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Summary of PhD Thesis

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

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Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

Contents

1. 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. Introduction

2.1. HIV-1 structure and genome

Human immunodeficiency virus (HIV-1), the aetiological agent of AIDS, is a lentivirus of retroviridae family. It is a spherical, enveloped virus of about 120 nm diameter. The two single-stranded positive sense RNAs constituting the genome and functional enzymes of HIV-1 are packed inside a conical capsid protein, which in turn is covered by a matrix protein. The outer bilayer lipid envelope is studded with glycoprotein trimer cap molecules (gp120) and transmembrane glycoprotein trimers (gp41) that connect gp120 with the lipid envelope. The genome of HIV-1 is approximately 9.8kb in length. 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.

2.2. HIV-1 life cycle and latency

The HIV-1 replication cycle begins with binding of HIV-1 gp120 to its receptor CD4 and co-receptors on target cells, CXCR4 (on T helper cells) or CCR5 (on Macrophages). Following the entry of HIV-1 into target cell, viral RNA is reverse-transcribed to DNA and gets incorporated into host genome. The integrated viral DNA (known as provirus) can either enter into a transcriptionally silent latent stage or continue to be actively transcribed. The transcribed viral genes are translated to produce viral proteins that are assembled to form immature viruses, and finally matured, new HIV-1 particles are released by budding (Freed, 2015).

Among the target cells of the HIV-1, resting CD4+ T cells are considered to be the most important reservoirs of latent HIV-1 in the body, because, mean half-life of resting CD4+ T cells can reach 44 months. It has been estimated that it would require over 60 years of combined antiretroviral therapy (cART) to eradicate the latent reservoirs from the body (Siliciano et al., 2003). When a small portion of infected active CD4+ T cells returns to a quiescent state, it results in a stable latent state (Chun et al., 1995). However, a recent study has shown that latