μl/ml)	0	1.25	2.5	5.0	7.5
Apoptosis (%)	7.40 ± 0.84	8.24 ± 1.09	9.55 ± 1.09	11.90 ± 1.11	15.00 ± 1.32
MTT (%)	100.00 ± 0.00	97.04 ± 4.12	92.00 ± 7.27	82.48 ± 13.66	62.54 ± 9.45

Jurkat

HA (μl/ml)	0	1.25	2.5	5.0	7.5
Apoptosis (%)	9.95 ± 2.59	10.32 ± 2.71	13.47 ± 2.93	21.18 ± 0.85	27.57 ± 1.42
MTT (%)	100.00 ± 0.00	96.08 ± 3.75	81.02 ± 9.02	67.83 ± 7.37	36.18 ± 4.22

Fig. 2. Heme arginate inhibits HIV-1 growth during acute infection. (A and B) 0.2×10^6/ml of A3.01 or Jurkat cells were infected or mock-infected with HIV-1 and grown in 24-well plates in the presence of indicated concentrations of heme arginate (HA). The concentrations 1.25 and 2.5 μl/ml of HA correspond to 31.25 and 62.5 μg/ml of hemin or 48 and 96 μM hemin, respectively. At indicated days after infection (p.i.), aliquots of culture supernatants and cells were collected for further analysis, the cells were split 1:4, and supplemented with fresh medium and HA. (A) p24 antigen in culture supernatants determined by ELISA. (B) Apoptosis of the cells characterized by flow cytometry of cells fixed with 1% paraformaldehyde. Graphs (A and B) represent mean of two experiments performed in duplicates ±SD (C) Cytotoxicity of heme arginate. 0.06×10^6/ml of A3.01 and Jurkat cells were treated with increasing concentrations of HA. After 2 days of incubation, apoptosis and cytotoxicity were characterized using flow cytometry and the MTT assay, respectively. The results represent means of three (A3.01) or two experiments (Jurkat) performed in duplicates ±SD.

concentrations of HA used were selected based on preliminary experiments and on the estimated distribution volume of HA *in vivo*. At day 4 p.i., both 2.5 and 1.25 μl/ml of HA decreased the levels of p24 antigen in the culture supernatants to about half of the levels of the untreated controls in both cell lines. At later time points, the concentration of HA 2.5 μl/ml kept the levels of p24 antigen very low, close to the detection limit of the assay; the concentration of HA 1.25 μl/ml decreased the levels of the p24 antigen significantly also, with an increase in p24 antigen levels at days 10 and 13 p.i.

In an additional series of experiments, we determined the viability of HIV-infected and mock-infected cells in the presence of 1.25 and 2.5 μl/ml of HA during the time course experiment. As shown in Fig. 2B, cell viability determined by the analysis of a FSC-A × SSC-A dot plot decreased only in HIV-infected, untreated cells. In contrast, both HA-treated infected and mock-infected cells revealed a viability comparable to untreated mock-infected cells up to the 13 days p.i.

Finally, we characterized the effects of HA on T-cell viability, growth, and cytotoxicity in actively dividing A3.01 and Jurkat cells during a 48 h experiment, comparing flow cytometry and the MTT assay (Fig. 2C). Percentage of apoptotic cells was determined by analysis of a FSC-A × SSC-A dot plot. The cells were also analyzed after labeling with Hoechst 33342 and 7-AAD, yielding similar results (data not shown). It can be observed that the concentrations of HA 1.25 and 2.5 μl/ml that inhibit HIV-1 growth do not induce any increased apoptosis of A3.01 cells, while 2.5 μl/ml of HA increased apoptosis of Jurkat cells somewhat. Cytotoxicity and growth inhibitory properties of HA were characterized by activity of mitochondrial dehydrogenases using the MTT assay. 1.25 μl/ml

of HA did not induce any significant decrease of this activity, while 2.5 μl/ml of HA somewhat decreased it in both cell lines. Based on flow cytometry assays, CC50 was determined as 42 and 17 μl/ml of HA (1612 and 636 μM hemin) in A3.01 and Jurkat cells, respectively; based on MTT test, CC50 was determined as 10.7 and 6.4 μl/ml of HA (412 and 244 μM hemin) in A3.01 and Jurkat cells, respectively.

3.2. Heme arginate inhibits reverse transcription of HIV-1

It has been previously published that heme inhibited activity of reverse transcriptase (Argyris et al., 2001; Levere et al., 1991; Staudinger et al., 1996). Therefore we also tested the effects of HA on reverse transcription as presented in Fig. 3. The results of PCR performed on DNA isolated at 48 h after infection using primers specific for HIV LTR/gag demonstrate the inhibitory effects of HA on levels of reverse transcripts that were comparable to those of AZT. On the other hand, levels of a house-keeping gene GAPDH were found comparable in all samples.

3.3. Heme arginate potentiates PMA-stimulated reactivation of the HIV-1 provirus in ACH-2 cells

In contrast to reverse transcription, the effect of heme or hemin on reactivation of the HIV-1 provirus has not been previously studied. Therefore, we first determined the effects of HA on the stimulation of ACH-2 cells harboring an integrated HIV-1 provirus with PMA. Unexpectedly, results presented in Fig. 4 indicated that HA dose-dependently increased reactivation of the provirus in PMA-stimulated ACH-2 cells. In western blot analysis of the cells (Fig. 4A), levels of the p24 antigen as well as of p55, its precursor, were increased at 24 h after induction with PMA in the presence of HA. Similarly in ELISA analysis of culture supernatants, levels of the p24 antigen that reflect the p24 antigen and virions released from the cells (Fig. 4B) were increased at 24 h after induction, in dependence on the levels of HA. On the hand, HA alone was not found to stimulate reactivation of the HIV-1 provirus at any concentration tested (data not shown).

3.4. Heme arginate reactivates the latent HIV-1 "mini-virus" in clones of Jurkat cells

In order to confirm the stimulatory effects of HA on the reactivation of the latent provirus, we have used two clones of Jurkat cells harboring HIV-1 "mini-virus" consisting of the HIV-1 LTR-Tat-IRES-EGFP-LTR. The two clones were previously shown to differentially express EGFP and to contain different DNA modifications in the promoter region (Blazkova et al., 2009; Jordan et al., 2003). In agreement with the results in ACH-2 cells, western blot analysis of EGFP (Fig. 5A) revealed a stimulatory effect of HA on EGFP expression in PMA-stimulated A2 and H12 Jurkat cells. The effect of HA alone on EGFP expression was also stimulatory, albeit weaker than that in combination with PMA. In both experiments,

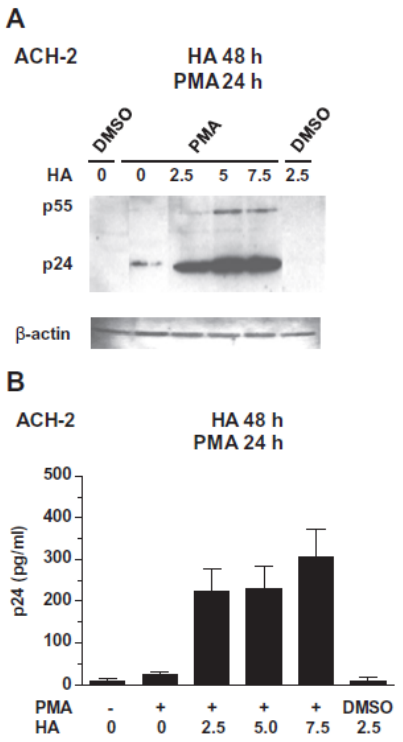


Fig. 4. HA potentiates PMA-stimulated reactivation of the HIV-1 provirus in ACH-2 cells. 0.5×10^6/ml of ACH-2 cells harboring an integrated HIV-1 provirus were pre-treated with increasing concentrations of HA for 24 h, and then stimulated with phorbol myristate acetate (PMA; final concentration 0.5 ng/ml) in the presence of HA. 24 h after stimulation, aliquots of culture supernatants and cells were collected for further analysis. (A) Western blot analysis of p24 antigen in the cells. The cells were lysed in Laemmli reducing sample buffer, and resolved by SDS–PAGE. Levels of p24, p55 and β-actin were characterized by western blot analysis and chemiluminescence, using a mouse monoclonal antibody ND-1 against p24 and a goat polyclonal antibody against β-actin, respectively. Representative results of two independent experiments. (B) p24 antigen in culture supernatants. Levels of p24 antigen were determined by ELISA. Graphs represent mean of two independent experiments performed in duplicates ± SD.

higher concentrations of HA (2.5 μl of HA/ml and higher) were cytotoxic, as indicated by decreased levels of the house-keeping gene β-actin.

The effects of HA and PMA on the expression of EGFP were also studied using flow cytometry (Fig. 5B, Supplementary data Table S1) and confirmed the results of western blot analysis. HA alone as well as in combination with PMA dose-dependently stimulated the expression of EGFP. However, H12 cells revealed a

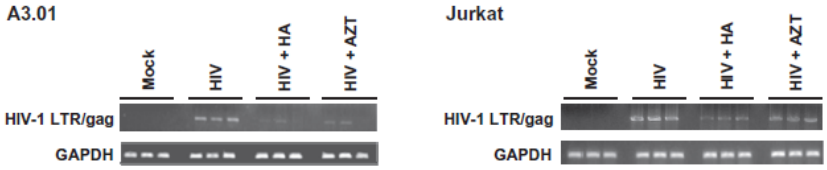


Fig. 3. HA inhibits reverse transcription of HIV-1. 0.5×10^6/ml of A3.01 or Jurkat cells were mock-infected or infected with a DNase-treated inoculum of HIV-1, and grown in 24-well plates in the presence of 2.5 μl/ml of HA or 10 μM AZT. 48 h after infection, the cells were collected and total DNA was isolated. HIV-1 reverse transcripts were determined by PCR using primers specific for HIV-1 LTR/gag and compared with control PCR detecting GAPDH. Representative results of two independent experiments performed in triplicates.

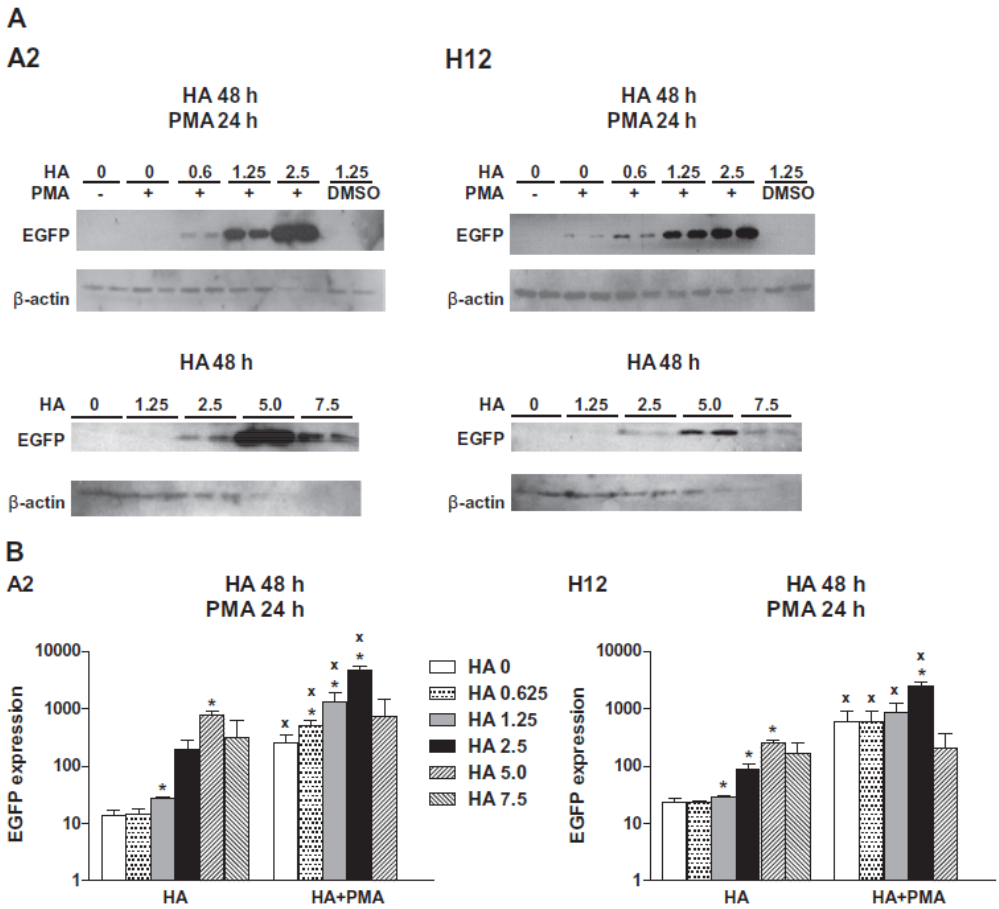


Fig. 5. HA reactivates the latent HIV-1 'mini-virus' and expression of EGFP. 0.5×10^6/ml (A) or 0.1×10^6/200 μl (B) of Jurkat cell clones A2 and H12 latently infected with a "mini-virus" containing the HIV-1 LTR-Tat-IRES-EGFP-LTR were pre-treated with increasing concentrations of HA for 24 h, and then stimulated with PMA (final concentration 0.5 ng/ml) or mock-treated in the presence of HA. Twenty four hours after stimulation, the cells were collected and used for further analysis. (A) Western blot analysis of EGFP in the cells. The cells were lysed in Laemmli reducing sample buffer, and resolved by SDS–PAGE. Levels of EGFP and β-actin were characterized by western blot analysis and chemiluminescence, using a rabbit polyclonal antibody against EGFP and a goat polyclonal antibody against β-actin, respectively. Representative results of two independent experiments. (B) Flow cytometric analysis of EGFP expression. The cells were analyzed by flow cytometry in FL1. The graphs show a quantification of EGFP expression calculated as the arithmetic mean of green fluorescence of green cell population × percentage of all EGFP-positive cells. *Increase in EGFP expression is statistically significant when compared to HA 0 in each treatment ($p < 0.05$). ×, Increase in EGFP expression in the presence of PMA is statistically significant when compared to the same concentration of HA without PMA ($p < 0.05$).

higher background expression of EGFP than A2 cells. Again, the increased expression of EGFP inversely correlated with cell viability, with a significant increase of apoptosis at concentrations of HA 2.5 μl/ml and higher.

3.5. Heme arginate with PMA stimulate expression of heme-oxygenase-1 in ACH-2 cells and the stimulatory effects on reactivation of the HIV-1 provirus can be inhibited by N-acetyl cysteine

Heme and hemin are well-established inducers of heme oxygenase-1 (HO-1; Maines et al., 1986; Wu and Wang, 2005), the enzyme degrading heme into carbon monooxide, biliverdin and Fe^{2+} (Tenhunen et al., 1969). The release of Fe^{2+} would catalyze production of the hydroxyl radical (Kruszewski, 2003), thus possibly leading to activation of the transcription factor NF-κB and reactivation of the HIV-1 provirus. Therefore, we have first determined the expression of HO-1 in ACH-2 cells. As demonstrated in Fig. 6A, HA induced a dose-dependent increase in HO-1 levels in the presence of PMA, i.e. under the conditions leading to the reactivation of HIV-1 provirus, while untreated cells revealed low background levels of HO-1 that were not affected by PMA alone. Consequently, we pretreated the cells with an anti-oxidative agent *N*-acetyl cysteine (NAC), precursor of the reduced glutathione (GSH). As shown in Fig. 6B, NAC decreased reactivation of the provirus in HA-pretreated, PMA-stimulated ACH-2 cells, as characterized by levels of the p24 antigen in the cells using western blot analysis. Additionally, it can be observed that NAC also decreased expression of the p24 antigen in cells treated with PMA only. On the other hand, ELISA analysis of culture supernatants (Fig. 6C) revealed that pretreatment with NAC decreased the levels of p24 antigen released

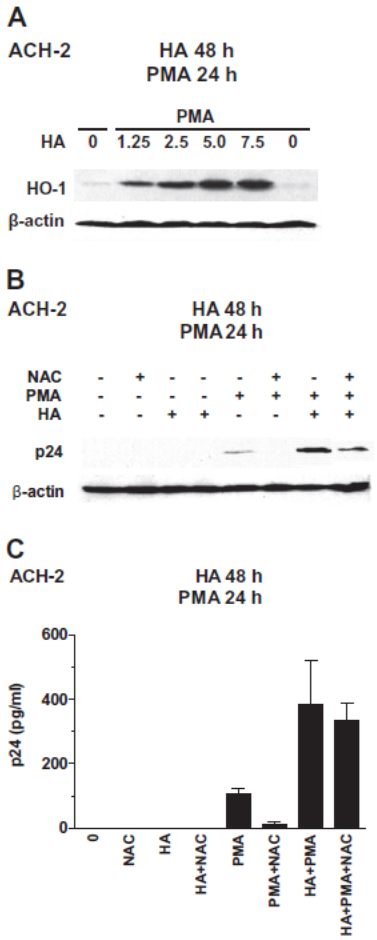


Fig. 6. (A) HA with PMA stimulate expression of heme oxygenase-1 in ACH-2 cells. 0.5×10^6/ml of ACH-2 cells harboring an integrated HIV-1 provirus were pre-treated with increasing concentrations of HA for 24 h, and then stimulated with PMA (final concentration 0.5 ng/ml) in the presence of HA. 24 h after stimulation, the cells were collected, lysed in Laemmli reducing sample buffer, and resolved by SDS–PAGE. Levels of HO-1 and β-actin were characterized by western blot analysis and chemiluminescence, using a rabbit polyclonal antibody against HO-1 and a rabbit polyclonal antibody against β-actin, respectively. (B) *N*-acetyl cysteine prevents reactivation of the HIV-1 provirus. 0.5×10^6/ml of ACH-2 cells were pre-treated with *N*-acetyl cysteine (NAC; final concentration 5 mM) for 4 h, treated with 5 μl/ml of HA for 24 h, and then stimulated with PMA in the presence of HA and/or NAC. Levels of p24 and β-actin were characterized by western blot analysis and chemiluminescence, using a mouse monoclonal antibody ND-1 against p24 and a rabbit polyclonal antibody against β-actin, respectively. (C) Levels of p24 antigen in culture supernatants were determined by ELISA. Representative results of two independent experiments ±SD.

by PMA-stimulated ACH-2 cells, while it was not sufficient to significantly decrease p24 release by HA-pretreated, PMA-stimulated cells.

3.6. Expression of heme-oxygenase-1 in clones of Jurkat cells and the effects of tin protoporphyrin IX and N-acetyl cysteine

We have also studied the levels of HO-1 in A2 and H12 Jurkat cells. In these cells, HO-1 was found expressed already in untreated cells and the addition of either HA or HA and PMA did not increase its levels (Fig. 7A and data not shown). On the contrary, increasing concentrations of HA led to a decrease of HO-1 levels in A2 and H12 cells, in parallel with a cytotoxic effect of HA demonstrated by decreasing levels of β-actin. Consequently, we explored the effect of NAC in these cells. Similarly to the effects observed in ACH-2 cells, pretreatment with NAC decreased the levels of EGFP in A2 and H12 cells treated with both HA and PMA, as well as in cells treated with PMA only (Fig. 7B; expression of EGFP induced by HA only could be observed in longer exposures). Finally, we studied the effect of an inhibitor of HO-1, tin protoporphyrin IX (SnPP; Devadas and Dhawan, 2006). SnPP strongly stimulated expression of EGFP in cells treated with HA alone (Fig. 7C); it also somewhat increased levels of EGFP in HA- and PMA-treated cells, while it did not affect or somewhat decreased the levels of EGFP in PMA-stimulated cells. On the other hand, SnPP alone did not stimulate any expression of EGFP in untreated cells.

The effects of SnPP and NAC on the expression of EGFP were further studied using flow cytometry (Fig. 7D, Supplementary data Table S2), providing a more quantitative assessment of EGFP expression. The results revealed similar tendencies as the western blot analysis. Additionally, SnPP seemed to decrease basal expression of EGFP in otherwise untreated A2 cells, while it did not affect it in untreated H12 cells. On the other hand, NAC did not affect expression of EGFP in untreated A2 cells, while it decreased it in untreated H12 cells. Also, NAC decreased expression of EGFP stimulated by all the combinations of HA, SnPP and PMA, suggesting that these effects were mediated by an increased redox stress. It should be also noted that in contrast to A2 cells, the H12 cells reveal a higher background expression of EGFP in untreated cells, and in general respond with a smaller fold-increase than A2 cells. Finally, heme arginate decreased the cell viability somewhat, while SnPP with HA decreased it relatively more. In parallel with the effects on EGFP expression, NAC restored the cell viability in all cases.

3.7. Comparison of the effects of heme arginate with other HIV-1 reactivating agents

Finally, we have compared the stimulatory effects of HA with the effects of several other inducers of HIV-1 reactivation during a 48 h experiment. As shown in Fig. 8A, even 5 μl of HA/ml did not stimulate reactivation of HIV-1 in ACH-2 cells, as characterized by western blot analysis of the p24 Ag, while a 48 h treatment led to a comparable increase in expression of p24 Ag in cells stimulated with PMA only as well as with PMA and HA. Stimulation of the cells with 10 U/ml of TNF-α led to an even higher expression of p24 Ag, while 1 U/ml induced a relatively smaller expression of p24 Ag. On the other hand, any concentration of phytohemagglutinin A tested (PHA; 0.5, 2.5; 5 μg/ml) alone or in combination with 1 μM ionomycine did not yield a positive signal of p24 Ag in western blot analysis (Fig. 8A and data not shown).

ELISA analysis of culture supernatants revealed similar changes in levels of the p24 antigen as the western blot analysis (Fig. 8B). However, it is obvious that the overall release of p24 by ACH-2 cells stimulated with PMA for 48 h was stronger than by ACH-2 cells stimulated with PMA and HA for the same time period. This effect is possibly due to the death of the PMA- and HA-stimulated cells or to the inhibitory effects of CO and bilirubin on HIV-1 reactivation as discussed below.

The same stimulatory agents were also used for treatment of A2 and H12 cells for 48 h. As shown in Fig. 8C, expression of EGFP was stimulated with HA alone weakly in both cells, very strongly with PMA and even more strongly with PMA and HA. The stimulation with 10 U/ml of TNF-α or 0.5–1 μg/ml PHA was comparable to the effect of PMA, while the stimulation with 1 U/ml TNF-α induced a relatively weaker expression of EGFP. It can be observed that the effect of 1 U/ml TNF-α was comparable to the effect of HA (2.5 μl/ml) in H12 cells, while it was stronger in A2 cells.

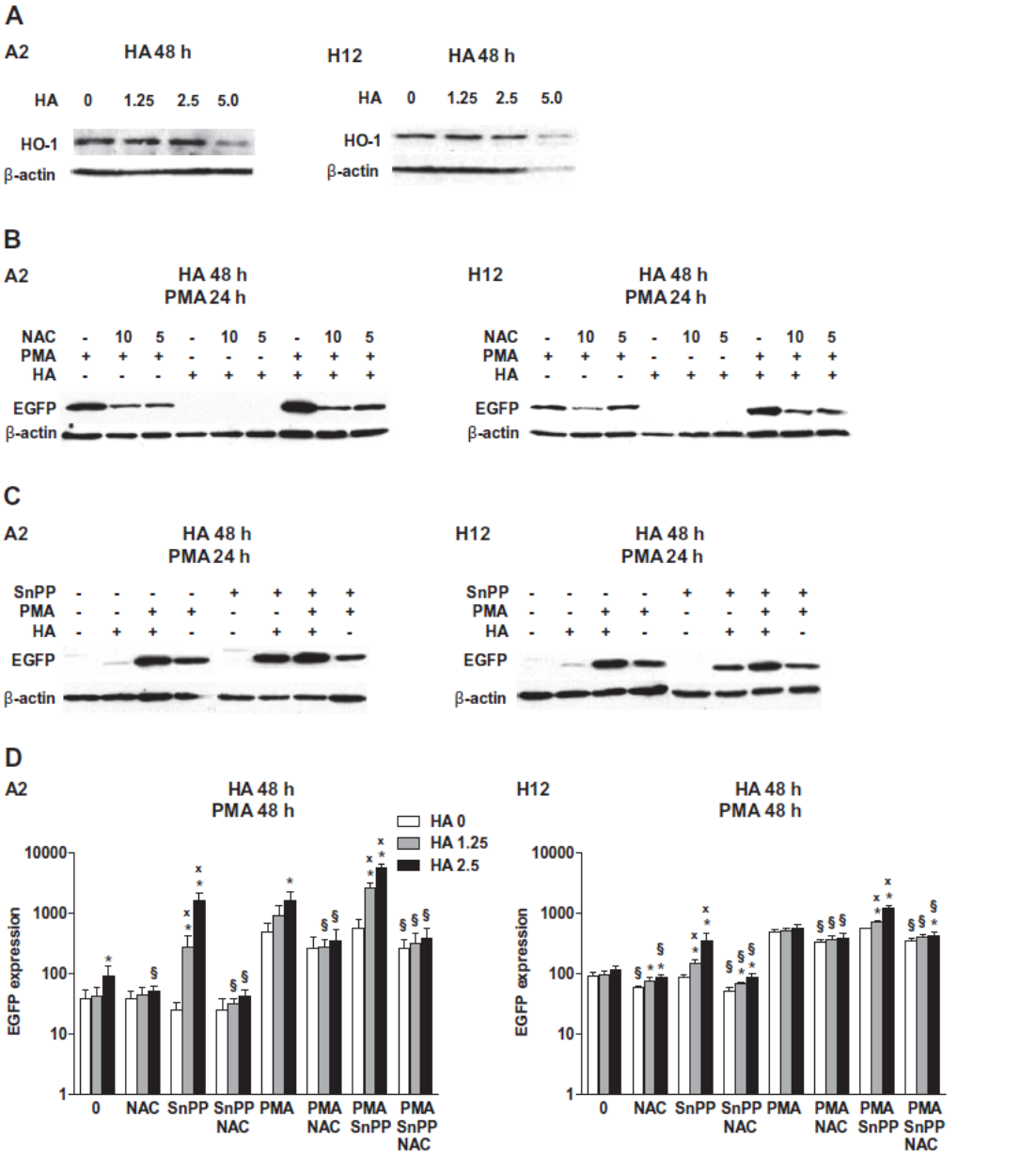


Fig. 7. (A) Expression of heme oxygenase-1 in A2 and H12 cells. 0.5×10^6/ml of Jurkat cell clones A2 and H12 latently infected with a "mini-virus" containing the HIV-1 LTR-Tat-IRES-EGFP-LTR were treated with increasing concentrations of HA for 48 h. The cells were collected, lysed in Laemmli reducing sample buffer, and resolved by SDS–PAGE. Levels of HO-1 and β-actin were characterized by western blot analysis and chemiluminescence, using a rabbit polyclonal antibody against HO-1 and a rabbit polyclonal antibody against β-actin, respectively. (B) *N*-acetyl cysteine prevents reactivation of HIV-1 'mini-virus' and expression of EGFP. 0.5×10^6/ml of A2 and H12 cells were pre-treated with *N*-acetyl cysteine (NAC; final concentrations 5 and 10 mM) for 4 h and treated with 2.5 μl/ml of HA in the presence or absence of PMA (final concentration 0.5 ng/ml) for 48 h. Levels of EGFP and β-actin were characterized by western blot analysis and chemiluminescence, using a rabbit polyclonal antibody against EGFP and a rabbit polyclonal antibody against β-actin, respectively. The levels of EGFP expressed in the absence of HA were visible only in longer exposures. Representative results of two independent experiments. (C) Effect of SnPP on reactivation of HIV-1 'mini-virus' and expression of EGFP. 0.5×10^6/ml of A2 and H12 cells were pre-treated with SnPP (final concentration 6.25 μM) for 30–45 min and treated with 2.5 μl/ml of HA in the presence or absence of PMA (final concentration 0.5 ng/ml) for 48 h. Levels of EGFP and β-actin were characterized by western blot analysis and chemiluminescence, using a rabbit polyclonal antibody against EGFP and a rabbit polyclonal antibody against β-actin, respectively. Representative results of two independent experiments. (D) Flow cytometric analysis of EGFP expression. 0.1×10^6/200 μl of A2 and H12 cells were pre-treated with *N*-acetyl cysteine (NAC; final concentrations 5 mM) for 4 h, then with SnPP (final concentration 6.25 μM) for 30–45 min, and treated with 1.25 or 2.5 μl/ml of HA in the presence or absence of PMA (final concentration 0.5 ng/ml) for 48 h. The cells were analyzed by flow cytometry in FL1. The graphs show a quantification of EGFP expression calculated as the arithmetic mean of green fluorescence of green cell population × percentage of all EGFP-positive cells. *Increase in EGFP expression is statistically significant when compared to HA 0 in each treatment ($p < 0.05$). ×, Increase in EGFP expression in the presence of SnPP is statistically significant when compared to the same treatment without SnPP ($p < 0.05$). §Decrease in EGFP expression in the presence of NAC is statistically significant when compared to the same treatment without NAC ($p < 0.05$).

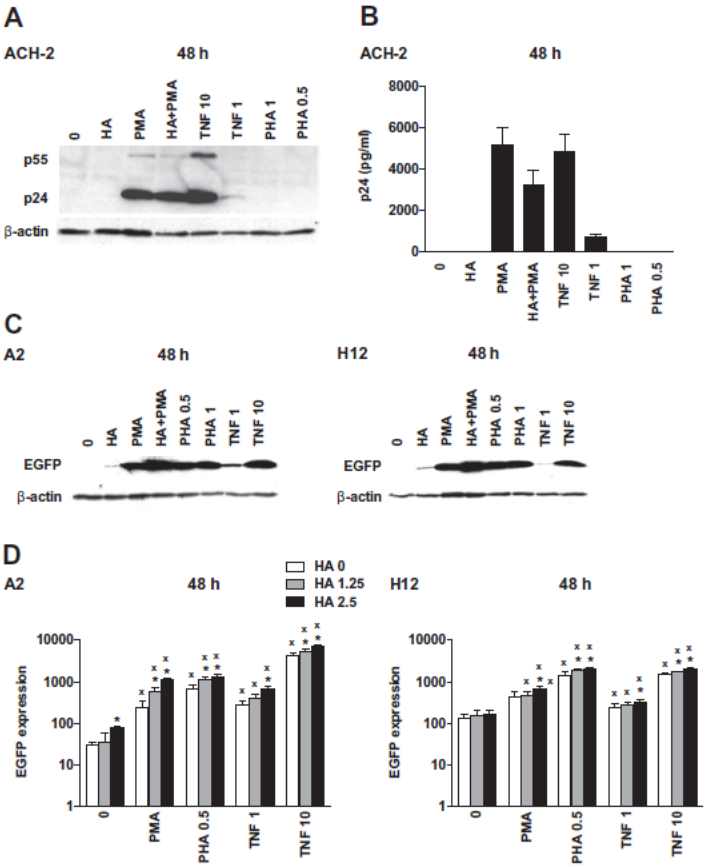


Fig. 8. Effects of TNF-α and phytohemagglutinin. (A and B) ACH-2 cells. 0.5×10^6/ml of ACH-2 cells harboring an integrated HIV-1 provirus were treated with 5 μl/ml of HA, PMA (final concentration 0.5 ng/ml), HA and PMA, TNF-α (final concentration 1 and 10 U/ml) or phytohemagglutinin (PHA; final concentration 0.5 and 1 μg/ml). 48 h after the treatment, cells were collected and used for further analysis. (A) p24 antigen in the cells. The cells were lysed in Laemmli reducing sample buffer, and resolved by SDS–PAGE. Levels of p24, p55 and β-actin were characterized by western blot analysis and chemiluminescence, using a mouse monoclonal antibody ND-1 against p24 and a rabbit polyclonal antibody against β-actin, respectively. (B) p24 antigen in culture supernatant. Levels of p24 antigen in culture supernatants were determined by ELISA. Results of two independent experiments ±SD. (C and D) A2 and H12 cells. 0.5×10^6/ml (C) or 0.1×10^6/200 μl (D) of A2 and H12 cells were treated with 1.25 μl/ml (D) or 2.5 μl/ml (C–E) of HA, PMA (final concentration 0.5 ng/ml), HA and PMA, PHA (final concentration 0.5 and 1 μg/ml) or TNF-α (final concentration 1 and 10 U/ml). 48 h after the treatment, cells were collected and used for further analysis. (C) western blot analysis of EGFP in the cells. The cells were lysed in Laemmli reducing sample buffer, and resolved by SDS–PAGE. Levels of EGFP and β-actin were characterized by western blot analysis and chemiluminescence, using a rabbit polyclonal antibody against EGFP and a rabbit polyclonal antibody against β-actin, respectively. Representative results of two independent experiments. (D) Flow cytometric analysis of EGFP expression. The cells were analyzed by flow cytometry in FL1. The graphs show a quantification of EGFP expression calculated as the arithmetic mean of green fluorescence of green cell population × percentage of all EGFP-positive cells. *Increase in EGFP expression is statistically significant when compared to HA 0 in each treatment ($p < 0.05$). ×, Increase in EGFP expression in the presence of the stimulatory agent is statistically significant when compared to the same concentration of HA without the agent ($p < 0.05$).

The stimulatory effects of individual agents on the expression of EGFP were also studied using flow cytometry (Fig. 8D, Supplementary data Table S3). Again, these results reveal similar tendencies as western blot analysis, but as mentioned above, H12 cells reveal a higher background expression of EGFP in untreated cells than A2 cells, and in general respond with a smaller fold-increase than A2 cells. Based on various criteria used in this analysis, it can be concluded that A2 cells are more responsive to TNF-α than H12 cells. When analyzing the cell viability, neither PMA nor TNF-α alone or in combination with HA were found to decrease it. On the other hand, PHA reduced cell viability relatively strongly.

3.8. Heme arginate does not affect expression of CD69 in A3.01 cells

In addition to the previous studies, we have explored the ability of T-cells to get activated by PMA in the presence of HA. The A3.01 cells were stimulated with PMA and expression of CD69 on the cell surface was determined. In these assays, HA revealed no negative effects on the T-cell activation characterized by this activation marker at any concentration of PMA tested (1 and 10 ng/ml; data not shown), especially not even at the lowest concentration used throughout the experiments (0.5 ng/ml; Fig. 9). Importantly, HA alone did not stimulate any increased expression of CD69 on the cell surface either.

4. Discussion

In this paper, we demonstrate the overall inhibitory effects of heme arginate on HIV-1 replication in T-cell lines that were accompanied by the inhibition of reverse transcription, while we show that HA alone stimulated the reactivation of HIV-1 "mini-virus" and synergized with PMA or TNF-α in the reactivation of HIV-1

provirus. To our knowledge, this is the first work demonstrating the stimulatory effect of hemin on reactivation of the latent provirus.

Heme has been previously shown to inhibit replication of HIV-1 (Levere et al., 1991), specifically reverse transcriptase (Argyris et al., 2001). Further, heme derivative hemin has been demonstrated to inhibit HIV-1 growth in human PBMC-reconstituted NOD-SCID mice and to induce a dose-dependent inhibition of HIV-1 replication in tissue culture during a 7-day long infection (Devadas and Dhawan, 2006). Accordingly, we showed here the inhibitory effects of HA on HIV-1 replication and reverse transcription in acutely infected cells, characterized by levels of p24 and reverse transcripts, respectively.

Devadas and Dhawan (2006) also found hemin to induce expression of HO-1, and the inhibitory effects of hemin on HIV-1 replication could be reversed by certain concentrations of SnPP, the inhibitor of HO-1. Based on these results, it would be possible to conclude that the inhibition of HIV-1 growth was mediated by the action of HO-1. We also observed here a HA-induced expression of HO-1 in ACH-2 cells, while its levels were already increased in untreated A2 and H12 cells. However simultaneously, we observed HA-induced stimulatory effects on HIV-1 provirus and "mini-virus" reactivation in ACH-2 and A2, H12 cells, respectively. HA stimulated HIV-1 provirus reactivation in synergy with PMA or TNF-α, while it acted alone and/or in synergy with the two agents in A2 and H12 cells. Further, the effects of HA in A2 and H12 cells were increased by the addition of SnPP, the inhibitor of HO-1, and all the stimulatory effects could be inhibited by NAC. Thus based on our results, it can be suggested that in the experiments of Devadas and Dhawan (2006), the inhibitory effects of hemin on HIV-1 replication were in fact over-ridden by the increased redox stress due to inhibition of HO-1 by SnPP and the resulting increase in expression of the provirus.

Heme and hemin differ in the oxidation state of iron in the two compounds; they contain Fe^{2+} and Fe^{3+}, respectively. In the organism, heme is mostly bound as a prosthetic group in various heme proteins. In the presence of various oxidizing agents, the heme moiety is oxidized to hemin, while the oxidized heme proteins as well as the free hemin readily undergo reduction driven by CO, both in biological systems and *in vitro* (Bickar et al., 1984). Changes in the oxidation state of iron in heme moiety are also mediated by another ubiquitously present gas signaling agent, nitric oxide (Ascenzi et al., 2010; Kilbourn et al., 1994). It is thus impossible to strictly separate the effects of heme and hemin as their mutual balance is dynamically regulated. On the other hand, only heme can serve as a substrate of HO-1. As a hydrophobic compound, hemin inserts into plasma membranes and translocates inside the cells. Inside the cells, the free iron is released namely by the action of heme oxygenases, hydrogen peroxide or other non-specific degradation (Belcher et al., 2010), leading to the generation of the hydroxyl radical (Kruszewski, 2003) and activation of the redox-sensitive transcription factor NF-κB (Lander et al., 1993; Pantano et al., 2006). Heme also regulates levels and targeting of key enzymes involved in heme synthesis and degradation, non-specific synthase of 5-aminolevulinic acid (ALAS1), HO-1, and of oxidative stress response genes (Furuyama et al., 2007; Igarashi and Sun, 2006; Mense and Zhang, 2006).

In the time-course experiments presented in this paper, HA inhibited HIV-1 replication characterized by levels of p24 Ag. In similar time-course experiments, viability of the mock-infected and infected cells in the presence of HA was found comparable to the untreated mock-infected cells, while untreated infected cells succumbed to apoptosis. A long-term culture of the cells in the presence of HA in concentrations that inhibited HIV-1 replication did not therefore negatively affect cell growth and viability; on the contrary, HA protected the infected cells from dying. We cannot, though, exclude a possibility that a selection of HA-resistant cells could take place.

In contrast to the acutely infected cells, HA revealed stimulatory effects on HIV-1 provirus and "mini-virus" reactivation in ACH-2 and A2, H12 cells, respectively. In A2 and H12 cells, HA stimulated "mini-virus" reactivation even by itself, but its effects were much weaker than the effects of PMA, PHA, or TNF-α alone or in combination with HA. The overall EGFP expression as well as percentage of EGFP-positive cells were dose-dependent in all agents. During a 48 h-incubation period, stimulatory effects of HA and TNF-α were more or less comparable to HA and PMA in H12 cells, while A2 cells appeared to be more responsive to TNF-α (Fig. 8D). Both cell lines seemed to respond similarly to PHA. H12 cells revealed a higher background fluorescence of untreated cells than A2 cells, similarly to the published data (Blazkova et al., 2009), but in general, they responded to the individual inducers with a smaller fold-increase

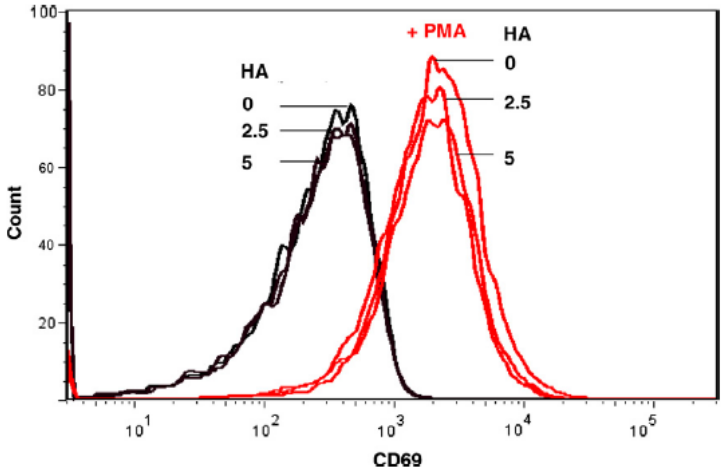


Fig. 9. Heme arginate does not affect expression of CD69 in A3.01 cells. 0.5×10^6/ml of A3.01 cells were treated with increasing concentrations of HA in the presence or absence of PMA (final concentration 0.5 ng/ml). At 24 h after stimulation, the cells were collected and stained with a mouse monoclonal antibody against human CD69 labeled with AlexaFluor-700 (dilution 1:50) followed by flow cytometry analysis. Representative results of two independent experiments performed in duplicates.

than A2 cells. Perhaps, the lower responsiveness of H12 cells might be due to a somewhat higher CpG methylation of the 5′ LTR region compared to A2 cells (Blazkova et al., 2009).

The observed effects of PMA on the HIV-1 provirus reactivation in ACH-2 cells were biphasic, possibly due to a low concentration of PMA used. During a 24 h-treatment, PMA stimulated the provirus reactivation only weakly, while a 48 h-treatment induced a 10-fold increase in the levels of p24 compared to a 24 h-treatment. Apparently, PMA was inducing the provirus reactivation indirectly. It seems to induce expression and/or activity of certain factors that in turn mediate reactivation of the provirus. Phorbol esters mimic the action of diacyl glycerols (DAG), activators of protein kinase C family proteins (PKC) and of several non-PKC targets. In addition to DAG or phorbolester, the full activation of PKC's requires also Ca^{2+} and acidic phospholipids, leading to a synergistic activation of two different ligand binding domains and to the appropriate membrane targeting (Brose and Rosenmund, 2002; Goel et al., 2007). PKC was also found to mediate expression of HO-1 stimulated by PMA or LPS (Devadas et al., 2010; Naidu et al., 2008). The effects of PMA in ACH-2 cells could be greatly potentiated with HA during a 24 h-treatment (Figs. 4 and 6). Possibly, HA could synergize with PMA by changing levels of cytoplasmic Ca^{2+}, membrane targeting of PKC's or by increasing the redox stress and changing the properties of zinc-finger-like repeats in C1 domain involved in PMA binding to its targets. Heme and PMA were independently shown to affect also other signal transduction pathways, e.g. Ras and MAPK, increasing chances for their synergistic action (Mense and Zhang, 2006; Sacks, 2006).

The exact mechanism of stimulation of HIV-1 reactivation by HA remains to be established, but a mechanism involving induction and/or activity of HO-1 along with release of Fe^{2+}, increased redox stress and activation of the redox-sensitive transcription factor NF-κB can be suggested (Belcher et al., 2010; Devadas and Dhawan, 2006; Kruszewski, 2003; Lander et al., 1993; Morse et al., 2009; Pantano et al., 2006). Our results indicate a HA-induced expression of HO-1 in ACH-2 cells, while HO-1 was found present already in untreated A2 and H12 cells. In all cell lines, LTR-driven expression could be inhibited by pretreatment of the cells with NAC, precursor of the potent anti-oxidant, GSH, suggesting that the effect of HA involved an increased redox stress. In fact, we have also detected increased production of free radicals by A3.01 and Jurkat cells in the presence of HA or PMA (unpublished results). Additionally, we have tested the effect of the inhibitor of HO-1, SnPP, in A2 and H12 cells. While SnPP was not found to affect basal expression of EGFP in either cell line, it strongly stimulated this expression in the presence of HA in both A2 and H12 cells. Most probably, EGFP expression could be stimulated by an increased redox stress imposed by HA that could not be counteracted by the anti-oxidative effects of HO-1 because of its inhibition by SnPP. Alternatively, electron transfer between the two porphyrin species and generation of ROS could take place. Again, the stimulatory effects of SnPP and HA on LTR-driven expression were inhibited by NAC.

The other two products of heme oxygenases, CO and biliverdin, further converted to bilirubin, reveal strong antioxidant and cytoprotective properties (Morse et al., 2009). The effect of HA addition thus can be shortly pro-oxidative and then anti-oxidative for a prolonged period of time. Thus, upon a massive induction of expression of HO-1 stimulated by HA and PMA in ACH-2 cells, the anti-oxidative effects could eventually prevail, and inhibit provirus reactivation during a longer incubation in the presence of HA, as suggested by the results presented in Fig. 8A and B. The situation seems to be different in A2 and H12 cells in which HO-1 was found expressed already in untreated cells and its levels were not further increased by any treatment; HO-1 thus could start to effectively degrade HA immediately after its addition. Apparently, the kinetics and balance between the pro-oxidative and anti-oxidative effects of HO-1 products might be different in these cells.

We have used A2 and H12 cells (Blazkova et al., 2009; Jordan et al., 2003) to characterize the effects of HA on LTR-driven expression, comparing western blot analysis detecting levels of EGFP and flow cytometry detecting fluorescence of EGFP. The flow cytometry results underestimate the numbers of EGFP-positive cells and/or levels of EGFP expressed, as high levels of EGFP are cytotoxic and dead cells loose EGFP fluorescence. Nevertheless, we assessed the overall expression of EGFP by the number of all EGFP-positive cells × arithmetic mean of green fluorescence of the green cell population. Using this approximation, the levels of EGFP expression were found increased even by treatment with 1.25 μl/ml of HA in most experiments, corresponding to the results of western blot analysis. The percentage of green (EGFP-positive) cells in samples treated with 1.25 μl/ml of HA used to be lower than in untreated cells, while the arithmetic mean and median of green fluorescence of all green and live green cells, respectively, were always higher. In higher concentrations of HA, as well as in other stimulatory treatments, all values were higher than in controls. In general in A2 and H12 cells, HA alone or in combination with other stimulatory agents increased LTR-driven EGFP expression as well as cell death. These tendencies seemed to be similar in ACH-2 cells. However, a long term incubation of A3.01 and Jurkat cells with HA did not significantly increase cell death. It is thus possible that the cytotoxicity of HA might be further increased due to expression of HIV or EGFP. In fact, it would be of advantage if latently infected cells were more prone to cell death induced by HA alone or in combinations. There might be several mechanisms involved in cell death induced by HA: first, a direct increase in ROS production due to a higher availability of heme and iron; second, an indirect cytotoxicity of HA that would further increase ROS production and HIV reactivation; third, the resulting increase in HIV reactivation would lead to the cell death. The excess of oxygen free radicals induces oxidation of proteins, lipids, lipoproteins, nucleic acids, carbohydrates and other cellular or viral targets. Thus, HA might not only stimulate expression of the provirus, but also affect the viability and infectivity of the released virions. A similar inhibition of HIV-1 by reactive oxygen species was indeed shown in the case of bleomycin (Georgiou et al., 2004).

Heme oxygenase has been suggested to exert various immunoregulatory effects on innate and adaptive immune cells, and to inhibit pathogenesis of several immune-mediated inflammatory diseases (Soares et al., 2009). Further, analysis of HO-1 promoter polymorphism revealed that Caucasian HIV-1-infected patients who maintain low levels of immune activation and control HIV-1 viral loads to undetectable levels are more likely to possess a specific microsatellite $(GT)_n$ repeat and two single nucleotide polymorphisms in HO-1 promoter region that favor enhanced HO-1 gene expression (Seu et al., 2009).

The ability of cells to become activated remained unaffected by HA as demonstrated by expression of the early activation marker CD69, characterized by flow cytometry. Since the activation of T-cells constitutes an essential component of immune responses to the virus itself as well as to other infections, we consider the finding that HA does not seem to generally decrease the activation of T-cells as important. Moreover, HA did not induce any global activation of T-cells either; this finding is significant as well, since a non-specific T-cell activation and release of proinflammatory cytokines should be avoided. The effect of HA thus could be compared to the effect of 5-hydroxynaphthalene-1,4-dione, a compound recently described to reactivate the latent provirus without cellular activation (Yang et al., 2009).

In vivo, HIV-1 infection can coincide with several conditions that lead to acute or chronic hemolysis that could cause a similar exposure to extracellular heme as does administration of HA. These

conditions include genetically determined glucose-6-phosphate dehydrogenase deficiencies, sickle cell anemia, thalasemia or other hemoglobinopathies as well as various other diseases involving hemolytic episodes or chronic hemolysis, especially malaria (Lopez et al., 2010; Pamplona et al., 2009). It would be worthwhile to determine a possible correlation of HIV-1/AIDS progression with these conditions. However, the situation is complex and therapeutic interventions, namely iron supplementation, could strongly affect the fine balance of pro-oxidative and anti-oxidative agents.

In clinics, HA is used to treat acute attacks of hepatic porphyrias. The mean maximum plasma levels of heme after a single dose of HA 3 mg/kg body weight was determined as 60 μg/ml (corresponds to 2.4 μl/ml of HA), with a plasma half-life of 10.8 h and a distribution volume of 3.4 L (Tokola et al., 1986). The concentrations of HA used throughout this paper are thus very close to the levels achieved in clinics. Additionally, 24–48 h after administration of the same dose of hemin, but in the form of Panhematin, plasma levels of HO-1 were increased 5-times, while its activity in venous leukocytes increased 15-times (Bharucha et al., 2010). Heme mediates a feedback inhibition of the rate-limiting enzyme in the heme synthetic pathway, synthase of 5-aminolevulinic acid. It also reconstitutes heme stores and function of various hemoproteins, namely hemoglobin, cytochrome P450, guanylate synthase, nitric oxide synthases, tryptophan dioxygenase, catalase and peroxidase. However, neither the exact pathogenesis of the neurovisceral symptoms in acute porphyrias, nor the precise mechanism of action of heme arginate are understood (http://www.porphyria.uct.ac.za/professional/prof-haem-therapy.htm; Herrick and McColl, 2005; Siegesmund et al., 2010). Nevertheless since HA has been approved for human use, it can be suggested that HA could be tested as a supplement of HAART in selected cases. For example its administration could be suggested as an additional measure in early stages of HIV/AIDS disease to release the virus from the existing latent pool, while inhibiting its dissemination to the new viral reservoirs. Since the levels of TNF-α and other cytokines are increased and/or dysregulated in HIV/AIDS, HA might synergize with these cytokines in provirus reactivation also *in vivo*. The suggestion of HA use in HIV/AIDS is further supported by a case of an HIV-positive individual that was administered one infusion of Normosang because of anemia. This patient then remained p24 negative for several months (Pavel Martasek, General Faculty Hospital in Prague, personal communication). Obviously, the use of HA should be tested first in animal models of retrovirus infection to assess its therapeutic potential against retroviruses more closely. Also, the administration of Normosang can be complicated by its adverse side effects. Vascular side effects of Normosang, especially on hemostasis, can occur, but they are reported to be much weaker than after administration of hematin (Panhaematin). Additionally, since hemin decreased HIV growth in humanized mice even when administered intraperitoneally (Devadas and Dhawan, 2006), it is possible that the i.p. or some other way of administration of Normosang would be also effective against HIV in humans. Repeated administrations of HA could lead to an iron overload. However, HIV/AIDS disease is often accompanied by the anemia due to a chronic immune activation, altered porphyrin metabolism caused by iron deficiency (Adetifa and Okomo, 2009; Fuchs et al., 1990) as well as by treatment with antiretrovirals (Bozzi et al., 2004; Fox et al., 1999). All these conditions would be improved by the administration of heme, while iron overload might not develop.

On the whole, these results suggest a possibility of an alternative approach to the management of HIV/AIDS disease. HA seems to possess a combination of two unique properties: it can help to reactivate the provirus from latent pools, and simultaneously prevent HIV-1 dissemination into new cells and/or expansion of the latent pool. In this way, HA could significantly prolong the latent stage of the disease and/or delay the depletion of CD4+ T-cells.

In conclusion, we demonstrate the inhibitory properties of heme arginate, Normosang, on HIV-1 reverse transcription and the overall replication on the one hand, and its stimulatory effects on reactivation of the latent provirus on the other hand. Altogether, the results suggest a new direction to explore in treatment of HIV/AIDS infection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2011.09.011.

References

Adachi, A., Gendelman, H.E., Koenig, S., Folks, T., Willey, R., Rabson, A., Martin, M.A., 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. 59, 284–291.
Adetifa, I., Okomo, U., 2009. Iron supplementation for reducing morbidity and mortality in children with HIV. Cochrane Database Syst Rev. CD006736.
Argyris, E.G., Vanderkooi, J.M., Paterson, Y., 2001. Mutagenesis of key residues identifies the connection subdomain of HIV-1 reverse transcriptase as the site of inhibition by heme. Eur. J. Biochem. 268, 925–931.
Ascenzi, P., Cao, Y., di Masi, A., Gullotta, F., De Sanctis, G., Fanali, G., Fasano, M., Coletta, M., 2010. Reductive nitrosylation of ferric human serum heme-albumin. FEBS J. 277, 2474–2485.
Belcher, J.D., Beckman, J.D., Balla, G., Balla, J., Vercellotti, G., 2010. Heme degradation and vascular injury. Antioxid. Redox Signal. 12, 233–248.
Bharucha, A.E., Kulkarni, A., Choi, K.M., Camilleri, M., Lempke, M., Brunn, G.J., Gibbons, S.J., Zinsmeister, A.R., Farrugia, G., 2010. First-in-human study demonstrating pharmacological activation of heme oxygenase-1 in humans. Clin. Pharmacol. Ther. 87, 187–190.
Bickar, D., Bonaventura, C., Bonaventura, J., 1984. Carbon monoxide-driven reduction of ferric heme and heme proteins. J. Biol. Chem. 259, 10777–10783.
Blazkova, J., Trejbalova, K., Gondois-Rey, F., Halfon, P., Philibert, P., Guiguen, A., Verdin, E., Olive, D., Van Lint, C., Hejnar, J., Hirsch, I., 2009. CpG methylation controls reactivation of HIV from latency. PLoS Pathog. 5, e1000554.
Bozzi, A., Brisdelli, F., D'Alessandro, A.M., D'Andrea, G., Lizzi, A.R., Rinaldi, A.C., Oratore, A., 2004. Effects of AZT on cellular iron homeostasis. Biometals 17, 443–450.
Brose, N., Rosenmund, C., 2002. Move over protein kinase C, you've got company: alternative cellular effectors of diacylglycerol and phorbol esters. J. Cell Sci. 115, 4399–4411.
Clouse, K.A., Powell, D., Washington, I., Poli, G., Strebel, K., Farrar, W., Barstad, P., Kovacs, J., Fauci, A.S., Folks, T.M., 1989. Monokine regulation of human immunodeficiency virus-1 expression in a chronically infected human T cell clone. J. Immunol. 142, 431–438.
Devadas, K., Dhawan, S., 2006. Hemin activation ameliorates HIV-1 infection via heme oxygenase-1 induction. J. Immunol. 176, 4252–4257.
Devadas, K., Hewlett, I.K., Dhawan, S., 2010. Lipopolysaccharide suppresses HIV-1 replication in human monocytes by protein kinase C-dependent heme oxygenase-1 induction. J. Leukoc. Biol. 87, 915–924.
Fox, P.A., Boag, F.C., Hawkins, D.A., Francis, N., 1999. Acute porphyria following commencement of indinavir. Aids 13, 622–623.
Fuchs, D., Artner-Dworzak, E., Hausen, A., Reibnegger, G., Werner, E.R., Werner-Felmayer, G., Dierich, M.P., Wachter, H., 1990. Urinary excretion of porphyrins is increased in patients with HIV-1 infection. Aids 4, 341–344.

Furuyama, K., Kaneko, K., Vargas, P.D., 2007. Heme as a magnificent molecule with multiple missions: heme determines its own fate and governs cellular homeostasis. Tohoku J. Exp. Med. 213, 1–16.
Georgiou, N.A., van der Bruggen, T., Oudshoorn, M., de Bie, P., Jansen, C.A., Nottet, H.S., Marx, J.J., van Asbeck, B.S., 2004. Mechanism of inhibition of the human immunodeficiency virus type 1 by the oxygen radical generating agent bleomycin. Antiviral Res. 63, 97–106.
Goel, G., Makkar, H.P., Francis, G., Becker, K., 2007. Phorbol esters: structure, biological activity, and toxicity in animals. Int. J. Toxicol. 26, 279–288.
Greene, W.C., 1991. The molecular biology of human immunodeficiency virus type 1 infection. N. Engl. J. Med. 324, 308–317.
Harlow, E., Lane, D., 1988. Immunoblotting. In: Harlow, E., Lane, D. (Eds.), Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York, pp. 471–510.
Herrick, A.L., McColl, K.E., 2005. Acute intermittent porphyria. Best Pract. Res. Clin. Gastroenterol. 19, 235–249.
Igarashi, K., Sun, J., 2006. The heme-Bach1 pathway in the regulation of oxidative stress response and erythroid differentiation. Antioxid. Redox Signal. 8, 107–118.
Jordan, A., Bisgrove, D., Verdin, E., 2003. HIV reproducibly establishes a latent infection after acute infection of T cells in vitro. EMBO J. 22, 1868–1877.
Kalbacova, M., Vrbacky, M., Humlova, Z., Melkova, Z., 2002. Protooncogene Bcl-2 induces apoptosis in several cell lines. Folia Biol. (Praha) 48, 15–27.
Kilbourn, R.G., Joly, G., Cashon, B., DeAngelo, J., Bonaventura, J., 1994. Cell-free hemoglobin reverses the endotoxin-mediated hyporesponsivity of rat aortic rings to alpha-adrenergic agents. Biochem. Biophys. Res. Commun. 199, 155–162.
Kruszewski, M., 2003. Labile iron pool: the main determinant of cellular response to oxidative stress. Mutat. Res. 531, 81–92.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
Lander, H.M., Sehajpal, P., Levine, D.M., Novogrodsky, A., 1993. Activation of human peripheral blood mononuclear cells by nitric oxide-generating compounds. J. Immunol. 150, 1509–1516.
Levere, R.D., Gong, Y.F., Kappas, A., Bucher, D.J., Wormser, G.P., Abraham, N.G., 1991. Heme inhibits human immunodeficiency virus 1 replication in cell cultures and enhances the antiviral effect of zidovudine. Proc. Natl Acad. Sci. USA 88, 1756–1759.
Lizard, G., Deckert, V., Dubrez, L., Moisant, M., Gambert, P., Lagrost, L., 1996. Induction of apoptosis in endothelial cells treated with cholesterol oxides. Am. J. Pathol. 148, 1625–1638.
Lopez, C., Saravia, C., Gomez, A., Hoebeke, J., Patarroyo, M.A., 2010. Mechanisms of genetically-based resistance to malaria. Gene 467, 1–12.
Maines, M.D., Trakshel, G.M., Kutty, R.K., 1986. Characterization of two constitutive forms of rat liver microsomal heme oxygenase. Only one molecular species of the enzyme is inducible. J. Biol. Chem. 261, 411–419.
Mehellou, Y., De Clercq, E., 2010. Twenty-six years of anti-HIV drug discovery: where do we stand and where do we go? J. Med. Chem. 53, 521–538.
Mense, S.M., Zhang, L., 2006. Heme: a versatile signaling molecule controlling the activities of diverse regulators ranging from transcription factors to MAP kinases. Cell Res. 16, 681–692.
Morse, D., Lin, L., Choi, A.M., Ryter, S.W., 2009. Heme oxygenase-1, a critical arbitrator of cell death pathways in lung injury and disease. Free Radic. Biol. Med. 47, 1–12.
Nabel, G., Baltimore, D., 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature 326, 711–713.
Naidu, S., Wijayanti, N., Santoso, S., Kietzmann, T., Immenschuh, S., 2008. An atypical NF-kappa B-regulated pathway mediates phorbol ester-dependent heme oxygenase-1 gene activation in monocytes. J. Immunol. 181, 4113–4123.
Olerup, O., Zetterquist, H., 1992. HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor–recipient matching in cadaveric transplantation. Tissue Antigens 39, 225–235.
Pace, G.W., Leaf, C.D., 1995. The role of oxidative stress in HIV disease. Free Radic. Biol. Med. 19, 523–528.
Pamplona, A., Hanscheid, T., Epiphanio, S., Mota, M.M., Vigario, A.M., 2009. Cerebral malaria and the hemolysis/methemoglobin/heme hypothesis: shedding new light on an old disease. Int. J. Biochem. Cell Biol. 41, 711–716.
Pantano, C., Reynaert, N.L., van der Vliet, A., Janssen-Heininger, Y.M., 2006. Redox-sensitive kinases of the nuclear factor-kappaB signaling pathway. Antioxid. Redox Signal. 8, 1791–1806.
Pyo, C.W., Yang, Y.L., Yoo, N.K., Choi, S.Y., 2008. Reactive oxygen species activate HIV long terminal repeat via post-translational control of NF-kappaB. Biochem. Biophys. Res. Commun. 376, 180–185.
Sacks, D.B., 2006. The role of scaffold proteins in MEK/ERK signalling. Biochem. Soc. Trans. 34, 833–836.
Schmidtmayerova, H., Alfano, M., Nuovo, G., Bukrinsky, M., 1998. Human immunodeficiency virus type 1 T-lymphotropic strains enter macrophages via a CD4- and CXCR4-mediated pathway: replication is restricted at a postentry level. J. Virol. 72, 4633–4642.
Seu, L., Burt, T.D., Williamson, D.W., Martin, J.N., Deeks, S.G., McCune, J.M., 2009. Heme oxygenase-1 promoter polymorphisms correlate with favorable virologic and immunological parameters in HIV-1 infection. Retrovirology 6, 109.
Siegesmund, M., van Serooskerken, A.M., Poblete-Gutierrez, P., Frank, J., 2010. The acute hepatic porphyrias: current status and future challenges. Best Pract. Res. Clin. Gastroenterol. 24, 593–605.
Soares, M.P., Marguti, I., Cunha, A., Larsen, R., 2009. Immunoregulatory effects of HO-1: how does it work? Curr. Opin. Pharmacol. 9, 482–489.
Staudinger, R., Abraham, N.G., Levere, R.D., Kappas, A., 1996. Inhibition of human immunodeficiency virus-1 reverse transcriptase by heme and synthetic heme analogs. Proc. Assoc. Am. Physicians 108, 47–54.
Tenhunen, R., Marver, H.S., Schmid, R., 1969. The enzymatic conversion of hemoglobin to bilirubin. Trans. Assoc. Am. Physicians. 82, 363–371.
Tokola, O., Tenhunen, R., Volin, L., Mustajoki, P., 1986. Pharmacokinetics of intravenously administered haem arginate. Br. J. Clin. Pharmacol. 22, 331–335.
Volin, L., Rasi, V., Vahtera, E., Tenhunen, R., 1988. Heme arginate: effects on hemostasis. Blood 71, 625–628.
Walmsley, S., Loutfy, M., 2002. Can structured treatment interruptions (STIs) be used as a strategy to decrease total drug requirements and toxicity in HIV infection? J. Int. Assoc. Physicians AIDS Care (Chic Ill) 1, 95–103.
Williams, S.A., Kwon, H., Chen, L.F., Greene, W.C., 2007. Sustained induction of NF-kappa B is required for efficient expression of latent human immunodeficiency virus type 1. J. Virol. 81, 6043–6056.
Wu, L., Wang, R., 2005. Carbon monoxide: endogenous production, physiological functions, and pharmacological applications. Pharmacol. Rev. 57, 585–630.
Yang, H.C., Xing, S., Shan, L., O'Connell, K., Dinoso, J., Shen, A., Zhou, Y., Shrum, C.K., Han, Y., Liu, J.O., Zhang, H., Margolick, J.B., Siliciano, R.F., 2009. Small-molecule screening using a human primary cell model of HIV latency identifies compounds that reverse latency without cellular activation. J. Clin. Invest. 119, 3473–3486.

11.2. Research article 2

Shankaran P., Madlenakova M., Hajkova V., Jilich D., Svobodova I., Horinek A., Fujikura Y., and Melkova Z. Effects of heme degradation products on reactivation of latent HIV-1. Acta Virologica. Submitted. (Manuscript)

EFFECTS OF HEME DEGRADATION PRODUCTS ON REACTIVATION OF LATENT HIV-1

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Running title:

Heme arginate, iron and HIV latency

Short Communication

Key words:

HIV-1 reactivation; iron; heme arginate; carbon monoxide; bilirubin; redox stress

SUMMARY

HIV-1 infection can be currently controlled by combined antiretroviral therapy, but a sterilizing cure is impossible as this therapy does not target persistent HIV-1 in latent reservoirs. Therefore, different latency reversing agents are intensively explored in various models.

We have previously observed that heme arginate, a drug approved for human use, reveals a strong synergism with PKC inducers in reactivation of the latent provirus. Heme is physiologically decomposed by heme oxygenases into 3 degradation products: iron (Fe^{2+}), carbon monoxide (CO) and biliverdin which is further converted to bilirubin by biliverdin reductase.

In this paper, we have studied the effects of individual heme-degradation products on latent HIV-1 reactivation in ACH-2 cells harboring integrated HIV-1 provirus and in H12 clone of Jurkat cells harboring HIV-minivirus expressing EGFP. We employed addition of ascorbate to generate Fe^{2+}, resulting in increased expression of both HIV-1 p24 Ag and EGFP in PMA-stimulated ACH-2 and H12 cells, respectively, as characterized on RNA and protein levels. On the other hand, addition of a CO-donor or bilirubin decreased the p24 expression. The reactivation of latent HIV-1 by iron or heme arginate was inhibited by antioxidant N-acetyl cysteine, or by an iron chelator desferrioxamine, suggesting that the effects were mediated by iron- or heme-induced redox stress. Finally, we demonstrated the synergistic effects of heme arginate and PMA on HIV-1 expression in peripheral blood mononuclear cells of HIV-infected patients cultured ex vivo. These results may constitute a new direction in the latent HIV-1 reactivation and therapy.

KEYWORDS

HIV-1 reactivation; iron; heme arginate; carbon monoxide; bilirubin; redox stress

ABBREVIATIONS

AIDS = *acquired immune deficiency syndrome;* cART = combined anti-retroviral therapy; CO = carbon monoxide; CORM-A1 = carbon monoxide releasing molecule-A1; DFO = desferrioxamine; EGFP = enhanced green fluorescent protein; FBS = fetal bovine serum; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; GSH = glutathione; HA = heme arginate; HIV-1 = human immunodeficiency virus-1; HO-1 = heme oxygenase-1; LTR = long terminal repeat; NAC = N-acetyl cysteine; PBMC's = peripheral blood mononuclear cells; PKC = protein kinase C; PMA = phorbol myristate acetate; RNS = reactive nitrogen species; ROS = reactive oxygen species; S.E.M. = standard error of mean; SIV = simian immunodeficiency *virus*

INTRODUCTION

HIV/AIDS pathogenesis inherently involves increased generation of reactive oxygen and nitrogen species (ROS, RNS) mediated by pro-inflammatory cytokines. An increased redox stress with decreased levels of GSH were found already in early stages of HIV infection (Pace and Leaf, 1995). Redox stress regulates signal transduction by activating redox-sensitive transcription factors like NF-kB and NFAT, as well as chromatin remodeling, namely by inhibiting histone deacetylases (Kennedy et al., 2012; Pantano et al., 2006; Rahman et al., 2004). HIV-1 LTR includes multiple upstream DNA regulatory elements that serve as binding sites for cellular transcription initiation factors and facilitate NF-kB and NFAT binding. ROS are involved in the reactivation of HIV from latent reservoirs through post-translational activation of NF-kB (Pyo et al., 2008). While activated, NF-kB facilitates HIV gene expression by directing recruitment of the histone acetyltransferases to the HIV-1 LTR (Gatignol, 2007). Iron and ascorbate-mediated redox stress affects methylation status of the promoters of genes involved in the redox stress response (Yara et al., 2013).

We have previously demonstrated in human T-cell lines that Normosang (heme arginate; HA), a hemin derivative approved for human use in treatment of acute porphyria, reactivated the latent provirus by itself or in synergy with PKC inducers like phorbol myristate acetate (PMA), TNF-alpha, prostratin or bryostatin-1 (Shankaran et al., 2011), and unpublished data). HA also induced expression of heme oxygenase-1 (HO-1) and inhibition of this enzyme increased the provirus reactivation. Antioxidant N-acetyl cysteine (NAC), precursor of GSH, inhibited the provirus reactivation, suggesting that the reactivation was mediated by ROS (Shankaran et al., 2011).

Heme is an efficient inhibitor of reverse transcriptase (Argyris et al., 2001; Levere et al., 1991; Shankaran et al., 2011), while its derivative hemin stimulates expression of various genes, especially HO-1 (Mense and Zhang, 2006). Free heme is toxic due to its ability to catalyze Fenton reaction generating hydroxyl radicals, highly reactive ROS (Shibahara, 2003). Heme from hemoglobin of aged erythrocytes is physiologically degraded by HO-1 in spleen, but the excess of heme and its

derivatives stimulates HO-1 expression ubiquitously (Sheftel et al., 2007; Soe-Lin et al., 2008). HO-1 breaks down heme into iron (Fe^{2+}), carbon monoxide (CO), and biliverdin that is consequently reduced to bilirubin by the action of biliverdin reductase. Fe^{2+} might induce a short-term pro-oxidative state, while CO and especially the redox cycle bilirubin/biliverdin/biliverdin reductase act as antioxidants. In general, HO-1 is considered an antioxidant enzyme with immunomodulatory properties (Otterbein et al., 2003).

In this paper, we have studied the effects of heme degradation products on PMA-induced reactivation of latent HIV-1 when added individually to latently infected T-cell lines. Further, we assessed the effects of antioxidant NAC and iron chelator desferrioxamine (DFO) and verified the synergistic effects of heme arginate and PMA in PBMC's of HIV+ patients' on combined antiretroviral therapy (cART) cultured ex vivo.

METHODS

Chemicals

All the media and growth supplements were purchased from Thermo Scientific, USA, Invitrogen Corporation (Carlsbad, CA) or PAA Laboratories GmbH (Pasching, Austria). Other chemicals used, including phorbol myristate acetate, ferric nitrate, ascorbic acid, carbon monoxide releasing molecule-A1 (CORM-A1), bilirubin, desferrioxamine mesylate salt (DFO) and N-Acetyl cysteine were purchased from Sigma-Aldrich (Germany) unless otherwise specified. Heme arginate (Normosang) was purchased from Orphan Europe (Paris, France). The chemicals for RNA and DNA isolation, PCR and real-time PCR were purchased from Top-Bio (Czech Republic), for ddPCR from Bio-Rad (Hercules, USA), primers and probes from IDT (Belgium) and Life Technologies (Carlsbad, CA, USA). TURBO DNA-free kit, Ambion, was from Life Technologies (Carlsbad, CA, USA).

Cell lines and primary cells and their treatment

Human T-cell line ACH-2 harboring an integrated HIV-1 provirus (clone #4; (Clouse et al., 1989)), their parental cell line A3.01, and the H12 clone of Jurkat cells latently infected with a ‘‘mini-virus’’ containing the HIV-1 LTR-Tat-IRES-EGFP-LTR

(Blazkova et al., 2009; Jordan et al., 2003; Shankaran et al., 2011), were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 12.5 mM Hepes, and antibiotics (penicillin $1x10^5$ U/l, streptomycin 100 mg/l; 10% FBS-RPMI). The cells were plated at final concentration of 0.5 x 10^6 cells/ml/well in 10% FBS-RPMI and then treated with iron (10 μM) and ascorbate (0.25 mM in ACH-2 cells; 0.25, 0.5, 1 and 2 mM in A3.01 and H12 cells; (Hermes-Lima et al., 2000; Zhang et al., 2008)) or with heme arginate (2.5 μl/ml), followed by PMA (0.5 ng/ml); in certain experiments, the cells were pre-treated with additional compounds as specified. The cells were collected after 24 h (ACH-2 cells) or 48 h (A3.01 and H12 cells).

Stock solutions of PMA and bilirubin were prepared in DMSO and freshly diluted in culture medium to 20x working solutions. Other working solutions were freshly prepared as follows: 100x Fe^{3+} (1 mM $FeNO_3$ in 50 mM Hepes pH=8 and 20 mM EDTA), 100x ascorbate (25 mM ascorbic acid in H_2O), 100x CORM-A1 (5 mM CORM-A1 in H_2O), 20x NAC (100 mM in RPMI 1640), 100x DFO (12.5 mM in H_2O). The compounds were added at final concentrations specified in each experiment.

PBMC's of HIV-infected patients on cART with undetectable viremia (< 50 copies/ml) were isolated by Ficoll gradient centrifugation (Histopaq, Sigma-Aldrich, Germany), differential blood count was determined by Advia 60 Hematology System (Bayer Healthcare, USA) and PBMC's were resuspended at final concentration of 5 x 10^6 lymphocytes/ml in 10% FBS-RPMI. PBMC's were plated at 1 ml/well in a 24-well plate and subjected to treatment with 2.5 μl of HA/ml and different concentrations of PMA for 18 h.

Western blot analysis

Cells were collected after 24 h of incubation, lysed in Laemmli reducing sample buffer, boiled and analyzed by SDS–PAGE and western blotting as previously described (Harlow, 1988; Laemmli, 1970; Shankaran et al., 2011), using chemiluminescence (West Femto, Thermo Fisher Scientific – Pierce, Rockford, IL). The cell lysates were resolved on a 12% SDS–PAGE and transferred to a PVDF membrane (Hybond, Bio-Rad, Hercules, USA). HIV-1 p24 was detected using a mouse monoclonal antibody ND-1 (dilution 1:500; Exbio, Prague, Czech Republic) and a peroxidase-conjugated goat anti-mouse IgG (dilution 1:20,000; Sigma Co.,

St.Louis, MO). β-actin was detected using a rabbit polyclonal antibody (dilution 1:10,000; Abcam, Cambridge, United Kingdom) and a peroxidase-conjugated goat anti-rabbit IgG (dilution 1:20,000, MP Biomedicals – Cappel, Solon, OH). The chemiluminescence was recorded using ChemiDoc MP system (BioRad, Hercules, USA) and the densitometry analysis was performed with ImageLab software version 5.0 (BioRad, Hercules, USA).

Flow cytometry analysis

Flow cytometer Canto II (Becton Dickinson) equipped with 3 lasers emitting at 488, 405 and 633 nm, and with 8 detectors was used. Flow cytometry measurements and subsequent analyses of the data were performed using the Diva 6 software (Becton Dickinson, Franklin Lakes, NJ). EGFP fluorescence was determined in FL1 (detecting at 515–545 nm) and expressed as the arithmetic mean of green fluorescence of green cell population x percentage of green cells. The results of each experiment performed in duplicate were then normalized to untreated cells (100%); the graphs represent mean and standard error of mean (S.E.M.) of 3-8 experiments. Live and apoptotic cells were distinguished based on their size and granulosity (FSC-A x SSC-A) and % of apoptotic cells was calculated (Shankaran et al., 2011).

RNA isolation and quantification

RNA was isolated using RNA Blue reagent by precipitation of the aqueous phase according to the manufacturer's protocol (Top-Bio, Czech Republic); RNA was solubilized in nuclease- and protease-free molecular biology grade water (Sigma-Aldrich, Germany) with the addition of RiboLock RNase inhibitor (Thermo Scientific, USA; final concentration 1 U/µl) and stored in -80ºC for further use. The concentration and purity of RNA was determined by measuring the absorbance at 260 and 280 nm using UV spectrophotometer Eppendorf BioSpectrometer (Eppendorff AG, Germany). The RNA isolated from ACH-2 cells was then treated with TURBO DNase (TURBO DNA-free kit, Ambion, Life Technologies) to remove contaminant genomic DNA according to the manufacturer's protocol. For HIV+ PBMC's, 18 µl of RNA (out of total 20 µl) were treated with 6 U of TURBO DNase; final volume after inactivation was 26 µl.

Quantification of HIV-1 RNA by droplet digital PCR

Absolute quantification of the HIV-1 RNA expressed in ACH-2 cells was performed using the QX100 droplet digital PCR system (Bio Rad, Hercules, USA) and One-Step RT ddPCR kit for probes (Bio Rad, Hercules, USA) according to the manufacturer's protocol. Twenty µl of the reaction mixture consisted of 10 µl of 2x one-step RT ddPCR super mix, 0.8 µl of 25 mM manganese acetate, 0.5 or 5 ng of DNase-treated RNA and primers and probes for HIV Gag or human GAPDH. Primers and probes used: HIV-1 Gag – forward primer GAG1 (5'-TCAGCCCAGAAGTAATACCCATGT-3') and reverse primer GAG2 (5'-CACTGTGTTTAGCATGGTGTTT-3'), final concentration 200 nM each, probe GAG3 (FAM-5'-ATTATCAGAAGGAGCCACCCCACAAGA-3'-BHQ1), final concentration 250 nM (Kiselinova et al., 2014; Pasternak et al., 2008); human GAPDH – 1x primer-probe Hs02758991_g1 VIC-MGB labelled (Life Technologies, Calsbard, CA, USA). The droplets were generated using the QX100 droplet generator (Bio Rad, Hercules, USA) by mixing 70µl of droplet generation oil for probes (Bio Rad, Hercules, USA) with 20 µl of ddPCR reaction mixture and PCR amplification was performed on the T100 thermal cycler (Bio Rad, Hercules, USA). The thermal cycling conditions were as follows: reverse transcription at 60ºC for 30 minutes, 95ºC for 5 min, 40 cycles consisting of denaturation at 94ºC for 30 s and annealing/extension at 60ºC for 60 s, followed by 10 min at 98ºC. The amplified droplets were read by the QX100 droplet reader (Bio Rad, Hercules, USA) and the data were analysed by QuantaSoft analysis software 1.6.6.0320 (Bio Rad, Hercules, USA). Copies of Gag RNA were standardized to human GAPDH as a reference gene.

Quantification of HIV-1 RNA by real-time PCR

RNA isolated from cultured and stimulated PBMC's of HIV-infected patients treated with cART was used for detection of cell-associated HIV-1 RNA using semi-nested 2-step RT-qPCR adapted from (Kiselinova et al., 2014; Pasternak et al., 2008). Sixteen µl of DNase-treated RNA was used for reverse transcription in a final volume of 40 µl using random hexamer primers (Premium Reverse Transcriptase, Thermo Scientific). Then, 20 µl of cDNA was amplified in a total volume of 100 µl using GAG1 and SK431 primers (final concentration 250 nM each; SK431 sequence: 5'-

TGCTATGTCAGTTCCCCTTGGTTCTCT-3'; (Pasternak et al., 2008)) and 0.05 U/µl of Blood Taq DNA polymerase (Top-Bio, Czech Republic) in 1x PCR Blue buffer (Top-Bio, Czech Republic) containing 2 mM $MgCl_2$. The thermal cycling conditions were as follows: initial denaturation at 95ºC for 5 min, 15 cycles consisting of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s, and extension at 72ºC for 60 s, followed by terminal extension at 72ºC for 10 min using the Mastercycler Gradient (Eppendorf AG, Hamburg, Germany). Finally, 10 µl of the first PCR product was used in duplicate for real-time PCR in a total volume of 50 µl containing GAG1 and GAG2 primers, GAG3 probe (final concentration 200 nM each; GAG3 sequence: FAM-5'-ATTATCAGAAGGAGCCACCCCACAAGA-3'-TAMRA; (Pasternak et al., 2008)), and 1x qPCR Blue buffer (Top-Bio, Czech Republic) using the Applied Biosystems 7300 Real-time PCR System (Applied Biosystems, Foster City, CA) and the universal cycling conditions. Four µl of the first PCR product was used for quantification of a reference gene in a 20 µl reaction also in duplicate. Four µl of DNase-treated RNA was used for a no-RT control and further processed in the way analogous to cDNA. The mean of technical duplicates was used for relative quantification of HIV Gag compared to GAPDH. Changes in expression of 10 additional house-keeping genes in healthy donors PBMCs after the individual treatments were determined using Real-time PCR kit for Human Reference Genes (Generi Biotech, Czech Republic).

Statistical analysis

Results are presented as means ± S.E.M. The data were analysed and graphically represented using GraphPad Prism 5.04 software. Statistical significance of differences between control and different treatments within each group were determined using a two-sample two-tailed unpaired Student's t-test with Welch's correction. Equality of variances was tested with F-test.

RESULTS

First, we aimed to determine the effects of iron-mediated redox stress on reactivation of the latent HIV-1 in ACH-2 cells harboring an integrated HIV-1 provirus. Since Fe^{2+} is quickly oxidized to Fe^{3+} in the aqueous environment, we employed ascorbate to generate Fe^{2+} in our system (Hermes-Lima et al., 2000; Zhang et al., 2008). We

have treated unstimulated or PMA-stimulated ACH-2 cells with Fe^{3+}, ascorbate and with a combination of Fe^{3+} and ascorbate. In unstimulated cells, none of the treatments induced p24 expression as detected by western blot analysis and chemiluminescence, recorded and analyzed digitally by the ChemiDoc system. In the PMA-stimulated cells, Fe^{3+} alone decreased the PMA-stimulated p24 levels, while ascorbate alone increased the PMA-stimulated p24 levels and this increase was partially inhibited by Fe^{3+} (Fig. 1 (a, b)). All the stimulatory effects on p24 expression were inhibited by pretreatment of the cells with an antioxidant NAC, precursor of GSH, or with an iron chelator desferrioxamine. These results suggest involvement of free radicals and/or iron in the reactivation of the latent HIV-1 by these compounds, including ascorbate. Interestingly, the effect of Fe^{3+} on PMA- and ascorbate/PMA-stimulated p24 expression was inhibitory in a way similar to NAC. Thus, Fe^{3+} alone appears to reveal final antioxidant effects in this system.

In order to further characterize the mechanism of iron and ascorbate action in ACH-2 cells, we have quantified levels of HIV-1 mRNA using 1-step RT-ddPCR. PMA alone increased background Gag mRNA levels 34-times, while addition of ascorbate or addition of iron with ascorbate increased the PMA-stimulated levels about 6- and 2-times more, respectively (Fig. 1 (c)). Again, Fe^{3+} alone inhibited PMA-stimulated p24 expression.

In addition, we have used a parental cell line of ACH-2 cells, A3.01, to determine effects of the individual treatments on induction of apoptosis using flow cytometry and changes in their size and granulosity (Fig. 1 (d)). PMA treatment increased the background apoptosis about twice, while addition of 2 and 1 mM ascorbate alone and with iron increased percentage of apoptosis 2-4-times more in both unstimulated and PMA-stimulated cells. Importantly, 0.25 mM ascorbate that was used in ACH-2 cells, did not induce any increase of apoptosis.

The other two degradation products of heme arginate are CO and biliverdin that is converted to bilirubin by the action of biliverdin reductase. We have used a CO-donor CORM-A1 to characterize the effects of CO and addition of bilirubin to PMA-stimulated ACH-2 cells. As shown in Fig. 2, increasing concentrations of both CORM-A1 and bilirubin dose-dependently decreased the levels of PMA-stimulated p24 levels. We have also tested the effects of biliverdin, but it did not reveal any appreciable effects (data not shown).

Additionally, we used the H12 clone of Jurkat cells harboring a latent HIV-1 “mini-virus” expressing EGFP under control of HIV-1 LTR (Shankaran et al., 2011), (Jordan et al., 2003). This clone was previously shown to possess an increased methylation of the HIV-1 LTR (Blazkova et al., 2009) and revealed similar responses to HA administration as ACH-2 cells, except that HA alone, in the absence of PMA, induced EGFP expression also (Shankaran et al., 2011). The combination of iron and ascorbate stimulated EGFP expression both with and without PMA, as determined by flow cytometry, while higher concentrations of ascorbate alone induced EGFP expression also in the unstimulated H12 cells (Fig. 3 (a)). In other concentrations, including 0.25 mM that was used in ACH-2 cells, ascorbate did not induce any reactivation. Further, we have determined the percentage of apoptosis induced by the individual treatments in these cells (Fig. 3 (b)). PMA treatment increased the background apoptosis about 5-times, while percentage of apoptosis induced by the individual treatments seemed to reveal a similar pattern as EGFP expression. Then, we tested the effects of CORM-A1 and bilirubin in H12 cells. CORM-A1 increased the levels of EGFP expression in PMA-stimulated H12 cells while pretreatment with of bilirubin did not seem to affect EGFP expression (data not shown). On the other hand, percentage of apoptosis was not affected either by CORM-A1 or bilirubin (around 5 and 25% in unstimulated and PMA-stimulated cells, respectively).

Previously, we have demonstrated a synergistic effect of heme arginate and PMA on reactivation of the latent HIV-1 in ACH-2 cells that was inhibited by the antioxidant NAC (Shankaran et al., 2011). In order to determine the role of iron in the HA-mediated effects, we have now pre-treated the ACH-2 cells with DFO before addition of HA and PMA. As shown in Fig. 4 (a, b), DFO decreased HA- and PMA-mediated reactivation of latent HIV-1 as characterized by levels of p24 antigen and western blot analysis. Of note, DFO decreased also the levels of control β-actin in the absence of HA in these experiments. Further, we have determined the levels of HIV-1 mRNA using 1-step RT-ddPCR (Fig. 4 (c)). As expected, DFO also decreased the levels of HA- and PMA-stimulated levels of HIV-1 mRNA, indicating the role of iron in PMA- and HA-mediated effects.

The results presented above were performed in cell lines which reveal different mutations and clonal bias. ACH-2 cells are known to have a mutation in the TAR region affecting the Tat-mediated transactivation (Emiliani et al., 1996; Venkatachari et al., 2015), while the expression of the latent provirus in different Jurkat clones is dependent on their integration site (Jordan 2001). Therefore, to validate the effects of HA and PMA on reactivation of the latent HIV-1, we decided to determine the effects of HA and PMA in primary cells isolated from the peripheral blood of HIV-infected patients on cART with undetectable plasma virus load. In the first approach, we have intentionally used PBMC's, as the wider spectrum of cell types is closer to the situation in vivo. As shown in Fig. 5, cell-associated HIV-1 RNA Gag characterized by a semi-nested 2-step RT-qPCR could be detected in all the samples at the time of isolation and went down during the 18 h-incubation ex vivo (based on relative quantification to GAPDH). PMA added at low concentrations somewhat increased the levels of HIV-1 RNA compared to untreated samples. HA alone increased the levels of HIV-1 RNA compared to untreated samples (1.7-4.5-fold), while HA synergized with PMA (2-12-fold increase compared to PMA-untreated samples) similarly as in ACH-2 cells. In these experiments, HIV-1 RNA levels were quantified relatively to GAPDH. In additional experiments, we have determined the effects of these treatments on 10 other house-keeping genes, with changes in expression of GAPDH and β-2 microglobulin being the smallest and comparable. Nevertheless, their expression was increased by PMA and HA with PMA, affecting the relative changes in HIV-1 RNA levels.

DISCUSSION

In summary, our results clearly point to the importance of iron and heme metabolism and their dysregulation in pathogenesis of HIV/AIDS. Further, the stimulatory effects of ascorbate warrant for a cautious use of antioxidants, vitamins and other food additives that could affect the redox state of iron.

Previously published results indicated that a longer pre-treatment with ascorbate suppressed the induction of HIV reactivation in latently infected T-cell lines (Clouse et al., 1989). However in our experiments, ascorbate added shortly before PMA stimulated latent HIV-1 reactivation in both ACH-2 and H12 cells; these effects could be attributed to a double faced character of ascorbate that can act as an anti- or pro-

oxidant, depending on its concentration (http://www.benbest.com/nutrceut/AntiOxidants.html#radicals). Further, the stimulatory effects of ascorbate but also those of PMA were inhibited by DFO, suggesting involvement of iron in these processes. In addition to direct effects of DFO on iron chelation, the inhibition by DFO might be explained also by previously described inhibitory effects of DFO on activation of NF-κB, the redox-sensitive transcription factor involved in both PMA signaling and HIV-1 reactivation (Chirullo et al., 2013; Jordan et al., 2003). On the other hand, addition of Fe^{3+}, similarly to NAC, was inhibitory to PMA- and ascorbate-stimulated latent HIV-1 reactivation; Fe^{3+} alone thus appears to reveal final antioxidant effects in this system, possibly due to its complex with EDTA. Evidently, the final outcome of chemical reactions and annihilations of individual compounds and free radicals, respectively, depends on many variables and can dynamically change in a biological system. Importantly, the concentrations of heme-degradation products used in this paper were comparable or lower than effective concentrations of heme arginate. On the other hand, effective concentrations of heme arginate are achievable in vivo (Tokola et al., 1986).

In ACH-2 cells, the effect of ascorbate or iron with ascorbate on induction of latent HIV expression was relatively lower at both RNA and protein levels than the effect of HA. Indeed, HA serves as a source of both iron and hemin. Heme similarly to iron generates ROS by Fenton reaction (Shibahara, 2003), while hemin is known to regulate expression of various genes (Furuyama et al., 2007; Mense and Zhang, 2006). On the other hand, pretreatment with a CO-donor or bilirubin dose-dependently decreased p24 levels in ACH-2 cells, while the CO donor increased EGFP expression and bilirubin was ineffective in H12 cells. These differences may be attributable to a different intracellular redox milieu, possibly affected by a constitutive expression of HO-1 (Shankaran et al., 2011). These results also suggest that the contribution of the individual heme-degradation products may vary in different cell types in vivo. In the first approach to verify the stimulatory effects of heme arginate we have intentionally used PBMC's as they are closer to the situation in vivo. Nevertheless, additional experiments on individual cell types, namely on isolated total and resting CD4+ cells, will better assess the magnitude of HA-stimulatory effects and help to delineate the underlying mechanisms.

HIV-1 replication and spread is closely connected with the induction of cell death and can be modulated by its onset (Cummins and Badley, 2013; Wang et al., 2016; Wang et al., 2011). While the general apoptosis due to toxicity of latency reversing agents is not desirable, elimination of the infected cells is a goal of all approaches aiming at curing HIV-1. Increased redox stress characterized by decreased levels of reduced glutathione is associated with HIV-1 replication (Pace and Leaf, 1995), while it was suggested as an approach to reactivate and kill latently infected cells by several authors (Iordanskiy and Kashanchi, 2016; Lewis et al., 2011; Shankaran et al., 2011). Importantly, central memory and transition memory T-cells, the main reservoir cells containing the latent HIV-1, were shown to be more susceptible to the redox stress and apoptosis (Chirullo et al., 2013).

HIV/AIDS, similarly to other chronic infections, has been reported to lead to immune-mediated anemia of chronic disease, iron deficiency anemia or their combination (Kerkhoff and Lawn, 2015; Minchella et al.), in which differential expression of hepcidin, hemojuvelin, ferroportin and other factors plays an important role (Drakesmith and Prentice, 2012; Krijt et al., 2004; Theurl et al., 2011; Xu et al., 2010). Consequently, iron supplements have often been administered. However, this can result in an increased labile iron pool (LIP) and reactivation, expression and dissemination of HIV-1. In fact, it has been recognized that iron plays a critical role in several steps of HIV-1 replication (Nekhai et al., 2013) and its levels are modulated by most nonpathogenic SIV (Koppensteiner et al., 2014). Several clinical studies also suggest that iron supplementation or hereditary defects leading to increased intracellular iron stores can fasten progression of HIV infection to AIDS in untreated patients (Gordeuk et al., 2001; McDermid et al., 2007; Rawat et al., 2009).

In our short-term experimental conditions, the stimulatory effects of heme arginate on HIV-expression clearly prevailed in ACH-2 and H12 cells as well as in HIV-infected PBMC's (Figs. 4, 5; (Shankaran et al., 2011)). On the other hand, heme arginate inhibits the acute HIV infection at the level of reverse transcription (Fig. 6 (Levere et al., 1991; Shankaran et al., 2011)). Thus, the ability of heme arginate to stimulate a short-term reactivation of the latent HIV while inhibiting reverse transcription and further replication of the new virus progeny could make it a useful

and safe agent in help to eliminate the HIV-1 latent pool in the presence of antiretroviral drugs.

Redox stress can affect epigenetic mechanisms regulating gene expression as well as activation of redox-sensitive transcription factors. Therefore, we propose a model in which heme arginate induces a redox stress leading to chromatin remodeling, binding of specific transcription factors to HIV-LTR and potentiation of HIV-1 expression induced by a PKC inducer. These results may constitute new direction in the latent HIV-1 reactivation and therapy.

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REFERENCES

Argyris, EG, Vanderkooi, JM, and Paterson, Y (2001): Mutagenesis of key residues identifies the connection subdomain of HIV-1 reverse transcriptase as the site of inhibition by heme. *Eur J Biochem* **268**, 925-31.

Blazkova, J, Trejbalova, K, Gondois-Rey, F, Halfon, P, Philibert, P, Guiguen, A, Verdin, E, Olive, D, Van Lint, C, Hejnar, J, and Hirsch, I (2009): CpG methylation controls reactivation of HIV from latency. *PLoS Pathog* **5**, e1000554.

Clouse, KA, Powell, D, Washington, I, Poli, G, Strebel, K, Farrar, W, Barstad, P, Kovacs, J, Fauci, AS, and Folks, TM (1989): Monokine regulation of human immunodeficiency virus-1 expression in a chronically infected human T cell clone. *J Immunol* **142**, 431-8.

Cummins, NW, and Badley, AD (2013): Anti-apoptotic mechanisms of HIV: lessons and novel approaches to curing HIV. *Cell Mol Life Sci* **70**, 3355-63.

Drakesmith, H, and Prentice, AM (2012): Hepcidin and the iron-infection axis. *Science* **338**, 768-72.

Emiliani, S, Van Lint, C, Fischle, W, Paras, P, Jr., Ott, M, Brady, J, and Verdin, E (1996): A point mutation in the HIV-1 Tat responsive element is associated with postintegration latency. *Proc Natl Acad Sci U S A* **93**, 6377-81.

Furuyama, K, Kaneko, K, and Vargas, PD (2007): Heme as a magnificent molecule with multiple missions: heme determines its own fate and governs cellular homeostasis. *Tohoku J Exp Med* **213**, 1-16.

Gatignol, A (2007): Transcription of HIV: Tat and cellular chromatin. *Adv Pharmacol* **55**, 137-59.

Gordeuk, VR, Delanghe, JR, Langlois, MR, and Boelaert, JR (2001): Iron status and the outcome of HIV infection: an overview. *J Clin Virol* **20**, 111-5.

Harlow, EL, D. (1988): Immunoblotting. *In: Harlow, E., Lane, D. (Eds.), Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York*, 471–510.

Hermes-Lima, M, Ponka, P, and Schulman, HM (2000): The iron chelator pyridoxal isonicotinoyl hydrazone (PIH) and its analogues prevent damage to 2-deoxyribose mediated by ferric iron plus ascorbate. *Biochim Biophys Acta* **1523**, 154-60.

Chirullo, B, Sgarbanti, R, Limongi, D, Shytaj, IL, Alvarez, D, Das, B, Boe, A, DaFonseca, S, Chomont, N, Liotta, L, Petricoin, EI, Norelli, S, Pelosi, E, Garaci, E, Savarino, A, and Palamara, AT (2013): A candidate anti-HIV reservoir compound, auranofin, exerts a selective 'anti-memory' effect by exploiting the baseline oxidative status of lymphocytes. *Cell Death Dis* **4**, e944.

Iordanskiy, S, and Kashanchi, F (2016): Potential of Radiation-Induced Cellular Stress for Reactivation of Latent HIV-1 and Killing of Infected Cells. *AIDS Res Hum Retroviruses* **32**, 120-4.

Jordan, A, Bisgrove, D, and Verdin, E (2003): HIV reproducibly establishes a latent infection after acute infection of T cells in vitro. *EMBO J* **22**, 1868-77.

Kennedy, KA, Sandiford, SD, Skerjanc, IS, and Li, SS (2012): Reactive oxygen species and the neuronal fate. *Cell Mol Life Sci* **69**, 215-21.

Kerkhoff, AD, and Lawn, SD (2015): Iron replacement therapy and anemia associated with chronic infectious diseases in sub-Saharan Africa. *Clin Infect Dis* **60**, 1438-9.

Kiselinova, M, Pasternak, AO, De Spiegelaere, W, Vogelaers, D, Berkhout, B, and Vandekerckhove, L (2014): Comparison of droplet digital PCR and seminested real-time PCR for quantification of cell-associated HIV-1 RNA. *PLoS One* **9**, e85999.

Koppensteiner, H, Hohne, K, Gondim, MV, Gobert, FX, Widder, M, Gundlach, S, Heigele, A, Kirchhoff, F, Winkler, M, Benaroch, P, and Schindler, M (2014): Lentiviral Nef suppresses iron uptake in a strain specific manner through inhibition of Transferrin endocytosis. *Retrovirology* **11**, 1.

Krijt, J, Vokurka, M, Chang, KT, and Necas, E (2004): Expression of Rgmc, the murine ortholog of hemojuvelin gene, is modulated by development and inflammation, but not by iron status or erythropoietin. *Blood* **104**, 4308-10.

Laemmli, UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-5.

Levere, RD, Gong, YF, Kappas, A, Bucher, DJ, Wormser, GP, and Abraham, NG (1991): Heme inhibits human immunodeficiency virus 1 replication in cell cultures and enhances the antiviral effect of zidovudine. *Proc Natl Acad Sci U S A* **88**, 1756-9.

Lewis, MG, DaFonseca, S, Chomont, N, Palamara, AT, Tardugno, M, Mai, A, Collins, M, Wagner, WL, Yalley-Ogunro, J, Greenhouse, J, Chirullo, B, Norelli, S, Garaci, E, and Savarino, A (2011): Gold drug auranofin restricts the viral reservoir in the monkey AIDS model and induces containment of viral load following ART suspension. *AIDS* **25**, 1347-56.

McDermid, JM, Jaye, A, Schim van der Loeff, MF, Todd, J, Bates, C, Austin, S, Jeffries, D, Awasana, AA, Whittlex, AA, and Prentice, A (2007): Elevated iron status strongly predicts mortality in West African adults with HIV infection. *J Acquir Immune Defic Syndr* **46**, 498-507.

Mense, SM, and Zhang, L (2006): Heme: a versatile signaling molecule controlling the activities of diverse regulators ranging from transcription factors to MAP kinases. *Cell Res* **16**, 681-92.

Minchella, PA, Armitage, AE, Darboe, B, Jallow, MW, Drakesmith, H, Jaye, A, Prentice, AM, and McDermid, JM Elevated Hepcidin Is Part of a Complex Relation That Links Mortality with Iron Homeostasis and Anemia in Men and Women with HIV Infection. *J Nutr* **145**, 1194-201.

Nekhai, S, Kumari, N, and Dhawan, S (2013): Role of cellular iron and oxygen in the regulation of HIV-1 infection. *Future Virol* **8**, 301-311.

Otterbein, LE, Soares, MP, Yamashita, K, and Bach, FH (2003): Heme oxygenase-1: unleashing the protective properties of heme. *Trends Immunol* **24**, 449-55.

Pace, GW, and Leaf, CD (1995): The role of oxidative stress in HIV disease. *Free Radic Biol Med* **19**, 523-8.

Pantano, C, Reynaert, NL, van der Vliet, A, and Janssen-Heininger, YM (2006): Redox-sensitive kinases of the nuclear factor-kappaB signaling pathway. *Antioxid Redox Signal* **8**, 1791-806.

Pasternak, AO, Adema, KW, Bakker, M, Jurriaans, S, Berkhout, B, Cornelissen, M, and Lukashov, VV (2008): Highly sensitive methods based on seminested real-time reverse transcription-PCR for quantitation of human immunodeficiency virus type 1 unspliced and multiply spliced RNA and proviral DNA. *J Clin Microbiol* **46**, 2206-11.

Pyo, CW, Yang, YL, Yoo, NK, and Choi, SY (2008): Reactive oxygen species activate HIV long terminal repeat via post-translational control of NF-kappaB. *Biochem Biophys Res Commun* **376**, 180-5.

Rahman, I, Marwick, J, and Kirkham, P (2004): Redox modulation of chromatin remodeling: impact on histone acetylation and deacetylation, NF-kappaB and pro-inflammatory gene expression. *Biochem Pharmacol* **68**, 1255-67.

Rawat, R, Humphrey, JH, Ntozini, R, Mutasa, K, Iliff, PJ, and Stoltzfus, RJ (2009): Elevated iron stores are associated with HIV disease severity and mortality among postpartum women in Zimbabwe. *Public Health Nutr* **12**, 1321-9.

Shankaran, P, Vlkova, L, Liskova, J, and Melkova, Z (2011): Heme arginate potentiates latent HIV-1 reactivation while inhibiting the acute infection. *Antiviral Res* **92**, 434-46.

Sheftel, AD, Kim, SF, and Ponka, P (2007): Non-heme induction of heme oxygenase-1 does not alter cellular iron metabolism. *J Biol Chem* **282**, 10480-6.

Shibahara, S (2003): The heme oxygenase dilemma in cellular homeostasis: new insights for the feedback regulation of heme catabolism. *Tohoku J Exp Med* **200**, 167-86.

Soe-Lin, S, Sheftel, AD, Wasyluk, B, and Ponka, P (2008): Nramp1 equips macrophages for efficient iron recycling. *Exp Hematol* **36**, 929-37.

Theurl, I, Schroll, A, Nairz, M, Seifert, M, Theurl, M, Sonnweber, T, Kulaksiz, H, and Weiss, G (2011): Pathways for the regulation of hepcidin expression in anemia of chronic disease and iron deficiency anemia in vivo. *Haematologica* **96**, 1761-9.

Tokola, O, Tenhunen, R, Volin, L, and Mustajoki, P (1986): Pharmacokinetics of intravenously administered haem arginate. *Br J Clin Pharmacol* **22**, 331-5.

Venkatachari, NJ, Zerbato, JM, Jain, S, Mancini, AE, Chattopadhyay, A, Sluis-Cremer, N, Bar-Joseph, Z, and Ayyavoo, V (2015): Temporal transcriptional response to latency reversing agents identifies specific factors regulating HIV-1 viral transcriptional switch. *Retrovirology* **12**, 85.

Wang, X, Mbondji-Wonje, C, Zhao, J, and Hewlett, I (2016): IL-1beta and IL-18 inhibition of HIV-1 replication in Jurkat cells and PBMCs. *Biochem Biophys Res Commun* **473**, 926-30.

Wang, X, Ragupathy, V, Zhao, J, and Hewlett, I (2011): Molecules from apoptotic pathways modulate HIV-1 replication in Jurkat cells. *Biochem Biophys Res Commun* **414**, 20-4.

Xu, M, Kashanchi, F, Foster, A, Rotimi, J, Turner, W, Gordeuk, VR, and Nekhai, S (2010): Hepcidin induces HIV-1 transcription inhibited by ferroportin. *Retrovirology* **7**, 104.

Yara, S, Lavoie, JC, Beaulieu, JF, Delvin, E, Amre, D, Marcil, V, Seidman, E, and Levy, E (2013): Iron-ascorbate-mediated lipid peroxidation causes epigenetic changes in the antioxidant defense in intestinal epithelial cells: impact on inflammation. *PLoS One* **8**, e63456.

Zhang, AS, Canonne-Hergaux, F, Gruenheid, S, Gros, P, and Ponka, P (2008): Use of Nramp2-transfected Chinese hamster ovary cells and reticulocytes from mk/mk mice to study iron transport mechanisms. *Exp Hematol* **36**, 1227-35.

FIGURE LEGENDS

Fig. 1 Effect of iron and ascorbate on PMA-stimulated reactivation of the latent HIV-1. ACH-2 cells pre-treated with 5 mM N-Acetyl cysteine (NAC) or with 125 and 62.5 µM desferrioxamine (DFO) for 4 h, and treated with 10 µM ferric nitrate (Fe), 0.25 mM ascorbate (Asc), and 0.5 ng/ml PMA for 24 h. **(a)** Western blot analysis of HIV-1 p24 antigen. **(b)** Digital quantification of the western blots presented in panel (a). **(c)** Droplet digital PCR (ddPCR) analysis of HIV-1 RNA. **(d)** Flow cytometric analysis of apoptosis in A3.01 cells, the parental cell line of ACH-2 cells. * Increase is statistically significant when compared to control in each group ($p<0.05$).

Fig. 2. Carbon monoxide and bilirubin dose-dependently inhibit PMA-stimulated reactivation of the latent HIV-1. ACH-2 cells pre-treated with CORM-A1 (2, 10 and 50 µM) for 5 min or with bilirubin (0.01, 0.1 and 1 µM) for 6 h, and treated with 0.5 ng/ml PMA for 24 h **(a, c)** Western blot analysis of HIV-1 p24 antigen. **(b, d)** Quantification of the western blots presented in panel (a, c), respectively.

Fig. 3. Flow cytometric analysis of EGFP expression. H12 cells treated with 10 µM ferric nitrate (Fe), 0.25 mM ascorbate (Asc), and 0.5 ng/ml PMA for 48 h. **(a)** Flow cytometric analysis of EGFP expression. **(b)** Percentage of apoptosis. [x,] * Increase is statistically significant when compared to control in each group ($p<0.1$, 0.05 respectively).

Fig. 4. Iron chelator prevents hem arginate- and PMA-stimulated reactivation of the latent HIV-1. ACH-2 cells pre-treated with 125 and 62.5 µM DFO for 4 h, and treated with 2.5 ul of heme arginate/ml and 0.5 ng/ml PMA for 24 h **(a)** Western blot analysis of HIV-1 p24 antigen. **(b)** Quantification of the western blots presented in panel (a). **(c)** ddPCR analysis of HIV-1 RNA.

Fig. 5. Heme arginate stimulates HIV-1 expression in human HIV+ PBMC's ex vivo. HIV+ human PBMC's treated with 2.5 ul of heme arginate/ml and 0.5 or 2.5 ng/ml PMA for 18 h. T0 – time 0, 0 – no treatment, HA – heme arginate 2.5 µl/ml, PMA 0.5 or 2.5 ng/ml, NP – not performed.

Fig. 6. Scheme of action of heme arginate in ACH-2 cells. Heme inhibits reverse transcription, leading to HIV-1 inhibition. Heme is decomposed by the action of HO-1 into Fe^{2+}, CO and biliverdin that is further converted to bilirubin by biliverdin reductase. Heme- and iron-mediated Fenton reaction generates reactive oxygen species (ROS), leading to reactivation of the latent HIV-1; these processes can be inhibited by the antioxidant N-Acetyl cysteine or iron chelator Desferrioxamine. CO and bilirubin inhibit reactivation of the latent HIV-1.

Fig. 1

(a)

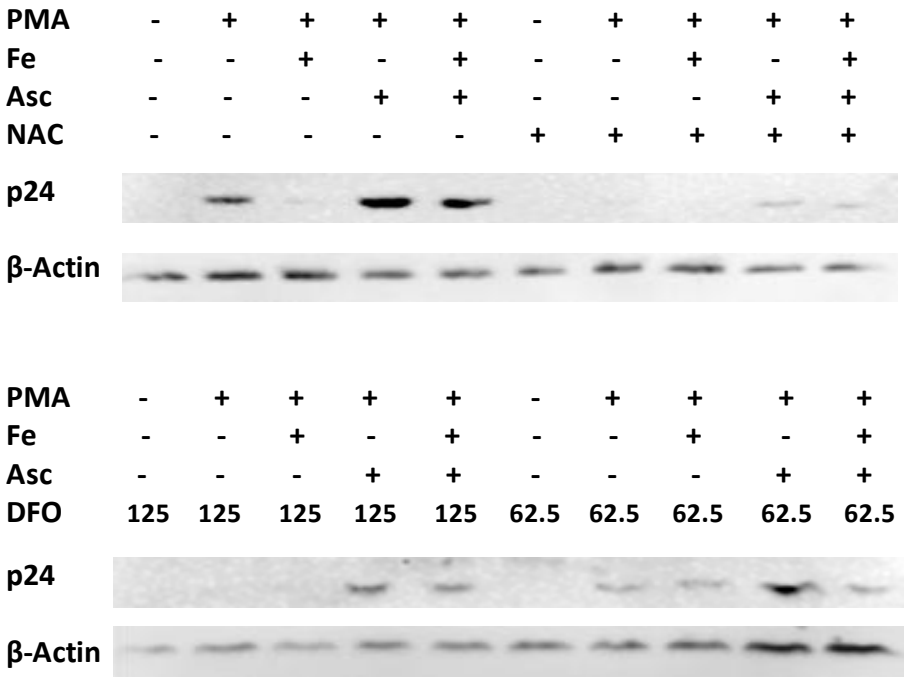

PMA - + + + + - + + + +
Fe - - + - + - - + - +
Asc - - - + + - - - + +
NAC - - - - - + + + + +
p24
β-Actin
PMA - + + + + - + + + +
Fe - - + - + - - + - +
Asc - - - + + - - - + +
DFO 125 125 125 125 125 62.5 62.5 62.5 62.5 62.5
p24
β-Actin

Fig. 1

(b)

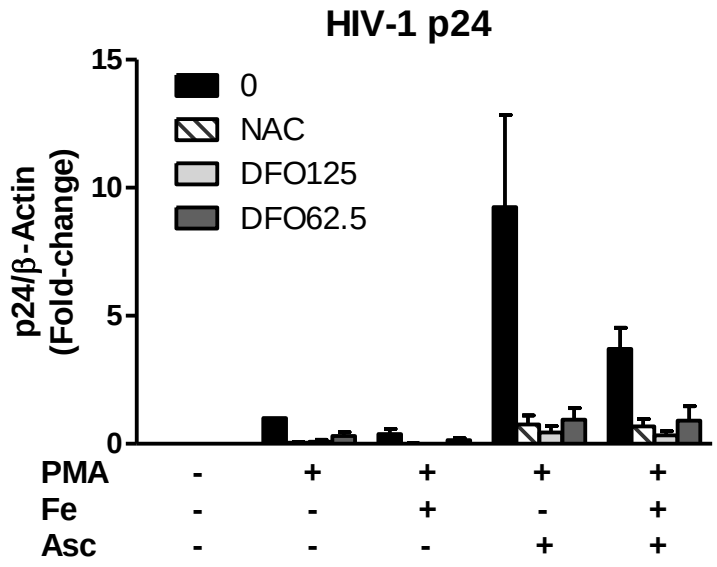

HIV-1 p24
p24/β-Actin (Fold-change)
15
10
5
0
0
NAC
DFO125
DFO62.5
PMA - + + + +
Fe - - + - +
Asc - - - + +

Fig. 1

(c)

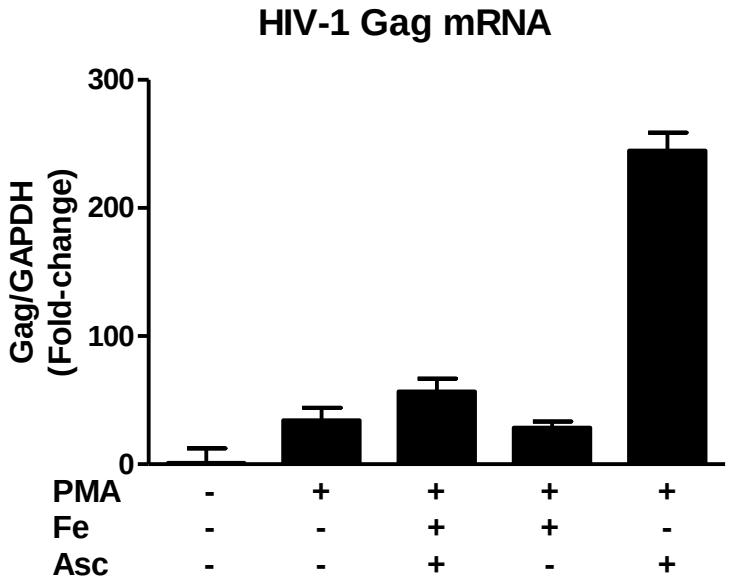

HIV-1 Gag mRNA
Gag/GAPDH (Fold-change)
300
200
100
0
PMA - + + + +
Fe - - + + -
Asc - - + - +

Fig. 1

(d)

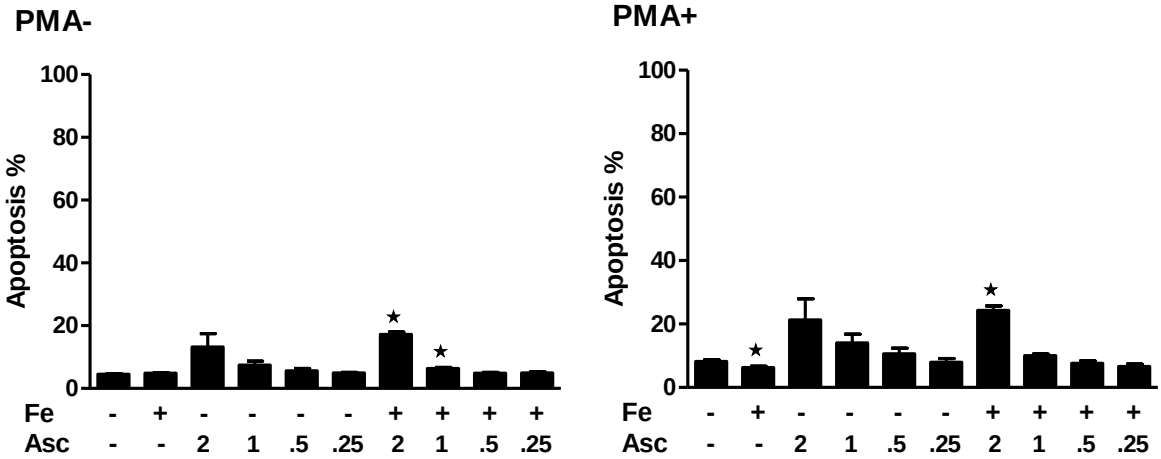

PMA-
PMA+
Apoptosis %
100
80
60
40
20
0
Fe - + - - - - + + + +
Asc - - 2 1 .5 .25 2 1 .5 .25

Fig. 2

(a)

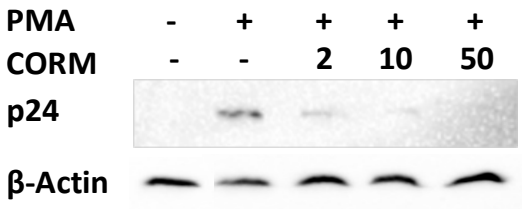

PMA - + + + +
CORM - - 2 10 50
p24
β-Actin

Fig. 2

(b)

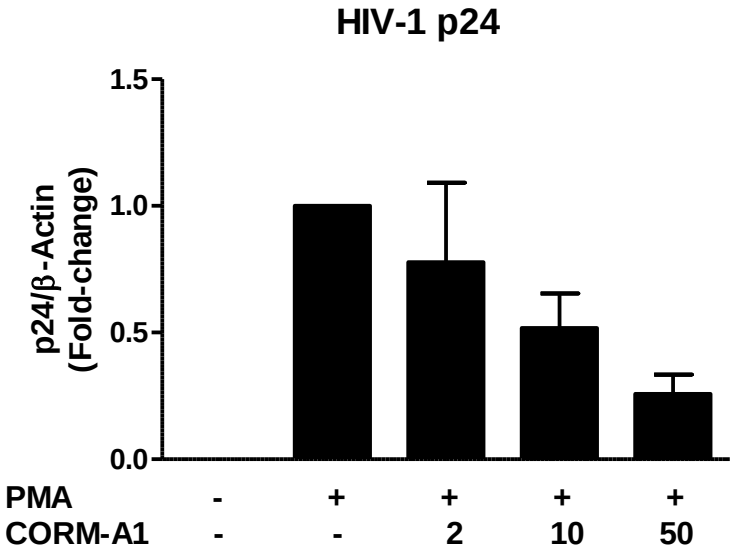

HIV-1 p24
p24/β-Actin (Fold-change)
1.5
1.0
0.5
0.0
PMA - + + + +
CORM-A1 - - 2 10 50

Fig. 2

(c)

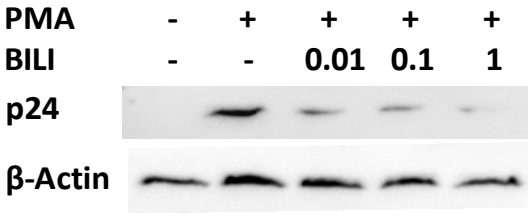

PMA - + + + +
BILI - - 0.01 0.1 1
p24
β-Actin

Fig. 2

(d)

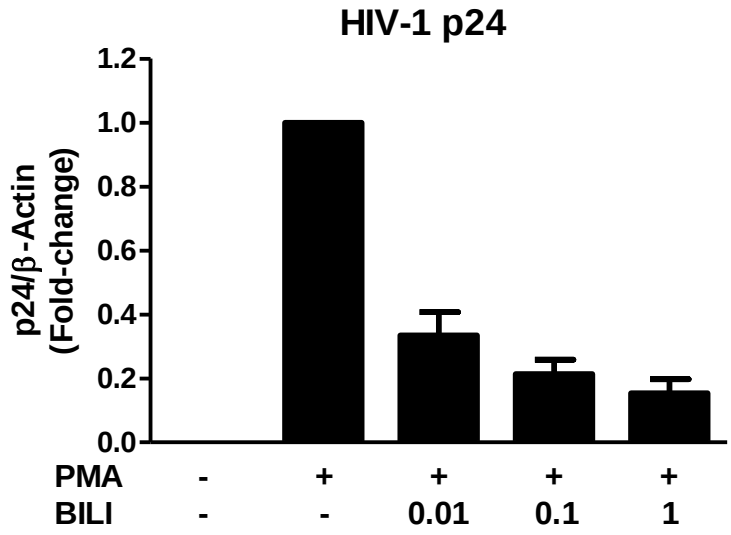

HIV-1 p24
p24/β-Actin (Fold-change)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
PMA - + + + +
BILI - - 0.01 0.1 1

Fig. 3

(a)

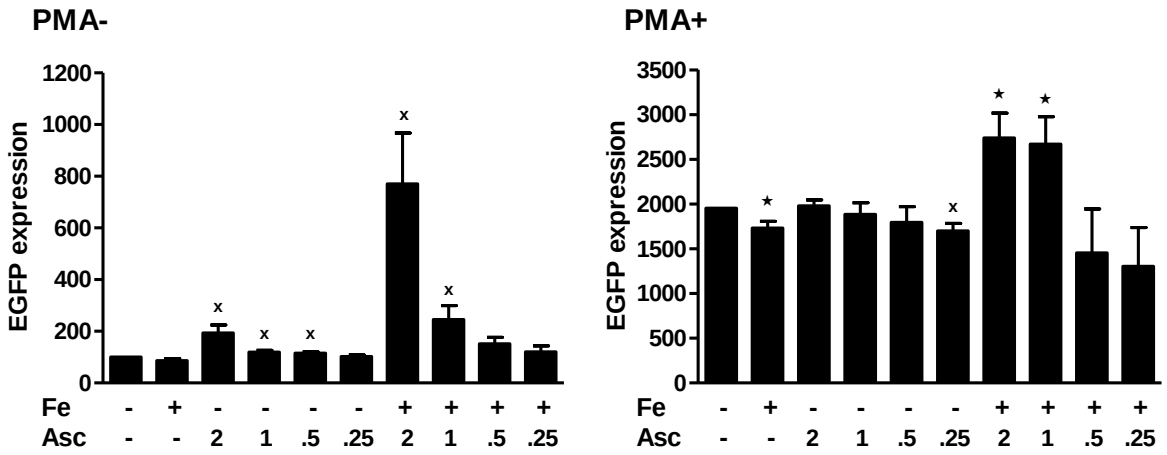

PMA-
PMA+
EGFP expression
1200
1000
800
600
400
200
0
3500
3000
2500
2000
1500
1000
500
Fe - + - - - - + + + +
Asc - - 2 1 .5 .25 2 1 .5 .25

Fig. 3

(b)

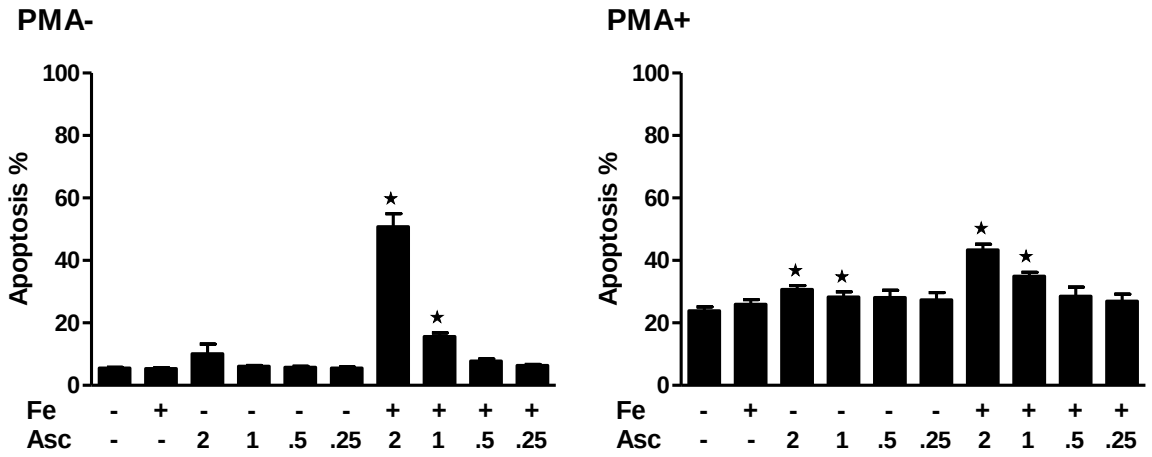

PMA-
PMA+
Apoptosis %
100
80
60
40
20
0
Fe - + - - - - + + + +
Asc - - 2 1 .5 .25 2 1 .5 .25

Fig. 4

(a)

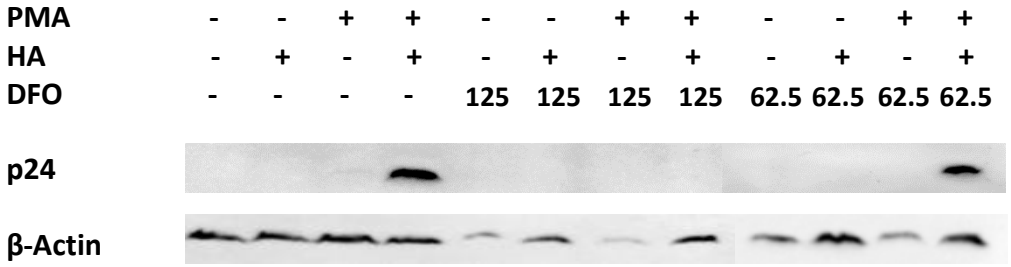

PMA - - + + - - + + - - + +
HA - + - + - + - + - + - +
DFO - - - - 125 125 125 125 62.5 62.5 62.5 62.5
p24
β-Actin

Fig. 4

(b)

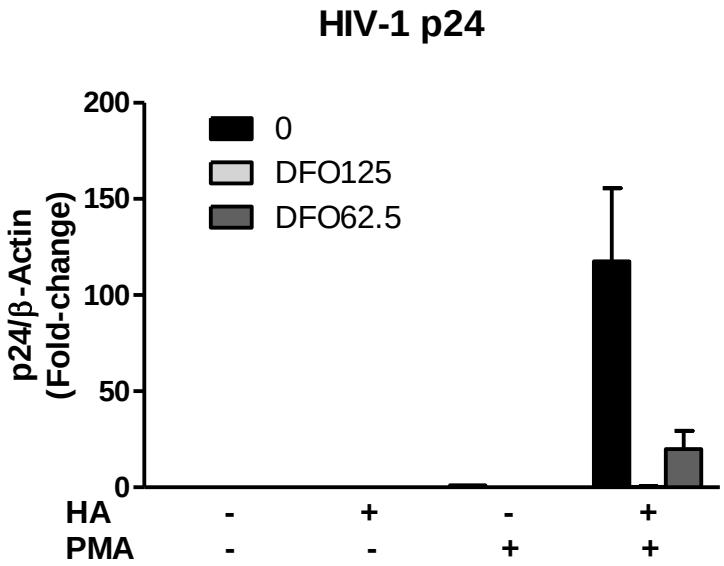

HIV-1 p24
p24/β-Actin (Fold-change)
200
150
100
50
0
0
DFO125
DFO62.5
HA - + - +
PMA - - + +

Fig. 4

(c)

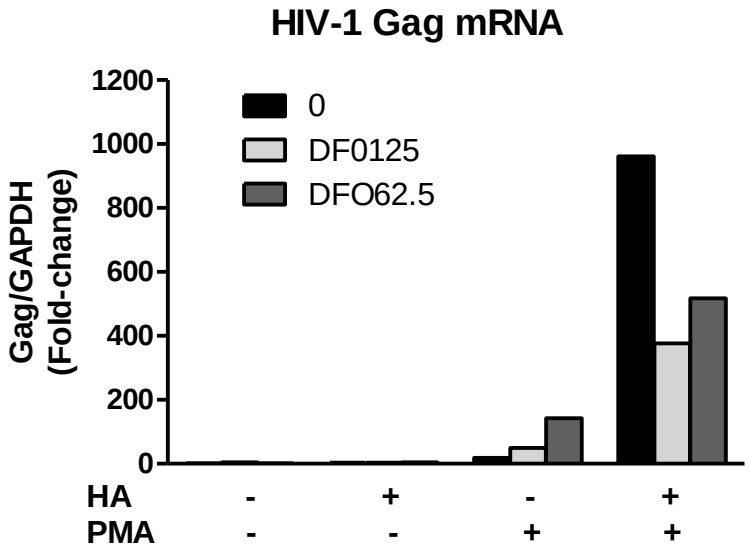

HIV-1 Gag mRNA
Gag/GAPDH (Fold-change)
1200
1000
800
600
400
200
0
0
DF0125
DFO62.5
HA - + - +
PMA - - + +

Fig. 5

(a)

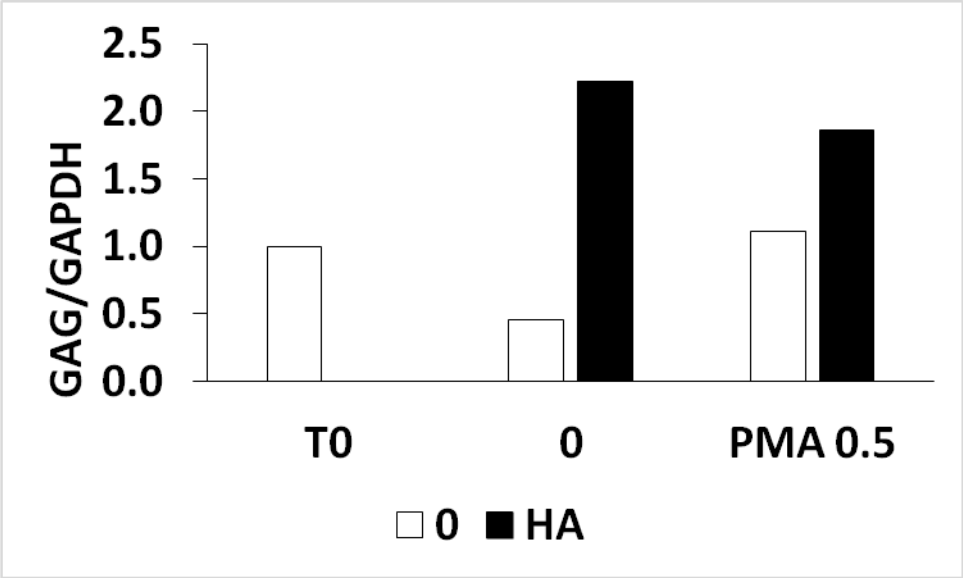

2.5
2.0
1.5
1.0
0.5
0.0
GAG/GAPDH
T0
0
PMA 0.5
0
HA

Fig. 5

(a)

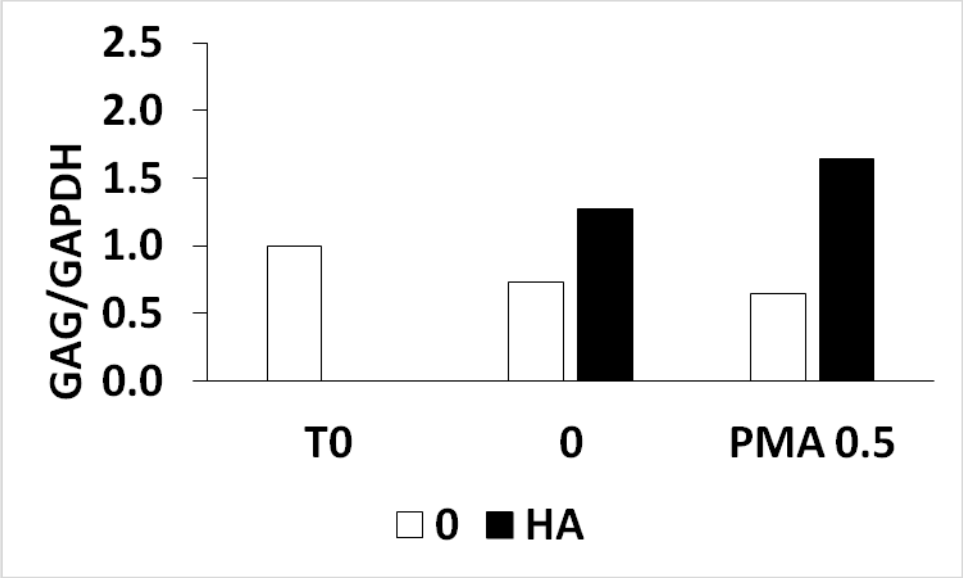

2.5
2.0
1.5
1.0
0.5
0.0
GAG/GAPDH
T0
0
PMA 0.5
0
HA

Fig. 5

(c)

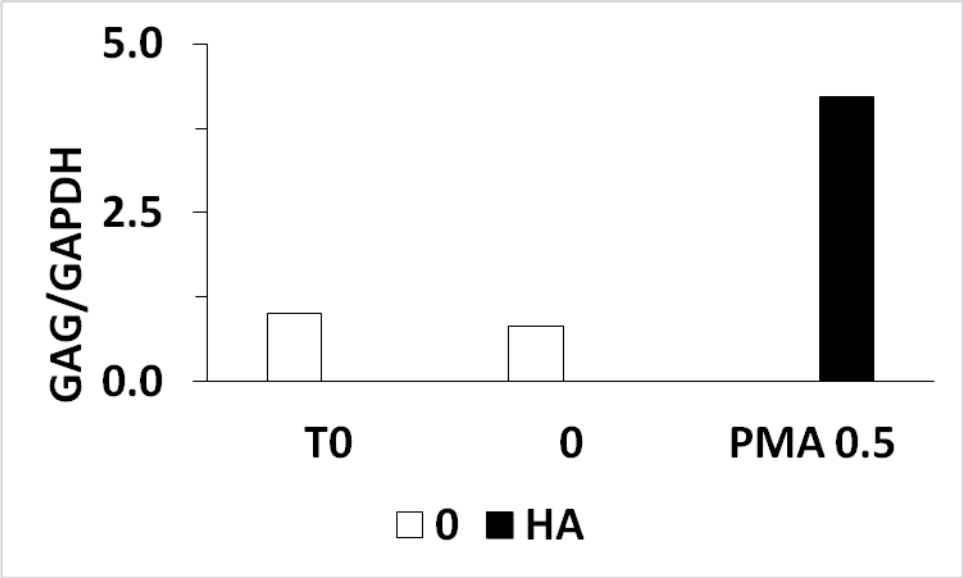

5.0
2.5
0.0
GAG/GAPDH
T0
0
PMA 0.5
0
HA

Fig. 5

(d)

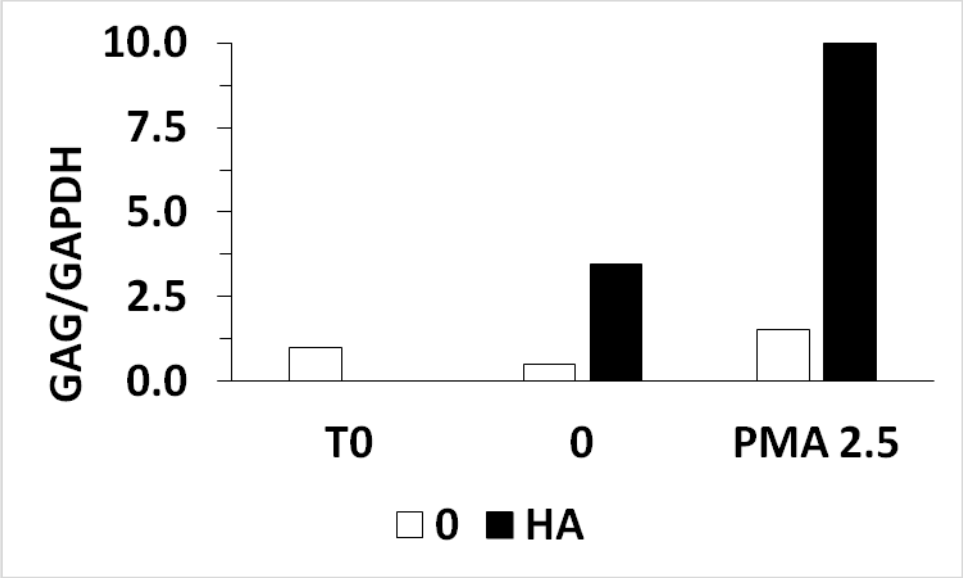
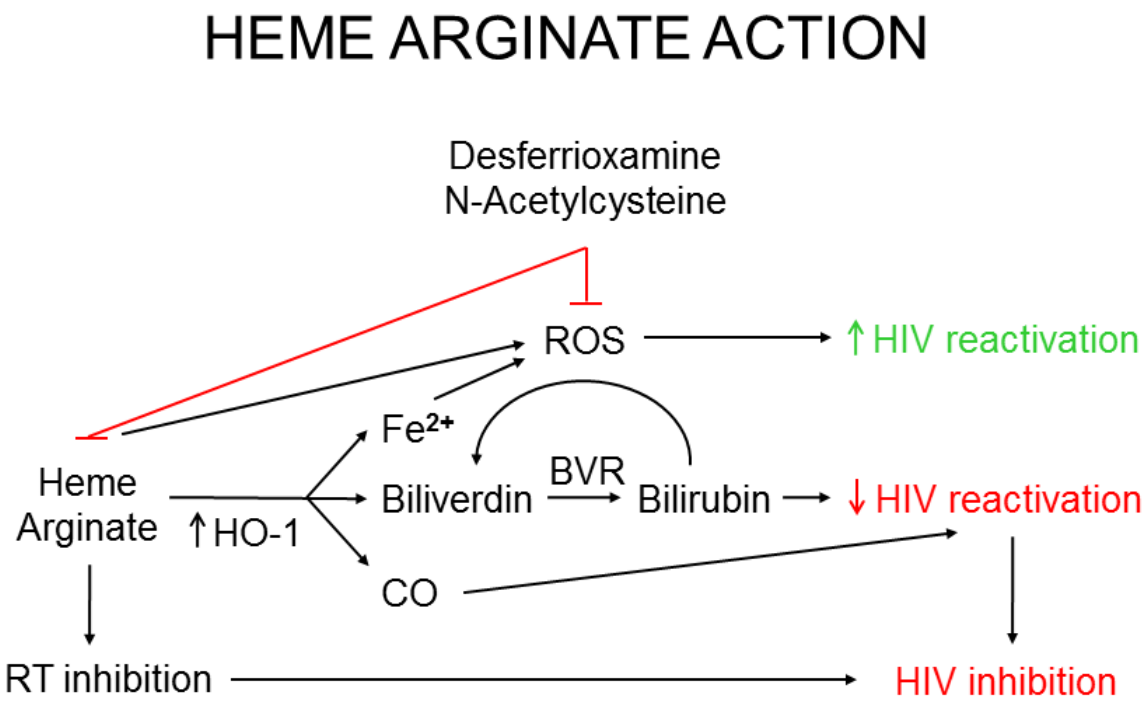

10.0
7.5
5.0
2.5
0.0
GAG/GAPDH
T0
0
PMA 2.5
0
HA

Fig. 6


HEME ARGINATE ACTION
Desferrioxamine
N-Acetylcysteine
ROS
↑HIV reactivation
Fe2+
Heme
Arginate
↑HO-1
Biliverdin
BVR
Bilirubin
↓HIV reactivation
CO
RT inhibition
HIV inhibition

11.3. Review article

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CURRENT VIEWS ON HIV-1 LATENCY, PERSISTENCE, AND CURE

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HIV-1 LATENCY, PERSISTENCE, AND CURE

Review

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Abstract

HIV-1 infection cannot be cured as it persists in latently infected cells that are not targeted by the immune system nor by available therapeutic approaches. Consequently, a lifelong therapy suppressing only the actively replicating virus is necessary. The latent reservoir has been defined and characterized in various experimental models and in human patients, allowing research and development of approaches targeting individual steps critical for HIV-1 latency establishment, maintenance, and reactivation. However, additional mechanisms and processes driving the remaining low-level HIV-1 replication in the presence of the suppressive therapy still remain to be identified and targeted.

Current approaches towards HIV-1 cure involve namely attempts to reactivate and purge HIV-latently infected cells (so called “shock and kill” strategy), as well as approaches involving gene therapy and/or gene editing and stem cell transplantation aiming at generation of cells resistant to HIV-1.

This review summarizes current views and concepts underlying different approaches aiming at functional or sterilizing cure of HIV-1 infection.

Key words:

HIV-1 latency, HIV-1 persistence, HIV-1 latency models, HIV-1 reactivation, HIV-1 cure, latent reservoir determination

Introduction

HIV/AIDS infection can be successfully treated and controlled with the combined antiretroviral therapy (cART) affecting different steps of HIV-1 replication cycle. Thus, HIV-1 infection could be viewed as a chronic disease with a relatively long life expectancy. However cART, which is still not available to all in need, cannot cure HIV infection due to the presence of latently infected reservoir cells. The latent proviral DNA cannot be recognized by the immune system nor targeted by cART. Consequently, a lifelong therapy is necessary, which is expensive and leads to various complications and treatment failures. Therefore, new approaches towards functional or sterilizing cure are intensively explored - namely attempts to reactivate and purge HIV-1 latently infected cells (so called “shock and kill” strategy), as well as approaches involving gene therapy and/or gene editing and stem cell transplantation aiming at generation of cells resistant to HIV-1. Ongoing research focuses especially on the mechanisms of establishment and maintenance of the latent reservoir, assessment of its size and composition, as well as on stimulation of the innate and specific immunity to promote HIV-1 clearance.

HIV-1 latency

Retrovirus replication cycle is specific by a step of reverse transcription and a consequent stable integration of the proviral DNA into the host cell genome. Depending on the activation status of the host cell, the HIV-1 provirus can be either immediately expressed and the virus replication cycle can proceed further or the provirus can become dormant and wait until the latently infected cell encounters the right stimulus (often a specific, possibly rare antigen) and becomes activated (Fig. 1). It is the very presence of the latently infected cells that makes HIV-1 infection incurable as these cells serve as a source of virus rebound after a discontinuation or failure of the antiretroviral therapy. After the activation of the host cell or specific changes in the epigenetic regulation, the chromatin status and availability of transcription and other factors change, and HIV-1 replication can restart.

HIV persistence during therapy

HIV-1 infects namely CD4+ T lymphocytes, but also myeloid cells like macrophages, microglia, astrocytes and dendritic cells, even though to a lesser extent. Acute infection of CD4+ T cells usually leads to cell death, but occasionally, these cells survive and revert back to a resting memory state. Alternatively, latency may be established directly in resting CD4+ T cells. Resting memory cells, including the latently infected ones, then persist and/or are replenished by homeostatic proliferation. In contrast, macrophages are resistant to cytopathic effects of HIV-1 infection, and support virus persistence in various anatomical sanctuaries as tissue resident macrophages (Kim and Siliciano 2016; Kumar et al. 2015; McKinstry et al. 2010).

After initiation of cART, plasma viremia and the level of HIV-1 infected cells in peripheral blood decays with a well characterized kinetics based on populations with a different turnover contributing to plasma viremia (Hilldorfer et al. 2012) (Fig. 2). A rapid decline of plasma viremia during first two phases indicates the efficiency of antiretroviral drugs. Phase I represents the turnover of the free virus (half-life from minutes to hours) and mainly of productively infected CD4+ T cells (half-life of 1-2 days; (Ho et al. 1995; Perelson et al. 1997; Perelson et al. 1996; Wei et al. 1995)). Consequently, Phase II corresponds to the decay of cells more resistant to HIV-induced cytopathic effect (partially activated T cells and cells of the monocyte-macrophage lineage with half-life of 2-4 weeks; (Perelson et al. 1997; Shan and Siliciano 2013)). The following Phase III with a very low decay kinetics (half-life of 273 days) proceeds after several years into Phase IV with a remaining stable low level plasma viremia that does not decrease any more (Hilldorfer et al. 2012; Maldarelli et al. 2007). Despite the very low plasma viremia below the limit of clinical assays (< 50 copies/ml), which is detectable with only ultrasensitive assays (about 3 copies/ml), cell-associated proviral DNA (prDNA) and RNA (caRNA) is commonly detected by PCR-based assays in PBMCs during this phase (Palmer et al. 2008; Palmer et al. 2003) (Fig. 2). The presence of the Phase IV indicates the existence of additional cell populations that do not succumb to virus-induced cytopathic effects and/or are either refractory to cART (proviral DNA or transcription cannot be targeted

by cART) or persist in anatomical sanctuaria that are not accessed by cART. Reactivation of the latently infected cells, that both persist and are replenished by homeostatic proliferation (Chomont et al. 2009; Kim and Siliciano 2016), is viewed as the most relevant source of the new HIV RNA (Shan and Siliciano 2013). However, lower levels of antiretrovirals (ARV) found in anatomical/immunological sanctuaria like brain, genital tract, gut mucosa or lymph nodes can support the ongoing HIV replication (Fletcher et al. 2014; Huang et al. 2016; Massanella et al. 2016). Lately, it was described that SIV/HIV-1 may continue to replicate within B-cell follicles due to follicular exclusion of CD8+ T cells and dysregulated responses of follicular regulatory T cells (TFR) and follicular T helper cells (TFH; (Fukazawa et al. 2015; Miles et al. 2015; Saison et al. 2014; Tran et al. 2008)). In addition to latently infected long-lived memory T-cells and tissue resident macrophages (Avalos et al. 2016) (Gludish et al. 2015), an evidence for existence of tissue resident memory T cells (TRM) is emerging (Farber 2015; Farber et al. 2014).

Another cause of the residual low-level HIV replication under cART is believed to involve the immune hyperactivation induced by various mechanisms. In addition to the residual productive infection that is likely to rise some immune responses, there is a much more frequent (95%) abortive infection of resting non-permissive CD4+ T cells. Due to the cytoplasmic DNA-sensing mechanisms like IFI-16, cGAS, and PQBP-1 (Gao et al. 2013; Thompson et al. 2014; Yoh et al. 2015) these cells might die by pyroptosis, a very inflammatory type of programmed cell death (Doitsh et al. 2014; Monroe et al. 2014). Further, HIV-1 replication and disruption of the intestinal barrier lead to microbial translocation, stimulation of innate immune responses and depletion of CD4 Th17 cells, a defect persisting even after initiation of cART (Chege et al. 2011; Schuetz et al. 2014). Finally, the activation and dysregulated functions of regulatory T cells (Tregs), critical modulators of immune responses, apparently also contribute the immune hyperactivation (Mendez-Lagares et al. 2014; Saison et al. 2014). The residual low-level HIV replication that leads to an elevated immune activation thus further stimulates HIV replication, generating a vicious cycle.

In summary, HIV persistence during cART is supported by homeostatic and antigen-induced proliferation of latently infected cells, ongoing replication in the

sanctuaries as well as by increased immune activation and inflammation (Chomont et al. 2009; Fukazawa et al. 2015; Kim and Siliciano 2016; Van Lint et al. 2013). Additionally, the latent reservoir is readily being replenished during episodes of viraemia due to a treatment failure or other conditions of incomplete or missing pharmaceutical control.

Models of HIV latency

Our knowledge of the mechanisms, maintenance and reactivation of the latent reservoir is based namely on various in vitro and in vivo models as the presence of latently infected cells in human body is very low, around 1-10 millions in the whole organism, and difficult to study (Crooks et al. 2015; Finzi et al. 1999; Massanella and Richman 2016; Siliciano et al. 2003).

Individual models of HIV latency reproduce to some extent certain aspects of the complex situation in vivo and allow for studies of certain latently infected populations or specific aspects of latently infected cells. Models used to study HIV-1 latency in vitro include HIV-infected cell lines (immortalized lymphocytic or monocytic cells like J-Lat, ACH-2, U1; (Clouse et al. 1989; Folks et al. 1989; Folks et al. 1987; Jordan et al. 2003), primary cell models (derived from HIV-1 negative donor PBMCs by infection with a particular HIV-1 isolate or recombinant) and resting CD4 cells (derived from HIV-1 infected patients; (Spina et al. 2013)). While the cell lines are commonly available and easy to handle, they reveal several aspects that often make them behave differently from the situation in vivo (namely clonality based on the integration site or mutations of specific HIV-1 or host-cell sequences).

Primary cell models might seem to be closer to the situation in vivo while the percentage of latently infected cells available for further studies is much higher than in vivo. Different primary cell models developed by different laboratories use either resting or activated CD4+ T cells that are infected with wild-type (typically NL4-3) or recombinant HIV-1 (often expressing a fluorescent reporter gene like EGFP) and treated with combinations of several cytokines or chemokines. After establishment of the infection, latency is induced by ARVs followed by reactivation by different means. The individual models differ in the target cell population, percentage of latently

infected cells generated, type and time of readout (Bosque and Planelles 2009; Gondois-Rey et al. 2006; Lassen et al. 2012; Saleh et al. 2007; Spina et al. 2013; Tyagi et al. 2010; Yang et al. 2009b). Nevertheless, it is questionable how closely these models reflect the situation in vivo and how much they are biased due to the experimental setup and mode of readout.

Resting CD4 cells derived from HIV-1 infected patients cultured and stimulated ex vivo thus seem to be the most relevant in vitro model. However their use is limited by a very low presence of latently infected cells, high background rate of defective integrated proviruses and difficulties of any analysis due to a very high background of uninfected cells (problems with sensitivity and specificity).

\The in vivo models of HIV latency include namely different types of humanized mice and macaques infected with HIV, SIV or various recombinants. The advantage of humanized mice consists in their relative affordability and ease of handling; on the other hand, their preparation is tedious while graft-versus-host disease (GvHD) and other differences, namely due to their genetic background, the way of immune reconstitution with human tissues, and lineage precursors may limit their use and relevance of the results.

Humanized mice used for HIV research were originally based on SCID (severe combined immunodeficiency) mice that were irradiated and then transplanted with foetal human thymus and liver (SCID-hu Thy/Liv). In this model, latent infection is established during thymopoiesis (deactivation phase), leading to generation of latently infected naïve CD4+ T-cells. Among other limitations, this model does not provide an efficient peripheral reconstitution and human cells are found in relatively small numbers (Brooks et al. 2001; Marsden et al. 2012).

Lately, BLT (bone marrow-liver-thymus) mice are considered as a better model for complex studies of HIV reservoirs and latency reversing agents (LRA) as they provide a robust peripheral reconstitution. These mice are most commonly based on irradiated NSG (NOD/SCID-gamma chain null) mice transplanted with foetal human thymus and liver and then reconstituted with bone marrow or purified CD34+ stem cells (Donahue and Wainberg 2013). This particular combination results

in high-level systemic reconstitution of all human leukocyte lineages with improved T-cell maturation and selection in a thymic environment and in generation of latently infected naïve and resting and CD4+ T-cells (Denton et al. 2012). There are also modifications of these mice using reconstitution with only discrete cell types like T cells or macrophages (ToM, T-cell only mice; MoM, myeloid-only mice; (Honeycutt et al. 2013; Honeycutt et al. 2016)), allowing studies of the role of the individual cell types in the establishment of latent reservoir or reactivation.

Although the use of BLT mouse model is valuable for the HIV studies, its important limitation consists in the development of GvHD, typically around 6 months after engraftment (Karpel et al. 2015). Further, these mice are unable to develop proper HIV-specific adaptive immune responses consisting in high levels of hyper-mutated, class-switched IgG antibodies as human cells of non-haematopoietic origin, namely those giving rise to stromal cells, are not transplanted and secondary lymphoid organs do not properly develop (Malhotra et al. 2013; Wang et al. 2011).

Yet another BLT model, based on human Rag2$^{-/-}\gamma c^{-/-}$ mice (Choudhary et al. 2012; Traggiai et al. 2004; Zhang et al. 2007) was recently developed and led to identification of central memory CD4+ T-cells (TCM) as the main latently infected population after suppressive cART similarly as in human (Choudhary et al. 2012; Donahue and Wainberg 2013). This model was further improved by generation of humanized triple knock-out (TKO) BLT mice on a C57Bl/6 background (C57BL/6 Rag2$^{-/-}\gamma c^{-/-}$ CD47$^{-/-}$) in which the GvDH was much reduced, complement was functional and secondary lymphoid organs with a well organized architecture and virus-specific immune responses were developed (Lavender et al. 2013).

A model closest to HIV-1 infection in human is infection of non-human primates (NHP), namely rhesus macaques, with various SIV strains. It is believed that different SIVs crossed the species barrier into humans many times; namely SIVsmm naturally infecting sooty mangabeys and SIVcpz infecting chimpanzees resulted in HIV-2 and certain clades of HIV-1 (Hirsch et al. 1989; Huet et al. 1990; Sharp and Hahn 2011).

The NHP models reveal many important features comparable to HIV-1 infected humans like anatomy, physiology, immune system, infectious agent itself, and susceptibility to antiretroviral treatment (Gardner and Luciw 2008; Policicchio et al. 2016). The use of NHP models has been essential for understanding pathogenesis of HIV/AIDS as well as for studies of different therapeutic and vaccination approaches. Many conclusions could be also inferred by comparing immune responses to SIV infection in the natural and unnatural hosts, soothy mangabeys and rhesus macaques, respectively, in which the SIV infection results in non-pathogenic or pathogenic conditions. There is a high viremia in both hosts, but a very little loss of CD4+ T cells and preservation of architecture and function of lymph nodes in the natural host. In the gut of the natural host, CD4+ T cells are only moderately depleted and numbers and functions of Th17 cells remain preserved, while microbial translocation is lacking. Further, TFH and TCM are only weakly infected (Ploquin et al. 2016).

Additionally, recombinant viruses like RT-SHIV or SHIV containing different parts of HIV-1 genome can be used to overcome certain differences between HIV-1 and SIVs e.g. in studies involving inhibitors specific for human reverse transcriptase (Jiang et al. 2009; Ndung'u et al. 2001). One important difference in comparison with HIV-1 is that most SIVs (and HIV-2) encode a vpx protein that allows virus infection also in non-dividing cells as it targets for degradation cellular SAMHD-1, an enzyme that would hydrolyze dNTPs and thus decrease reverse transcription (Hofmann et al. 2012). Despite many advantages, a major drawback of using macaques consists in their cost and ethical and legal regulations of their use.

Obviously, the most relevant approach to study HIV latency and reactivation in HIV-infected human patients is in clinical studies. However, performance of clinical studies is strictly regulated, expensive and must be preceded by extensive pre-clinical testing (including animal models). Additionally, human studies never provide really homogeneous and reproducible experimental settings with all necessary controls.

In summary, the results obtained in various in vitro and in vivo models and in human patients indicate that the latent HIV-1 reservoir is represented mainly by latently infected resting CD4+ T cells, long-lived central memory $CD4^+$ T cells (TCM cells) and transitional memory $CD4^+$ T cells (TTM cells). In addition to these cells, latent reservoir may comprise also other cell populations like CD34+ hematopoietic progenitor cells, naïve CD4+ T cells, CD4+ memory stem cells (TSCM cells) or $\gamma\delta$ T cells, as well as myeloid cells like macrophages and dendritic cells (Buzon et al. 2014; Carter et al. 2010; Chomont et al. 2009; Honeycutt et al. 2016; Soriano-Sarabia et al. 2015; Wightman et al. 2010). The importance of these cells constituting the latent reservoir consists in their different survival and stability as well as in different requirements for signaling and activation, and thus HIV-1 provirus induction (Archin et al. 2014).

Assessment of the size of the latent reservoir

Under suppressive cART, plasma viremia (virion-associated unspliced RNA) is undetectable or below the detection limit of common commercial assays (<50 copies/ml). However, markers of immune hyperactivation persist and cell-associated HIV RNA can be readily detected both in peripheral blood and tissues like lymph nodes, tonsils, gut or testes. Thus, in order to determine the effect of treatment intensification strategies and namely efficiency of LRA explored for therapeutic reactivation, availability of specific and sensitive methods allowing an accurate assessment of the size of the latent reservoir is crucial (Fig. 3).

The latently infected cells can be defined as cells harboring quiescent, replication-competent provirus. A gold standard in determination of latently infected cells is a quantitative virus outgrowth assay (qVOA) that is very material-, labor- and cost-demanding. It requires large volumes of patients blood to isolate sufficient numbers of highly purified latently infected resting CD4+ T cells, and plate them in serial dilutions along with activated donor PBMCs (CD8 negative) or with a MOLT4/CCR5 cell line. It requires 2-3-week incubation with changes of medium and other additives (Finzi et al. 1997; Massanella and Richman 2016; Siliciano and Siliciano 2005). However, this assay apparently underestimates the size of the latent

pool, as it has been already demonstrated that consecutive rounds of stimulation/reactivation of the isolated cells could lead to increased proportion of the reactivated proviruses. The replication competent, but non-inducible proviruses bias further this assay (Bruner et al. 2015; Ho et al. 2013). An analogue of this assay, Mouse virus outgrowth assay (MVOA), allows to determine virus outgrowth in humanized mice. It has been recently described to detect latently infected cells with higher sensitivity than the standard qVOA as a large number of cells can be used and a GvHD promotes HIV-1 reactivation. Nevertheless, it provides only qualitative results (Metcalf Pate et al. 2015).

At the other side is a PCR-based determination of the cell-associated DNA, which apparently overestimates the size of the latent pool, as most of the integrated proviruses are mutated or incapable of reactivation for unknown reasons (Ho et al. 2013; Sanchez et al. 1997). Depending on the primer/probe sequences, total, integrated or 2-LTR DNA can be commonly detected (Murray et al. 2014; Pasternak et al. 2013). Finally, determination of cell-associated RNA (caRNA) using various combinations of PCR-based techniques like semi-nested RT-qPCR or ddPCR (Bullen et al. 2014; Kiselinova et al. 2014) seems to provide a more relevant marker of viral persistence and/or reactivation. However, it is important to use approaches distinguishing between commonly present prematurely terminated short gag transcripts, multiply-spliced transcripts (msRNA) and unspliced RNA (usRNA). Levels of usRNA are generally higher, and therefore better detectable than msRNA. On the other hand, determination of msRNA (e.g tat/rev; (Pasternak et al. 2008)) or correctly terminated viral transcripts (using primers/probe detecting the polyadenylated tail; (Shan et al. 2013) better correlate with the ability of the cell to produce infectious viruses (Bullen et al. 2014; Pasternak et al. 2013).

Based on the PCR detection of tat/rev spliced transcripts, a new quantitative assay allowing single-cell based determination of the inducible viral reservoir called TILDA (Tat/rev Induced Limiting Dilution Assay) was recently presented. The advantage of this assay is use of only 10 ml of blood, serial limiting dilutions allowing detection of even single positive cell, and a good correlation with qVOA (Procopio et al. 2015).

A disadvantage of all PCR-based assays is their inability to differentiate between RNA that might remain retained in the nucleus and for this or other reasons not to be translated into a protein, and RNA giving rise to HIV proteins that could be presented on the cell surface or constitute new virions (Lassen et al. 2006). Therefore, determination of cell-free RNA (cfRNA) or p24 Ag in culture supernatant better estimates virion production. On the other hand, proportion of released virions and detectable cfRNA is much lower in comparison with caRNA (1.5 and 7%, respectively; (Cillo et al. 2014).

This problem can be partially solved also by a new assay termed Prime Flow RNA that combines cell-based detection of proteins with antibodies and detection of intracellular RNA with specific probes. It was reported to detect 1 infected cell in 10^4-10^5 cells (comparable numbers found in peripheral blood of patients on cART) (Romerio and Zapata 2015).

Apparently, highly sensitive assay for determination of HIV proteins in culture supernatant is necessary for a better assessment of the efficiency of LRA. One such fully automated assay based on a Quanterix Simoa technology could detect low levels of p24 Ag in cell lysates (3 pg/ml; (Howell et al. 2015).

Mechanisms of establishment and maintenance of the latent reservoir

There are two types of HIV latency. A pre-integration latency that occurs after infection of non-permissive cells, which is short-lived as unintegrated viral DNA is recognized by cytoplasmic DNA sensors like cGAS or IFI-16 leading to activation of interferon and inflammasome responses (Gao et al. 2013; Thompson et al. 2014; Yoh et al. 2015). On the other hand, the post-integration latency occurring in cells that become quiescent after the infection is the cause of virus persistence. The latent reservoir is established very early during the HIV-1 infection and its size can be limited by an early introduction of cART. It consists of both T-cells and myeloid cells (Marban et al. 2007; Wires et al. 2012) and it is very stable with half-life determined by qVOA of about 44 months (Crooks et al. 2015; Siliciano et al. 2003).

A key determinant of the future fate of the HIV-1 provirus is the site of its integration. Most commonly, sites of HIV-1 insertion are found in intragenic regions of actively transcribed genes (Lewinski et al. 2006; Serrao and Engelman 2016). Perhaps for this reason, the majority of repressed but inducible proviruses is also located within the introns of the expressed genes (Lewinski et al. 2005; Shan et al. 2011). The establishment of HIV latency is thus regulated independently of the control of expression of the target host genes (Mbonye and Karn 2014).

Insertion of the HIV-1 provirus in the actively transcribed genes may result in transcriptional interference, contributing to the regulation of HIV-1 latency. Divergent orientation of the cellular promoter and viral LTR can lead to the lack of recruitment of transcription factors, while convergent promoters may lead to a collision of the transcription machinery and premature termination of HIV-1 transcription. Parallel orientation of the HIV LTR located downstream of the cellular promotor can lead to the viral promotor occlusion by a readtrough transcription from the cellular gene, displacing key transcription factors on the HIV LTR (Han et al. 2008; Lenasi et al. 2011). In latently infected cells, a preference for a parallel orientation of the promotors was observed, while there was no preference in acutely or persistently infected cells, suggesting that transcriptional interference may be one of the important factors in the establishment and maintenance of HIV-1 latency (Shan et al. 2011).

HIV-1 itself does not encode any specific transcription repressors, but high levels of HIV-1 transcription activator Tat and its interactions with a cellular cofactor p-TEFb, resolving promoter-proximal pausing of RNA polymerase II (RNAP II), are absolutely critical for the provirus expression (Yamada et al. 2006). In the absence of Tat-pTEFb complex, transcription efficiency decreases drastically and only short Gag transcripts are generated (Kao et al. 1987; Lassen et al. 2004; Price 2000). Transcriptional silencing of the proviruses thus results from a series of epigenetic and non-epigenetic changes occurring at the promoter region and from processes during the transcription initiation and elongation phases that decrease levels of Tat and availability and/or binding of cellular factors (Mbonye and Karn 2014).

In the activated CD4+ T cells, which are productively infected most often, the intracellular milieu with high levels of cellular transcription factors, namely transcription initiation factors NF-κB, NFAT, and AP-1, drives HIV expression (Mbonye and Karn 2014). However, as the host cell returns to the resting memory phenotype, cytoplasmic sequestration of these factors causes a significant decrease of transcription initiation at the HIV LTR and allows transcriptional silencing of the provirus, possibly with help of transcriptional inhibitors (Bodor 2006; He and Margolis 2002; Tyagi and Karn 2007).

The chromatin structure of the proviral 5‘ LTR is a critical parameter in control of HIV expression. Regardless of the site of insertion, 5‘ HIV LTR is occupied by nucleosomes Nuc-0 and Nuc-1 in specific positions at the start site, imposing a block on RNAP II initiation (Verdin et al. 1993). Several negative DNA-binding factors factors (e.g. CBF1, YY1, LSF, BRD2, p50 homodimer (He and Margolis 2002; Karn 2013; Tyagi and Karn 2007; Williams et al. 2006)) then facilitate recruiting of other repressor complexes and histone and DNA-modifying enzymes at both core promoter and enhancer regions. Histones of the nucleosomes at the 5‘LTR of silent proviruses are deacetylated and trimethylated, which is a feature of the repressive heterochromatin. Further, histone methyltransferases (HMTs) can be found associated with the latent proviral LTR (du Chene et al. 2007; Friedman et al. 2011; Imai et al. 2010; Keedy et al. 2009). Namely, a dominant HMT EZH2 constitutes part of the polycomb repressive complex PRC2 which serves as a binding platform for additional chromatin-modifying enzymes, histone deacetylases (HDACs) and DNA methyltransferases (DNMTs)(Cheng et al. 2011; Friedman et al. 2011; Tae et al. 2011; Vire et al. 2006). Methylation of DNA (CpG islands; (Bednarik et al. 1990)) at transcription start site has been suggested to be the most stable modification of the latent provirus LTR. It might stabilize DNA and prevent provirus reactivation (Blazkova et al. 2009; Kauder et al. 2009). Lately, relative frequency of proviruses with a higher LTR DNA methylation was suggested to be increased by a prolonged ARV treatment or multiple rounds of reactivation (Trejbalova et al. 2016).

An additional block in HIV expression may consist in post-transcriptional processes inhibiting HIV-1 protein expression. Namely, both unspliced and spliced

HIV-1 transcripts may be retained in nuclei. The export of HIV-1 transcritps is supported by binding of Rev to RRE (Rev response element) present in partially spliced and unspliced genomic HIV-1 RNAs and by the interactions with Exportin 1 (Crm-1), a nuclear export factor. Further association with PTB (polypyrimidine tract binding protein) and related factors seems to affect the export efficiency. Thus, unavailability of either factor may promote the retention of HIV-1 RNAs in the nucleus. (Kula et al. 2013; Lassen et al. 2004; Lassen et al. 2006; Zolotukhin et al. 2003).

Further, several cellular miRNAs have been reported to modulate HIV-1 expression by targeting essential cellular cofactors involved in HIV-1 transcription like PCAF and cyclin T1. The former is targeted by miR175p and miR-20a, while the latter by miR 27b and miR198 (Sung and Rice 2009; Triboulet et al. 2007). There are also several cellular miRNAs (e.g. miR-28, miR-125b, miR-150, miR-223, and miR-382) that recognize the 3'-end of HIV-1 mRNAs, repressing their expression in resting CD4+ T cells (Huang et al. 2007). Finally, there are miRNAs derived from viral sequences (vmiRNAs), namely from TAR and Nef (miRTAR5p/3p and miRN367, respectively; (Bennasser et al. 2004; Klase et al. 2007; Omoto et al. 2004; Schopman et al. 2012). Both cellular and viral miRNAs may cause HIV-1 RNA degradation or inefficient expression of HIV-1 proteins.

Mechanisms of reactivation

The reactivation of the functional, inducible latent provirus depends on the chromatin status and availability of the cellular transcription factors. Upon appropriate stimulation and nuclear translocation, NF-κB and NFAT can bind to HIV LTR if the chromatin landscape of the promoter region is favorable (Bhatt and Ghosh 2014). The accessibility of the HIV LTR can be affected by methylation of CpG islands (Bednarik et al. 1990) or binding of other transcription factors like Sp1 that are able to promote the chromatin configuration favorable for binding of the main transcription factors. In fact, Sp1 is required for the formation of the pre-initiation complex and interacts with NF-κB (Perkins et al. 1993). NF-κB and NFAT probably bind in a mutually exclusive, possibly sequential way, as their HIV-1 LTR-binding

sites overlap (Giffin et al. 2003; Mbonye and Karn 2014). NF-κB is found in cell lines and primary naïve T-cells, while NFAT is typically present in memory T-cells (Dienz et al. 2007). In primary memory T-cells, NFAT and Lck are required for optimal latent virus reactivation and HIV-1 can be activated in an NF-kB-independent way by transcription factor DVII-Ets-1, without causing significant T cell activation (Bosque et al. 2011; Bosque and Planelles 2009; Yang et al. 2009a). On the other hand, NFAT is dispensable in Jurkat cell models. NF-κB is commonly activated by PMA or TNF-α, while NFAT is activated by calcium/calcineurin signaling (Bosque and Planelles 2009; Chan et al. 2013; Kim et al. 2011) (Bosque and Planelles,2009; Kim etal.,2011; Chan etal.,2013).

Both NF-κB and NFAT recruit CBP/p300 and other histon acetyl transferases (HATs) (Garcia-Rodriguez and Rao 1998) to further acetylate Nuc-1 and to attract SWI/SNF chromatin remodeling complex. After the minimal initiation and transcription through the TAR element, RNAP II pauses. If Tat and p-TEFb, composed of CDK9 and cyclin T1 (Herrmann and Rice 1995; Wei et al. 1998), are available to bind and form a complex with TAR RNA hairpin and the transcription machinery, kinase activity of p-TEFb mediates phosphorylation of negative elongation factors (DSIF and NELF) and of RNAP II and allows formation of a superelongation complex and continuation of transcription further into the elongation phase. Thus, Tat transactivation and its interaction with p-TEFb is absolutely necessary for the efficient transcription elongation of the HIV provirus. When not complexed with Tat, pTEFb is sequestered and held inactive in the transcriptionally inactive 7SK RNP complex containing 7SK small nuclear RNA (7SK snRNA), inhibitory factor HEXIM1 and RNA binding proteins LARP7 and MePCE. Yet, Tat may be out-competed by BRD4 in binding to p-TEFb, leading to p-TEFb targeting to transcription of cellular genes (Mbonye and Karn 2014).

Latency reversal and purging HIV-1

Latently infected cells represent the major barrier to HIV cure of HIV-1 (Donahue and Wainberg 2013). The major reservoir is considered to reside in resting

memory T cells and therefore this population is in the focus of most efforts to decrease the size of the latent reservoir (Bruner et al. 2015).

The initial attempts to eradicate HIV-1 latently infected cells were first described by Fauci et al. (Chun et al. 1998; Chun and Fauci 1999). Later, the term “shock and kill” strategy was introduced (Archin et al. 2012; Deeks 2012). In short, it consists in the attempts to reactivate a dormant provirus silently present in latently infected cells, namely long-lived memory CD4 T-cell, that would lead to death of the cells harboring the latent provirus and decrease the size of the latent pool in the presence of cART. Originally, it was assumed that virus reactivation could be achieved with a single agent, namely HDAC inhibitors (HDACi) or PKC inducers, and that the replication of the reactivated virus and the virus-induced cytopathic effects would be sufficient to kill the host cells, and thus decrease the size of the latent reservoir. Today, it is largely accepted that combinations of two or more agents with different mechanism of action together with an additional stimulation of anti-HIV immune responses would be necessary. Namely, improvement of cytotoxic responses using engineered dual-affinity (DART) and broadly specific antibodies (bNAbs) towards HIV-1 are intensively investigated (Caskey et al. 2015; Sloan et al. 2015). Still, there are doubts if the HIV cure can be ever achieved because of unknown fraction of the cells remaining refractory to the reactivation strategies. Further, it has been suggested that a prolonged antiretroviral treatment together with random or intentional cycles of reactivation might lead to an increased methylation of HIV-LTR and thus stabilization of the latent provirus (Trejbalova et al. 2016).

Activity of LRA is usually identified in vitro in tissue culture screens involving various cell lines and primary cells. The efficiency of the selected compounds must then be verified in vivo. Most intensively investigated LRA, namely those in clinical trials, involve compounds in use or under development for treatment of various cancers and other diseases. These are especially HDACi and PKC agonists (NF-κB inducers). There are 11 HDACs subdivided in four classes (Mottamal et al. 2015; Wang et al. 2009). Particularly HDACs 1-3 (Class I) seem to be important in maintaining HIV latency (Keedy et al. 2009). HDACi non-specifically activate transcription of many genes by increasing acetylation of the promotor regions,

including HIV LTR. Increased acetylation should modify chromatin status and allow for binding of transcription factors, namely NF-κB and NFAT. Among these, vorinostat (SAHA) has been the most extensively explored, but newer compounds with higher potency like givinostat, panobinostat or romidepsin seem more promising. Nevertheless, none of these agents alone was able to induce viral outgrowth from cells isolated from HIV-1-infected patients on cART (Bullen et al. 2014). On the other hand in clinical studies, vorinostat and some other compounds were able to increase levels of caRNA, but the size of the latent reservoir was not found decreased, suggesting that combination with other agents or strategies increasing immunological killing of the infected cells would be necessary (Rasmussen et al. 2013). Other classes of chromatin-modifying enzyme inhibitors include inhibitors of histone methyltransferases (HMTi) like Chaetocin, BIX-01294 or GSK343, or inhibitors of DNA methyltransferases (DNMTi) like 5-aza-2′deoxycytidine. However, they are more likely to be effective in combination with other LRA (Kumar et al. 2015).

The other important class of LRA represent PKC agonists that induce activation and nuclear translocation NF-κB and p-TEFb. They can also trigger activation of MAPK and nuclear translocation of AP1. The natural PKC inducer effective in HIV-1 reactivation is TNF-α, a cytokine found increased in untreated HIV-1 infection. However, due to its pro-inflammatory pleiotropic effects, its use as a LRA in vivo cannot be considered. Similarly the other very effective agent of this class, phorbol myristyl acetate (PMA), cannot be considered for therapy as it reveals a strong tumorigenic potential (Kumar et al. 2015). However, newer PKC inducers include nontumorigenic phorbol ester prostratin (Biancotto et al. 2004; Kulkosky et al. 2001), macrolide lactone bryostatin-1 (Mehla et al. 2010) or diterpenoids ingenol B and ingenol-3-angelate (Jiang et al. 2014; Jiang et al. 2015). PKC inducers downregulate expression of cell surface receptors CD4, CXCR4 or CCR5 in uninfected cells, thus limiting the spread of the newly released virus (Hezareh et al. 2004; Jiang et al. 2014; Mehla et al. 2010). Further, PKC was reported to phosphorylate HEXIM1, suggesting that PKC inducers might affect also this inhibitory protein (Fujinaga et al. 2012). Hexim phosphorylation is commonly mediated by AKT kinase that can be stimulated by HMBA (Contreras et al. 2007),

leading to the release of p-TEFb from the inhibitory complex 7SK RNP. Yet another compound affecting availability of p-TEFb is JQ1, bromodomain inhibitor affecting factors BRD2 and BRD4 (Boehm et al. 2013).

Disulfiram, the inhibitor of acetaldehyde dehydrogenase used in therapy of alcohol abuse, induces degradation of PTEN, again allowing AKT-mediated phosphorylation of HEXIM1 (Doyon et al. 2013; Xing et al. 2011). This compound has been described as an effective LRA in vitro in a relatively artificial model of primary CD4+ T cells immortalized with the Bcl-2 protooncogene. However, the expectations of its potency were not fulfilled as there was no significant effect on the size of the latent reservoir found in vivo (Spivak et al. 2014).

Apart of these mainstream studies, there are many other approaches toward latency reversal and HIV cure. Of these, use of a pro-oxidant gold-based drug Auranofin is of interest as it was shown to induce a partially selective killing of TCM and TTM CD4+ T cells, the main reservoir cells containing the latent HIV-1 (Lewis et al. 2011). Consequently, it was demonstrated that TCM and TTM CD4+ T lymphocytes are more susceptible to the redox stress and apoptosis (Chirullo et al. 2013).

Our laboratory has demonstrated independently that Normosang, a hemin containing compound used to treat acute hepatic porphyria, could strongly potentiate reactivation of the latent provirus induced by PKC inducers like PMA, TNF-α, prostratin or Bryostatin-1, while it inhibits HIV-1 replication during the acute infection through its effect on reverse transcription. The stimulatory effects of Normosang are mediated by a heme/iron-mediated Fenton reaction resulting in the increased redox stress, while there was no effect on the activation status of the cells (Melkova et al. 2015a; Melkova et al. 2015b; Shankaran et al. 2011). Based on our results, we propose a model in which redox-modulating agents induce chromatin remodeling, affect binding of specific transcription factors to HIV-LTR and potentiate HIV-1 expression induced by PKC or other inducers (Melkova et al. 2015a; Melkova et al. 2015b).

Historically, there is a case of one HIV+ patient that was administered Normosang and consequently remained p24-negative for several months (Pavel Martasek, Faculty General Hospital Prague, personal communication). We suggest that the outcome in this particular patient resulted from the inhibition of reverse transcription by heme (Levere et al. 1991) together with a short-term reactivation and death of the infected cells due to heme/iron-mediated redox stress. Consequently, a stable heme degradation product, antioxidant bilirubin, might have mediated prolonged inhibitory effects on HIV-1 reactivation (Melkova et al. 2015a; Melkova et al. 2015b). This scenario would be compatible with the hypothesis of (Chirullo et al. 2013) that auranofin decreases numbers of latently infected TCM and TTM CD4+ T cells by its pro-oxidant effects, and thus reduces size of the HIV latent pool.

The idea of possible involvement and use of redox stress to purge the latent reservoir is further supported by work of (Iordanskiy et al. 2015) showing that ionizing radiation alone was sufficient to activate the HIV-1 LTR and to effectively kill the infected T cells. Consequently, this group proposed that a low-dose ionizing radiation could be used therapeutically to reactivate and kill latently infected reservoir cells (Iordanskiy and Kashanchi 2016). Additionally in the Berlin Patient, the only known case of HIV-1 cure, high-dose irradiation was used after bone marrow transplantation from donor harboring CCR5□32 mutation with missing CCR5 expression, while irradiation was omitted in Boston patients in which virus rebound occurred (Henrich et al. 2013; Hutter et al. 2015).

Finally, any common infection involves increased generation of reactive oxygen and nitrogen species. Co-infections are well-known to increase HIV-1 replication and promote its spread (Modjarrad and Vermund 2010), while decreased levels of GSH, indicator of an increased redox stress, have been described early in HIV infection (Pace and Leaf 1995). Reactive oxygen and nitrogen species were shown to stimulate activation of the redox-sensitive transcription factor NF-κB and LTR-driven expression of reporter genes (Jimenez et al. 2001; Pyo et al. 2008). Thus, increased generation of free radicals is likely to be helpful in the attempts to eliminate the latent HIV-1 in the presence of cART.

A major concern when considering testing of LRA in vivo is the induction of immune hyperstimulation upon reactivation of the latent HIV-1 and development of a harmful cytokine storm. On the other hand, it is not clear if any significant latency reversal is achievable without increased cytokine levels (Marsden et al. 2015). However, specific anti-HIV immune responses are generally hampered or dysfunctional. Further, it was shown that both HDACi (romidepsin, panobinostat) and PKC agonists (prostratin and Bryostatin-1, but not Ingenol B) inhibited HIV-specifc T cell proliferation (Clutton et al. 2015).

Based on clinical studies, it has been widely accepted that stimulation or improved function of immune responses in addition to LRA would be necessary in order to achieve death of reactivated cells and thus decrease the size of the latent reservoir. Synergistic effects of different agents should also decrease the doses and/or negative side effects of individual compounds.

Summary/Conclusions

In conclusion, in view of the currently described sources of the remaining low-level HIV-1 replication in the presence of suppressive cART, additional mechanisms and processes promoting HIV-1 persistence remain to be identified. Any attempt to achieve HIV-1 cure should involve combination of several LRA with different mechanism of action together with stimulation or improvement of immune responses towards infected cells. To validate the efficiency of individual approaches, development of sufficiently sensitive and specific methods allowing accurate determination of the size of the latent reservoir and its changes are necessary.

References

Archin NM et al. (2012) Administration of vorinostat disrupts HIV-1 latency in patients on antiretroviral therapy Nature 487:482-485 doi:10.1038/nature11286

Archin NM, Sung JM, Garrido C, Soriano-Sarabia N, Margolis DM (2014) Eradicating HIV-1 infection: seeking to clear a persistent pathogen Nature reviews Microbiology 12:750-764 doi:10.1038/nrmicro3352

Avalos CR et al. (2016) Quantitation of Productively Infected Monocytes and Macrophages of SIV-Infected Macaques Journal of virology doi:10.1128/JVI.00290-16

Bednarik DP, Cook JA, Pitha PM (1990) Inactivation of the HIV LTR by DNA CpG methylation: evidence for a role in latency The EMBO journal 9:1157-1164

Bennasser Y, Le SY, Yeung ML, Jeang KT (2004) HIV-1 encoded candidate micro-RNAs and their cellular targets Retrovirology 1:43 doi:10.1186/1742-4690-1-43

Bhatt D, Ghosh S (2014) Regulation of the NF-kappaB-Mediated Transcription of Inflammatory Genes Frontiers in immunology 5:71 doi:10.3389/fimmu.2014.00071

Biancotto A et al. (2004) Dual role of prostratin in inhibition of infection and reactivation of human immunodeficiency virus from latency in primary blood lymphocytes and lymphoid tissue Journal of virology 78:10507-10515 doi:10.1128/JVI.78.19.10507-10515.2004

Blazkova J et al. (2009) CpG methylation controls reactivation of HIV from latency PLoS pathogens 5:e1000554 doi:10.1371/journal.ppat.1000554

Bodor J (2006) Regulation of HIV-1 and IL-2 transcription by inducible CAMP early repressor (ICER) Retrovirology 3 Suppl 1:S81

Boehm D et al. (2013) BET bromodomain-targeting compounds reactivate HIV from latency via a Tat-independent mechanism Cell cycle 12:452-462 doi:10.4161/cc.23309

Bosque A, Famiglietti M, Weyrich AS, Goulston C, Planelles V (2011) Homeostatic proliferation fails to efficiently reactivate HIV-1 latently infected central memory CD4+ T cells PLoS pathogens 7:e1002288 doi:10.1371/journal.ppat.1002288

Bosque A, Planelles V (2009) Induction of HIV-1 latency and reactivation in primary memory CD4+ T cells Blood 113:58-65 doi:10.1182/blood-2008-07-168393

Brooks DG, Kitchen SG, Kitchen CM, Scripture-Adams DD, Zack JA (2001) Generation of HIV latency during thymopoiesis Nature medicine 7:459-464 doi:10.1038/86531

Bruner KM, Hosmane NN, Siliciano RF (2015) Towards an HIV-1 cure: measuring the latent reservoir Trends in microbiology 23:192-203 doi:10.1016/j.tim.2015.01.013

Bullen CK, Laird GM, Durand CM, Siliciano JD, Siliciano RF (2014) New ex vivo approaches distinguish effective and ineffective single agents for reversing HIV-1 latency in vivo Nature medicine 20:425-429 doi:10.1038/nm.3489

Buzon MJ et al. (2014) HIV-1 persistence in CD4+ T cells with stem cell-like properties Nature medicine 20:139-142 doi:10.1038/nm.3445

Carter CC, Onafuwa-Nuga A, McNamara LA, Riddell Jt, Bixby D, Savona MR, Collins KL (2010) HIV-1 infects multipotent progenitor cells causing cell death and establishing latent cellular reservoirs Nature medicine 16:446-451 doi:10.1038/nm.2109

Caskey M et al. (2015) Viraemia suppressed in HIV-1-infected humans by broadly neutralizing antibody 3BNC117 Nature 522:487-491 doi:10.1038/nature14411

Chan JK, Bhattacharyya D, Lassen KG, Ruelas D, Greene WC (2013) Calcium/calcineurin synergizes with prostratin to promote NF-kappaB dependent activation of latent HIV PloS one 8:e77749 doi:10.1371/journal.pone.0077749

Chege D et al. (2011) Sigmoid Th17 populations, the HIV latent reservoir, and microbial translocation in men on long-term antiretroviral therapy Aids 25:741-749 doi:10.1097/QAD.0b013e328344cefb

Cheng AS et al. (2011) EZH2-mediated concordant repression of Wnt antagonists promotes beta-catenin-dependent hepatocarcinogenesis Cancer research 71:4028-4039 doi:10.1158/0008-5472.CAN-10-3342

Chirullo B et al. (2013) A candidate anti-HIV reservoir compound, auranofin, exerts a selective 'anti-memory' effect by exploiting the baseline oxidative status of lymphocytes Cell death & disease 4:e944 doi:10.1038/cddis.2013.473
Chomont N et al. (2009) HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation Nature medicine 15:893-900 doi:10.1038/nm.1972
Choudhary SK, Archin NM, Cheema M, Dahl NP, Garcia JV, Margolis DM (2012) Latent HIV-1 infection of resting CD4(+) T cells in the humanized Rag2(-)/(-) gammac(-)/(-) mouse Journal of virology 86:114-120 doi:10.1128/JVI.05590-11
Chun TW, Engel D, Mizell SB, Ehler LA, Fauci AS (1998) Induction of HIV-1 replication in latently infected CD4+ T cells using a combination of cytokines The Journal of experimental medicine 188:83-91
Chun TW, Fauci AS (1999) Latent reservoirs of HIV: obstacles to the eradication of virus Proceedings of the National Academy of Sciences of the United States of America 96:10958-10961
Cillo AR, Sobolewski MD, Bosch RJ, Fyne E, Piatak M, Jr., Coffin JM, Mellors JW (2014) Quantification of HIV-1 latency reversal in resting CD4+ T cells from patients on suppressive antiretroviral therapy Proceedings of the National Academy of Sciences of the United States of America 111:7078-7083 doi:10.1073/pnas.1402873111
Clouse KA et al. (1989) Monokine regulation of human immunodeficiency virus-1 expression in a chronically infected human T cell clone Journal of immunology 142:431-438
Clutton G, Archin N, Xu Y, Margolis DM, Goonetilleke N (2015) Differential effects of HIV latency-reversing agents on T cell phenotype and function: implications for HIV cure Journal of Virus Eradication 1:8
Contreras X, Barboric M, Lenasi T, Peterlin BM (2007) HMBA releases P-TEFb from HEXIM1 and 7SK snRNA via PI3K/Akt and activates HIV transcription PLoS pathogens 3:1459-1469 doi:10.1371/journal.ppat.0030146
Crooks AM et al. (2015) Precise Quantitation of the Latent HIV-1 Reservoir: Implications for Eradication Strategies The Journal of infectious diseases 212:1361-1365 doi:10.1093/infdis/jiv218
Deeks SG (2012) HIV: Shock and kill Nature 487:439-440 doi:10.1038/487439a
Denton PW et al. (2012) Generation of HIV latency in humanized BLT mice Journal of virology 86:630-634 doi:10.1128/JVI.06120-11
Dienz O et al. (2007) Accumulation of NFAT mediates IL-2 expression in memory, but not naive, CD4+ T cells Proceedings of the National Academy of Sciences of the United States of America 104:7175-7180 doi:10.1073/pnas.0610442104
Doitsh G et al. (2014) Cell death by pyroptosis drives CD4 T-cell depletion in HIV-1 infection Nature 505:509-514 doi:10.1038/nature12940
Donahue DA, Wainberg MA (2013) Cellular and molecular mechanisms involved in the establishment of HIV-1 latency Retrovirology 10:11 doi:10.1186/1742-4690-10-11
Doyon G, Zerbato J, Mellors JW, Sluis-Cremer N (2013) Disulfiram reactivates latent HIV-1 expression through depletion of the phosphatase and tensin homolog Aids 27:F7-F11 doi:10.1097/QAD.0b013e3283570620
du Chene I et al. (2007) Suv39H1 and HP1gamma are responsible for chromatin-mediated HIV-1 transcriptional silencing and post-integration latency The EMBO journal 26:424-435 doi:10.1038/sj.emboj.7601517
Farber D (2015) Tissue localization of human T cell responses Journal of Virus Eradication 1:6
Farber DL, Yudanin NA, Restifo NP (2014) Human memory T cells: generation, compartmentalization and homeostasis Nature reviews Immunology 14:24-35 doi:10.1038/nri3567
Finzi D et al. (1999) Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy Nature medicine 5:512-517 doi:10.1038/8394

Finzi D et al. (1997) Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy Science 278:1295-1300
Fletcher CV et al. (2014) Persistent HIV-1 replication is associated with lower antiretroviral drug concentrations in lymphatic tissues Proceedings of the National Academy of Sciences of the United States of America 111:2307-2312 doi:10.1073/pnas.1318249111
Folks TM, Clouse KA, Justement J, Rabson A, Duh E, Kehrl JH, Fauci AS (1989) Tumor necrosis factor alpha induces expression of human immunodeficiency virus in a chronically infected T-cell clone Proceedings of the National Academy of Sciences of the United States of America 86:2365-2368
Folks TM, Justement J, Kinter A, Dinarello CA, Fauci AS (1987) Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line Science 238:800-802
Friedman J, Cho WK, Chu CK, Keedy KS, Archin NM, Margolis DM, Karn J (2011) Epigenetic silencing of HIV-1 by the histone H3 lysine 27 methyltransferase enhancer of Zeste 2 Journal of virology 85:9078-9089 doi:10.1128/JVI.00836-11
Fujinaga K, Barboric M, Li Q, Luo Z, Price DH, Peterlin BM (2012) PKC phosphorylates HEXIM1 and regulates P-TEFb activity Nucleic acids research 40:9160-9170 doi:10.1093/nar/gks682
Fukazawa Y et al. (2015) B cell follicle sanctuary permits persistent productive simian immunodeficiency virus infection in elite controllers Nature medicine 21:132-139 doi:10.1038/nm.3781
Gao D et al. (2013) Cyclic GMP-AMP synthase is an innate immune sensor of HIV and other retroviruses Science 341:903-906 doi:10.1126/science.1240933
Garcia-Rodriguez C, Rao A (1998) Nuclear factor of activated T cells (NFAT)-dependent transactivation regulated by the coactivators p300/CREB-binding protein (CBP) The Journal of experimental medicine 187:2031-2036
Gardner MB, Luciw PA (2008) Macaque models of human infectious disease ILAR journal / National Research Council, Institute of Laboratory Animal Resources 49:220-255
Giffin MJ, Stroud JC, Bates DL, von Koenig KD, Hardin J, Chen L (2003) Structure of NFAT1 bound as a dimer to the HIV-1 LTR kappa B element Nature structural biology 10:800-806 doi:10.1038/nsb981
Gludish DW, Mwandumba HC, Jambo KC, Amie SM, Russel DG (2015) The human lung is a site of productive HIV infection during long-term ART: novel tools to study ART-durable HIV reservoirs Journal of Virus Eradication 1:7
Gondois-Rey F et al. (2006) R5 variants of human immunodeficiency virus type 1 preferentially infect CD62L- CD4+ T cells and are potentially resistant to nucleoside reverse transcriptase inhibitors Journal of virology 80:854-865 doi:10.1128/JVI.80.2.854-865.2006
Han Y et al. (2008) Orientation-dependent regulation of integrated HIV-1 expression by host gene transcriptional readthrough Cell host & microbe 4:134-146 doi:10.1016/j.chom.2008.06.008
He G, Margolis DM (2002) Counterregulation of chromatin deacetylation and histone deacetylase occupancy at the integrated promoter of human immunodeficiency virus type 1 (HIV-1) by the HIV-1 repressor YY1 and HIV-1 activator Tat Molecular and cellular biology 22:2965-2973
Henrich TJ et al. (2013) Long-term reduction in peripheral blood HIV type 1 reservoirs following reduced-intensity conditioning allogeneic stem cell transplantation The Journal of infectious diseases 207:1694-1702 doi:10.1093/infdis/jit086
Herrmann CH, Rice AP (1995) Lentivirus Tat proteins specifically associate with a cellular protein kinase, TAK, that hyperphosphorylates the carboxyl-terminal domain of the large subunit of RNA polymerase II: candidate for a Tat cofactor Journal of virology 69:1612-1620

Hezareh M et al. (2004) Mechanisms of HIV receptor and co-receptor down-regulation by prostratin: role of conventional and novel PKC isoforms Antiviral chemistry & chemotherapy 15:207-222
Hilldorfer BB, Cillo AR, Besson GJ, Bedison MA, Mellors JW (2012) New tools for quantifying HIV-1 reservoirs: plasma RNA single copy assays and beyond Current HIV/AIDS reports 9:91-100 doi:10.1007/s11904-011-0104-6
Hirsch VM, Olmsted RA, Murphey-Corb M, Purcell RH, Johnson PR (1989) An African primate lentivirus (SIVsm) closely related to HIV-2 Nature 339:389-392 doi:10.1038/339389a0
Ho DD, Neumann AU, Perelson AS, Chen W, Leonard JM, Markowitz M (1995) Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection Nature 373:123-126 doi:10.1038/373123a0
Ho YC et al. (2013) Replication-competent noninduced proviruses in the latent reservoir increase barrier to HIV-1 cure Cell 155:540-551 doi:10.1016/j.cell.2013.09.020
Hofmann H et al. (2012) The Vpx lentiviral accessory protein targets SAMHD1 for degradation in the nucleus Journal of virology 86:12552-12560 doi:10.1128/JVI.01657-12
Honeycutt JB, Wahl A, Archin N, Choudhary S, Margolis D, Garcia JV (2013) HIV-1 infection, response to treatment and establishment of viral latency in a novel humanized T cell-only mouse (TOM) model Retrovirology 10:121 doi:10.1186/1742-4690-10-121
Honeycutt JB et al. (2016) Macrophages sustain HIV replication in vivo independently of T cells The Journal of clinical investigation 126:1353-1366 doi:10.1172/JCI84456
Howell BJ et al. (2015) Developing and applying ultrasensitive p24 protein immunoassay for HIV latency Journal of Virus Eradication 1:5
Huang J et al. (2007) Cellular microRNAs contribute to HIV-1 latency in resting primary CD4+ T lymphocytes Nature medicine 13:1241-1247 doi:10.1038/nm1639
Huang Y et al. (2016) Antiretroviral drug transporters and metabolic enzymes in human testicular tissue: potential contribution to HIV-1 sanctuary site The Journal of antimicrobial chemotherapy doi:10.1093/jac/dkw046
Huet T, Cheynier R, Meyerhans A, Roelants G, Wain-Hobson S (1990) Genetic organization of a chimpanzee lentivirus related to HIV-1 Nature 345:356-359 doi:10.1038/345356a0
Hutter G, Bodor J, Ledger S, Boyd M, Millington M, Tsie M, Symonds G (2015) CCR5 Targeted Cell Therapy for HIV and Prevention of Viral Escape Viruses 7:4186-4203 doi:10.3390/v7082816
Imai K, Togami H, Okamoto T (2010) Involvement of histone H3 lysine 9 (H3K9) methyltransferase G9a in the maintenance of HIV-1 latency and its reactivation by BIX01294 J Biol Chem 285:16538-16545 doi:10.1074/jbc.M110.103531
Iordanskiy S, Kashanchi F (2016) Potential of Radiation-Induced Cellular Stress for Reactivation of Latent HIV-1 and Killing of Infected Cells AIDS research and human retroviruses 32:120-124 doi:10.1089/AID.2016.0006
Iordanskiy S et al. (2015) Therapeutic doses of irradiation activate viral transcription and induce apoptosis in HIV-1 infected cells Virology 485:1-15 doi:10.1016/j.virol.2015.06.021
Jiang G et al. (2014) Reactivation of HIV latency by a newly modified Ingenol derivative via protein kinase Cdelta-NF-kappaB signaling Aids 28:1555-1566 doi:10.1097/QAD.0000000000000289
Jiang G et al. (2015) Synergistic Reactivation of Latent HIV Expression by Ingenol-3-Angelate, PEP005, Targeted NF-kB Signaling in Combination with JQ1 Induced p-TEFb Activation PLoS pathogens 11:e1005066 doi:10.1371/journal.ppat.1005066
Jiang Y, Tian B, Saifuddin M, Agy MB, Emau P, Cairns JS, Tsai CC (2009) RT-SHIV, an infectious CCR5-tropic chimeric virus suitable for evaluating HIV reverse

transcriptase inhibitors in macaque models AIDS research and therapy 6:23 doi:10.1186/1742-6405-6-23
Jimenez JL, Gonzalez-Nicolas J, Alvarez S, Fresno M, Munoz-Fernandez MA (2001) Regulation of human immunodeficiency virus type 1 replication in human T lymphocytes by nitric oxide Journal of virology 75:4655-4663 doi:10.1128/JVI.75.10.4655-4663.2001
Jordan A, Bisgrove D, Verdin E (2003) HIV reproducibly establishes a latent infection after acute infection of T cells in vitro The EMBO journal 22:1868-1877 doi:10.1093/emboj/cdg188
Kao SY, Calman AF, Luciw PA, Peterlin BM (1987) Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product Nature 330:489-493 doi:10.1038/330489a0
Karn J (2013) A new BET on the control of HIV latency Cell cycle 12:545-546 doi:10.4161/cc.23679
Karpel ME, Boutwell CL, Allen TM (2015) BLT humanized mice as a small animal model of HIV infection Current opinion in virology 13:75-80 doi:10.1016/j.coviro.2015.05.002
Kauder SE, Bosque A, Lindqvist A, Planelles V, Verdin E (2009) Epigenetic regulation of HIV-1 latency by cytosine methylation PLoS pathogens 5:e1000495 doi:10.1371/journal.ppat.1000495
Keedy KS, Archin NM, Gates AT, Espeseth A, Hazuda DJ, Margolis DM (2009) A limited group of class I histone deacetylases acts to repress human immunodeficiency virus type 1 expression Journal of virology 83:4749-4756 doi:10.1128/JVI.02585-08
Kim M, Siliciano RF (2016) Reservoir expansion by T-cell proliferation may be another barrier to curing HIV infection Proceedings of the National Academy of Sciences of the United States of America 113:1692-1694 doi:10.1073/pnas.1600097113
Kim YK, Mbonye U, Hokello J, Karn J (2011) T-cell receptor signaling enhances transcriptional elongation from latent HIV proviruses by activating P-TEFb through an ERK-dependent pathway Journal of molecular biology 410:896-916 doi:10.1016/j.jmb.2011.03.054
Kiselinova M, Pasternak AO, De Spiegelaere W, Vogelaers D, Berkhout B, Vandekerckhove L (2014) Comparison of droplet digital PCR and seminested real-time PCR for quantification of cell-associated HIV-1 RNA PloS one 9:e85999 doi:10.1371/journal.pone.0085999
Klase Z et al. (2007) HIV-1 TAR element is processed by Dicer to yield a viral micro-RNA involved in chromatin remodeling of the viral LTR BMC molecular biology 8:63 doi:10.1186/1471-2199-8-63
Kula A, Gharu L, Marcello A (2013) HIV-1 pre-mRNA commitment to Rev mediated export through PSF and Matrin 3 Virology 435:329-340 doi:10.1016/j.virol.2012.10.032
Kulkosky J, Culnan DM, Roman J, Dornadula G, Schnell M, Boyd MR, Pomerantz RJ (2001) Prostratin: activation of latent HIV-1 expression suggests a potential inductive adjuvant therapy for HAART Blood 98:3006-3015
Kumar A, Darcis G, Van Lint C, Herbein G (2015) Epigenetic control of HIV-1 post integration latency: implications for therapy Clinical epigenetics 7:103 doi:10.1186/s13148-015-0137-6
Lassen KG, Bailey JR, Siliciano RF (2004) Analysis of human immunodeficiency virus type 1 transcriptional elongation in resting CD4+ T cells in vivo Journal of virology 78:9105-9114 doi:10.1128/JVI.78.17.9105-9114.2004
Lassen KG, Hebbeler AM, Bhattacharyya D, Lobritz MA, Greene WC (2012) A flexible model of HIV-1 latency permitting evaluation of many primary CD4 T-cell reservoirs PloS one 7:e30176 doi:10.1371/journal.pone.0030176
Lassen KG, Ramyar KX, Bailey JR, Zhou Y, Siliciano RF (2006) Nuclear retention of multiply spliced HIV-1 RNA in resting CD4+ T cells PLoS pathogens 2:e68 doi:10.1371/journal.ppat.0020068

Lavender KJ et al. (2013) BLT-humanized C57BL/6 Rag2-/-gammac-/-CD47-/- mice are resistant to GVHD and develop B- and T-cell immunity to HIV infection Blood 122:4013-4020 doi:10.1182/blood-2013-06-506949
Lenasi T, Peterlin BM, Barboric M (2011) Cap-binding protein complex links pre-mRNA capping to transcription elongation and alternative splicing through positive transcription elongation factor b (P-TEFb) J Biol Chem 286:22758-22768 doi:10.1074/jbc.M111.235077
Levere RD, Gong YF, Kappas A, Bucher DJ, Wormser GP, Abraham NG (1991) Heme inhibits human immunodeficiency virus 1 replication in cell cultures and enhances the antiviral effect of zidovudine Proceedings of the National Academy of Sciences of the United States of America 88:1756-1759
Lewinski MK et al. (2005) Genome-wide analysis of chromosomal features repressing human immunodeficiency virus transcription Journal of virology 79:6610-6619 doi:10.1128/JVI.79.11.6610-6619.2005
Lewinski MK et al. (2006) Retroviral DNA integration: viral and cellular determinants of target-site selection PLoS pathogens 2:e60 doi:10.1371/journal.ppat.0020060
Lewis MG et al. (2011) Gold drug auranofin restricts the viral reservoir in the monkey AIDS model and induces containment of viral load following ART suspension Aids 25:1347-1356 doi:10.1097/QAD.0b013e328347bd77
Maldarelli F et al. (2007) ART suppresses plasma HIV-1 RNA to a stable set point predicted by pretherapy viremia PLoS pathogens 3:e46 doi:10.1371/journal.ppat.0030046
Malhotra D, Fletcher AL, Turley SJ (2013) Stromal and hematopoietic cells in secondary lymphoid organs: partners in immunity Immunological reviews 251:160-176 doi:10.1111/imr.12023
Marban C et al. (2007) Recruitment of chromatin-modifying enzymes by CTIP2 promotes HIV-1 transcriptional silencing The EMBO journal 26:412-423 doi:10.1038/sj.emboj.7601516
Margolis DM, Salzwedel K, Chomont N, Psomas C, Routy JP, Poli G, Lafeuillade A (2016) Highlights from the Seventh International Workshop on HIV Persistence during Therapy, 8–11 December 2015, Miami, Florida, USA Journal of Virus Eradication 2:57-65
Marsden MD et al. (2012) HIV latency in the humanized BLT mouse Journal of virology 86:339-347 doi:10.1128/JVI.06366-11
Marsden MD et al. (2015) Evaluation of HIV latency reversal using designed PKC modulators in humanized BLT mice Journal of Virus Eradication 1:1
Massanella M, Fromentin R, Chomont N (2016) Residual inflammation and viral reservoirs: alliance against an HIV cure Current opinion in HIV and AIDS 11:234-241 doi:10.1097/COH.0000000000000230
Massanella M, Richman DD (2016) Measuring the latent reservoir in vivo The Journal of clinical investigation 126:464-472 doi:10.1172/JCI80567
Mbonye U, Karn J (2014) Transcriptional control of HIV latency: cellular signaling pathways, epigenetics, happenstance and the hope for a cure Virology 454-455:328-339 doi:10.1016/j.virol.2014.02.008
McKinstry KK, Strutt TM, Swain SL (2010) The potential of CD4 T-cell memory Immunology 130:1-9 doi:10.1111/j.1365-2567.2010.03259.x
Mehla R et al. (2010) Bryostatin modulates latent HIV-1 infection via PKC and AMPK signaling but inhibits acute infection in a receptor independent manner PloS one 5:e11160 doi:10.1371/journal.pone.0011160
Melkova Z, Shankaran P, Hajkova V, Jilich D (2015a) Reactivation of latent HIV-1 by iron-mediated redox stressh Cytokine 76:92 doi:doi:10.1016/j.cyto.2015.08.166
Melkova Z, Shankaran P, Hajkova V, Jilich D, Fujikura Y (2015b) Effects of heme degradation products on reactivation of latent HIV-1 Journal of Virus Eradication 1:42
Mendez-Lagares G, Jaramillo-Ruiz D, Pion M, Leal M, Munoz-Fernandez MA, Pacheco YM, Correa-Rocha R (2014) HIV infection deregulates the balance between regulatory T

cells and IL-2-producing CD4 T cells by decreasing the expression of the IL-2 receptor in Treg Journal of acquired immune deficiency syndromes 65:278-282 doi:10.1097/QAI.0000000000000092
Metcalf Pate KA et al. (2015) A Murine Viral Outgrowth Assay to Detect Residual HIV Type 1 in Patients With Undetectable Viral Loads The Journal of infectious diseases 212:1387-1396 doi:10.1093/infdis/jiv230
Miles B et al. (2015) Follicular regulatory T cells impair follicular T helper cells in HIV and SIV infection Nature communications 6:8608 doi:10.1038/ncomms9608
Modjarrad K, Vermund SH (2010) Effect of treating co-infections on HIV-1 viral load: a systematic review The Lancet Infectious diseases 10:455-463 doi:10.1016/S1473-3099(10)70093-1
Monroe KM, Yang Z, Johnson JR, Geng X, Doitsh G, Krogan NJ, Greene WC (2014) IFI16 DNA sensor is required for death of lymphoid CD4 T cells abortively infected with HIV Science 343:428-432 doi:10.1126/science.1243640
Mottamal M, Zheng S, Huang TL, Wang G (2015) Histone deacetylase inhibitors in clinical studies as templates for new anticancer agents Molecules 20:3898-3941 doi:10.3390/molecules20033898
Murray JM et al. (2014) HIV DNA subspecies persist in both activated and resting memory CD4+ T cells during antiretroviral therapy Journal of virology 88:3516-3526 doi:10.1128/JVI.03331-13
Ndung'u T et al. (2001) Infectious simian/human immunodeficiency virus with human immunodeficiency virus type 1 subtype C from an African isolate: rhesus macaque model Journal of virology 75:11417-11425 doi:10.1128/JVI.75.23.11417-11425.2001
Omoto S et al. (2004) HIV-1 nef suppression by virally encoded microRNA Retrovirology 1:44 doi:10.1186/1742-4690-1-44
Pace GW, Leaf CD (1995) The role of oxidative stress in HIV disease Free radical biology & medicine 19:523-528
Palmer S et al. (2008) Low-level viremia persists for at least 7 years in patients on suppressive antiretroviral therapy Proceedings of the National Academy of Sciences of the United States of America 105:3879-3884 doi:10.1073/pnas.0800050105
Palmer S et al. (2003) New real-time reverse transcriptase-initiated PCR assay with single-copy sensitivity for human immunodeficiency virus type 1 RNA in plasma Journal of clinical microbiology 41:4531-4536
Pasternak AO, Adema KW, Bakker M, Jurriaans S, Berkhout B, Cornelissen M, Lukashov VV (2008) Highly sensitive methods based on seminested real-time reverse transcription-PCR for quantitation of human immunodeficiency virus type 1 unspliced and multiply spliced RNA and proviral DNA Journal of clinical microbiology 46:2206-2211 doi:10.1128/JCM.00055-08
Pasternak AO, Lukashov VV, Berkhout B (2013) Cell-associated HIV RNA: a dynamic biomarker of viral persistence Retrovirology 10:41 doi:10.1186/1742-4690-10-41
Perelson AS et al. (1997) Decay characteristics of HIV-1-infected compartments during combination therapy Nature 387:188-191 doi:10.1038/387188a0
Perelson AS, Neumann AU, Markowitz M, Leonard JM, Ho DD (1996) HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time Science 271:1582-1586
Perkins ND, Edwards NL, Duckett CS, Agranoff AB, Schmid RM, Nabel GJ (1993) A cooperative interaction between NF-kappa B and Sp1 is required for HIV-1 enhancer activation The EMBO journal 12:3551-3558
Ploquin MJ, Silvestri G, Muller-Trutwin M (2016) Immune activation in HIV infection: what can the natural hosts of simian immunodeficiency virus teach us? Current opinion in HIV and AIDS 11:201-208 doi:10.1097/COH.0000000000000238
Policicchio BB, Pandrea I, Apetrei C (2016) Animal Models for HIV Cure Research Frontiers in immunology 7:12 doi:10.3389/fimmu.2016.00012

Price DH (2000) P-TEFb, a cyclin-dependent kinase controlling elongation by RNA polymerase II Molecular and cellular biology 20:2629-2634
Procopio FA et al. (2015) A Novel Assay to Measure the Magnitude of the Inducible Viral Reservoir in HIV-infected Individuals EBioMedicine 2:872-881 doi:10.1016/j.ebiom.2015.06.019
Pyo CW, Yang YL, Yoo NK, Choi SY (2008) Reactive oxygen species activate HIV long terminal repeat via post-translational control of NF-kappaB Biochemical and biophysical research communications 376:180-185 doi:10.1016/j.bbrc.2008.08.114
Rasmussen TA et al. (2013) Comparison of HDAC inhibitors in clinical development: effect on HIV production in latently infected cells and T-cell activation Human vaccines & immunotherapeutics 9:993-1001 doi:10.4161/hv.23800
Romerio F, Zapata JC (2015) Detection and enrichment to near purity of rare HIV-1 infected cells by PrimeFlow RNA Journal of Virus Eradication 1:5
Saison J et al. (2014) Association between discordant immunological response to highly active anti-retroviral therapy, regulatory T cell percentage, immune cell activation and very low-level viraemia in HIV-infected patients Clinical and experimental immunology 176:401-409 doi:10.1111/cei.12278
Saleh S, Solomon A, Wightman F, Xhilaga M, Cameron PU, Lewin SR (2007) CCR7 ligands CCL19 and CCL21 increase permissiveness of resting memory CD4+ T cells to HIV-1 infection: a novel model of HIV-1 latency Blood 110:4161-4164 doi:10.1182/blood-2007-06-097907
Sanchez G, Xu X, Chermann JC, Hirsch I (1997) Accumulation of defective viral genomes in peripheral blood mononuclear cells of human immunodeficiency virus type 1-infected individuals Journal of virology 71:2233-2240
Schopman NC et al. (2012) Deep sequencing of virus-infected cells reveals HIV-encoded small RNAs Nucleic acids research 40:414-427 doi:10.1093/nar/gkr719
Schuetz A et al. (2014) Initiation of ART during early acute HIV infection preserves mucosal Th17 function and reverses HIV-related immune activation PLoS pathogens 10:e1004543 doi:10.1371/journal.ppat.1004543
Serrao E, Engelman AN (2016) Sites of retroviral DNA integration: From basic research to clinical applications Critical reviews in biochemistry and molecular biology 51:26-42 doi:10.3109/10409238.2015.1102859
Shan L, Rabi SA, Laird GM, Eisele EE, Zhang H, Margolick JB, Siliciano RF (2013) A novel PCR assay for quantification of HIV-1 RNA Journal of virology 87:6521-6525 doi:10.1128/JVI.00006-13
Shan L, Siliciano RF (2013) From reactivation of latent HIV-1 to elimination of the latent reservoir: the presence of multiple barriers to viral eradication BioEssays : news and reviews in molecular, cellular and developmental biology 35:544-552 doi:10.1002/bies.201200170
Shan L et al. (2011) Influence of host gene transcription level and orientation on HIV-1 latency in a primary-cell model Journal of virology 85:5384-5393 doi:10.1128/JVI.02536-10
Shankaran P, Vlkova L, Liskova J, Melkova Z (2011) Heme arginate potentiates latent HIV-1 reactivation while inhibiting the acute infection Antiviral research 92:434-446 doi:10.1016/j.antiviral.2011.09.011
Sharp PM, Hahn BH (2011) Origins of HIV and the AIDS pandemic Cold Spring Harbor perspectives in medicine 1:a006841 doi:10.1101/cshperspect.a006841
Siliciano JD et al. (2003) Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4+ T cells Nature medicine 9:727-728 doi:10.1038/nm880
Siliciano JD, Siliciano RF (2005) Enhanced culture assay for detection and quantitation of latently infected, resting CD4+ T-cells carrying replication-competent virus in HIV-1-infected individuals Methods in molecular biology 304:3-15 doi:10.1385/1-59259-907-9:003

Sloan DD et al. (2015) Targeting HIV Reservoir in Infected CD4 T Cells by Dual-Affinity Re-targeting Molecules (DARTs) that Bind HIV Envelope and Recruit Cytotoxic T Cells PLoS pathogens 11:e1005233 doi:10.1371/journal.ppat.1005233

Soriano-Sarabia N et al. (2015) Peripheral Vgamma9Vdelta2 T Cells Are a Novel Reservoir of Latent HIV Infection PLoS pathogens 11:e1005201 doi:10.1371/journal.ppat.1005201

Spina CA et al. (2013) An in-depth comparison of latent HIV-1 reactivation in multiple cell model systems and resting CD4+ T cells from aviremic patients PLoS pathogens 9:e1003834 doi:10.1371/journal.ppat.1003834

Spivak AM et al. (2014) A pilot study assessing the safety and latency-reversing activity of disulfiram in HIV-1-infected adults on antiretroviral therapy Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 58:883-890 doi:10.1093/cid/cit813

Sung TL, Rice AP (2009) miR-198 inhibits HIV-1 gene expression and replication in monocytes and its mechanism of action appears to involve repression of cyclin T1 PLoS pathogens 5:e1000263 doi:10.1371/journal.ppat.1000263

Tae S, Karkhanis V, Velasco K, Yaneva M, Erdjument-Bromage H, Tempst P, Sif S (2011) Bromodomain protein 7 interacts with PRMT5 and PRC2, and is involved in transcriptional repression of their target genes Nucleic acids research 39:5424-5438 doi:10.1093/nar/gkr170

Thompson MR et al. (2014) Interferon gamma-inducible protein (IFI) 16 transcriptionally regulates type i interferons and other interferon-stimulated genes and controls the interferon response to both DNA and RNA viruses J Biol Chem 289:23568-23581 doi:10.1074/jbc.M114.554147

Traggiai E, Chicha L, Mazzucchelli L, Bronz L, Piffaretti JC, Lanzavecchia A, Manz MG (2004) Development of a human adaptive immune system in cord blood cell-transplanted mice Science 304:104-107 doi:10.1126/science.1093933

Tran TA et al. (2008) Resting regulatory CD4 T cells: a site of HIV persistence in patients on long-term effective antiretroviral therapy PloS one 3:e3305 doi:10.1371/journal.pone.0003305

Trejbalova K et al. (2016) Development of 5' LTR DNA methylation of latent HIV-1 provirus in cell line models and in long-term-infected individuals Clinical epigenetics 8:19 doi:10.1186/s13148-016-0185-6

Triboulet R et al. (2007) Suppression of microRNA-silencing pathway by HIV-1 during virus replication Science 315:1579-1582 doi:10.1126/science.1136319

Tyagi M, Karn J (2007) CBF-1 promotes transcriptional silencing during the establishment of HIV-1 latency The EMBO journal 26:4985-4995 doi:10.1038/sj.emboj.7601928

Tyagi M, Pearson RJ, Karn J (2010) Establishment of HIV latency in primary CD4+ cells is due to epigenetic transcriptional silencing and P-TEFb restriction Journal of virology 84:6425-6437 doi:10.1128/JVI.01519-09

Van Lint C, Bouchat S, Marcello A (2013) HIV-1 transcription and latency: an update Retrovirology 10:67 doi:10.1186/1742-4690-10-67

Verdin E, Paras P, Jr., Van Lint C (1993) Chromatin disruption in the promoter of human immunodeficiency virus type 1 during transcriptional activation The EMBO journal 12:3249-3259

Vire E et al. (2006) The Polycomb group protein EZH2 directly controls DNA methylation Nature 439:871-874 doi:10.1038/nature04431

Wang L, de Zoeten EF, Greene MI, Hancock WW (2009) Immunomodulatory effects of deacetylase inhibitors: therapeutic targeting of FOXP3+ regulatory T cells Nature reviews Drug discovery 8:969-981 doi:10.1038/nrd3031

Wang X, Cho B, Suzuki K, Xu Y, Green JA, An J, Cyster JG (2011) Follicular dendritic cells help establish follicle identity and promote B cell retention in germinal centers The Journal of experimental medicine 208:2497-2510 doi:10.1084/jem.20111449

Wei P, Garber ME, Fang SM, Fischer WH, Jones KA (1998) A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA Cell 92:451-462

Wei X et al. (1995) Viral dynamics in human immunodeficiency virus type 1 infection Nature 373:117-122 doi:10.1038/373117a0

Wightman F et al. (2010) Both CD31(+) and CD31(-) naive CD4(+) T cells are persistent HIV type 1-infected reservoirs in individuals receiving antiretroviral therapy The Journal of infectious diseases 202:1738-1748 doi:10.1086/656721

Williams SA, Chen LF, Kwon H, Ruiz-Jarabo CM, Verdin E, Greene WC (2006) NF-kappaB p50 promotes HIV latency through HDAC recruitment and repression of transcriptional initiation The EMBO journal 25:139-149 doi:10.1038/sj.emboj.7600900

Wires ES, Alvarez D, Dobrowolski C, Wang Y, Morales M, Karn J, Harvey BK (2012) Methamphetamine activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kappaB) and induces human immunodeficiency virus (HIV) transcription in human microglial cells Journal of neurovirology 18:400-410 doi:10.1007/s13365-012-0103-4

Xing S et al. (2011) Disulfiram reactivates latent HIV-1 in a Bcl-2-transduced primary CD4+ T cell model without inducing global T cell activation Journal of virology 85:6060-6064 doi:10.1128/JVI.02033-10

Yamada T, Yamaguchi Y, Inukai N, Okamoto S, Mura T, Handa H (2006) P-TEFb-mediated phosphorylation of hSpt5 C-terminal repeats is critical for processive transcription elongation Molecular cell 21:227-237 doi:10.1016/j.molcel.2005.11.024

Yang HC, Shen L, Siliciano RF, Pomerantz JL (2009a) Isolation of a cellular factor that can reactivate latent HIV-1 without T cell activation Proceedings of the National Academy of Sciences of the United States of America 106:6321-6326 doi:10.1073/pnas.0809536106

Yang HC et al. (2009b) Small-molecule screening using a human primary cell model of HIV latency identifies compounds that reverse latency without cellular activation The Journal of clinical investigation 119:3473-3486 doi:10.1172/JCI39199

Yoh SM et al. (2015) PQBP1 Is a Proximal Sensor of the cGAS-Dependent Innate Response to HIV-1 Cell 161:1293-1305 doi:10.1016/j.cell.2015.04.050

Zhang J, Scadden DT, Crumpacker CS (2007) Primitive hematopoietic cells resist HIV-1 infection via p21 The Journal of clinical investigation 117:473-481 doi:10.1172/JCI28971

Zolotukhin AS et al. (2003) PSF acts through the human immunodeficiency virus type 1 mRNA instability elements to regulate virus expression Molecular and cellular biology 23:6618-6630

FIGURE LEGEND

Fig. 1 Latency and reactivation (simplified scheme). HIV-1 latency is characterized by a repressed chromatin, presence of histone deacetylases (HDACs), histone methylases (HMTs) and DNA methylases (DMTs), and lack of transcription factors, resulting in a transcription block. Reactivation is associated with epigenetic changes that lead to open chromatin structure, namely presence of histone acetylases (HATs), nuclear translocation of transcription initiation factors NF-κB and NAFT, increased levels of Tat and formation of its complex with p-TEFb. Tat-p-TEFb complex binds to TAR RNA, resolving promoter-proximal pausing of RNAP II and allowing efficient transcription elongation. p-TEFb can be sequestered in the 7SK snRNP inhibitory complex.

Fig. 2 Comparison of HIV-1 RNA and DNA decay curves upon introduction of cART. After initiation of cART, plasma viremia and the level of HIV-1 infected cells in peripheral blood decays with a well characterized kinetics based on populations with different turnover contributing to plasma viremia. HIV-1 DNA reveals a relatively smaller decrease. Adapted from (Hilldorfer et al. 2012; Margolis et al. 2016).

Fig. 3 PCR-based detection of HIV-1 RNA and DNA. Determination of cell-associated RNA (caRNA) and proviral DNA (prDNA) using 2-step seminested (RT-) qPCR or using a combination of a limiting dilution assay and 1-step nested RT-PCR (e.g. TILDA; (Bullen et al. 2014; Kiselinova et al. 2014; Procopio et al. 2015)).

Fig.1

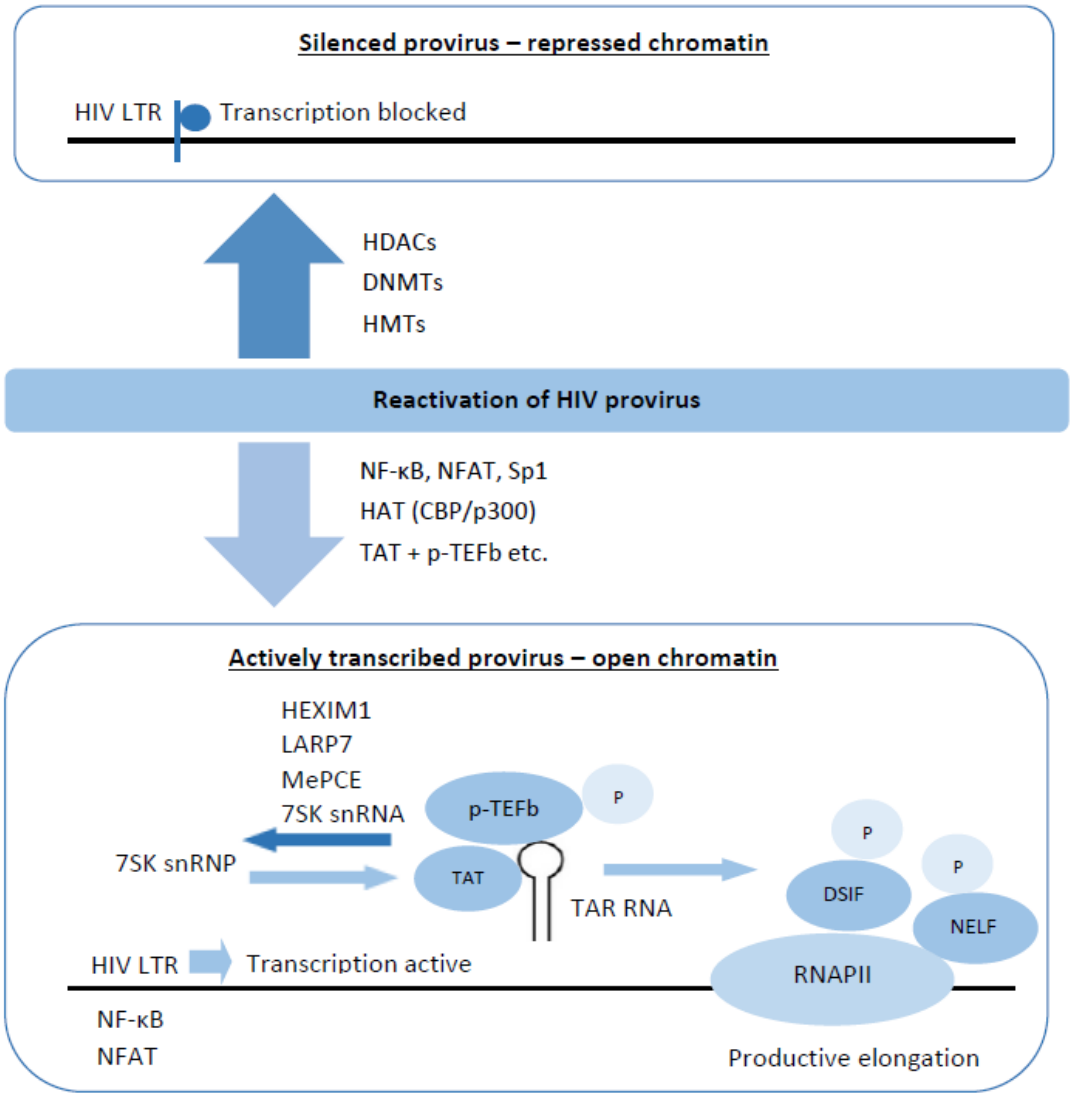

Silenced provirus – repressed chromatin
HIV LTR
Transcription blocked
HDACs
DNMTs
HMTs
Reactivation of HIV provirus
NF-κB, NFAT, Sp1
HAT (CBP/p300)
TAT + p-TEFb etc.
Actively transcribed provirus – open chromatin
HEXIM1
LARP7
MePCE
7SK snRNA
7SK snRNP
p-TEFb
P
TAT
TAR RNA
P
P
DSIF
NELF
RNAPII
HIV LTR
Transcription active
NF-κB
NFAT
Productive elongation

Fig.2

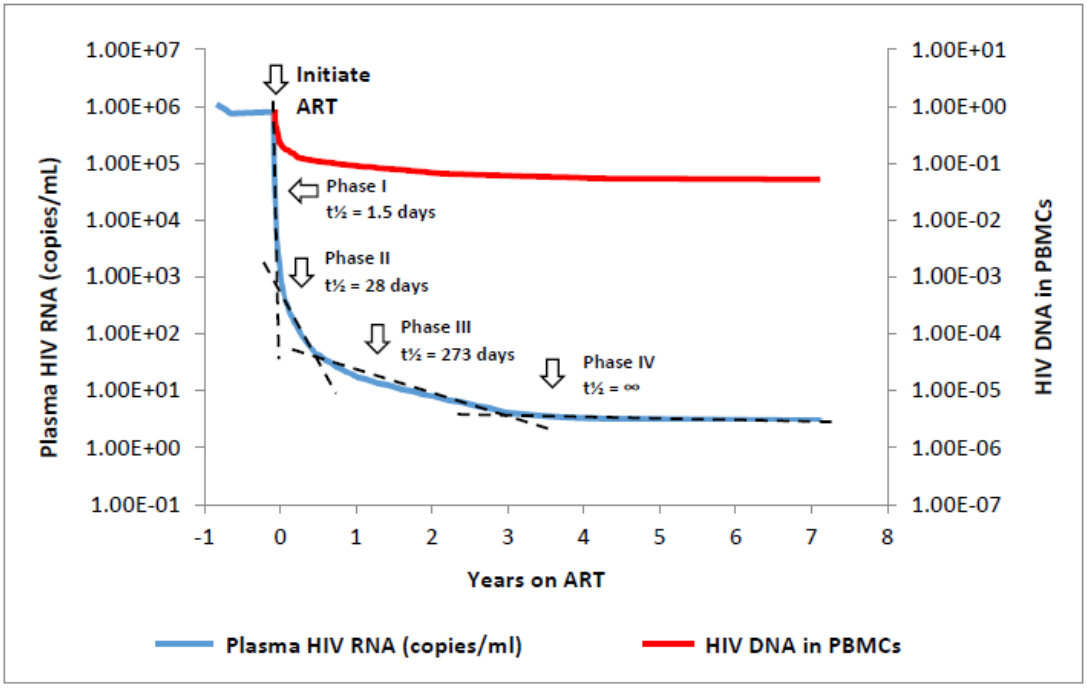
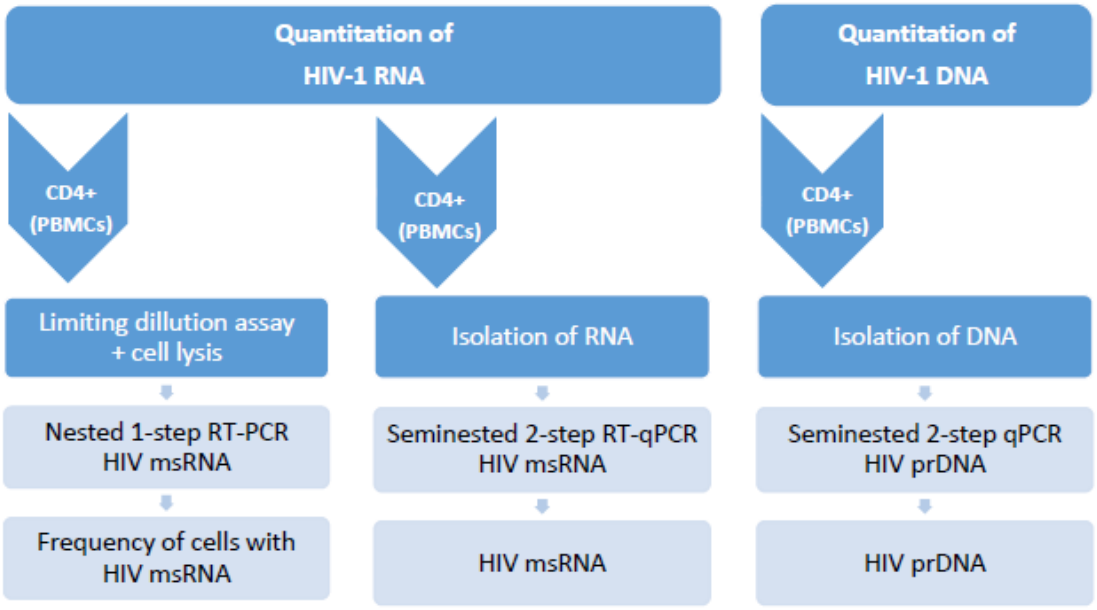

1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1.00E-01
Plasma HIV RNA (copies/mL)
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
1.00E-05
1.00E-06
1.00E-07
HIV DNA in PBMCs
Initiate ART
Phase I
t½ = 1.5 days
Phase II
t½ = 28 days
Phase III
t½ = 273 days
Phase IV
t½ = ∞
-1
0
1
2
3
4
5
6
7
8
Years on ART
Plasma HIV RNA (copies/ml)
HIV DNA in PBMCs

Fig.3


Quantitation of HIV-1 RNA
Quantitation of HIV-1 DNA
CD4+ (PBMCs)
CD4+ (PBMCs)
CD4+ (PBMCs)
Limiting dillution assay + cell lysis
Isolation of RNA
Isolation of DNA
Nested 1-step RT-PCR HIV msRNA
Seminested 2-step RT-qPCR HIV msRNA
Seminested 2-step qPCR HIV prDNA
Frequency of cells with HIV msRNA
HIV msRNA
HIV prDNA

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Prohlášení zájemce o nahlédnutí
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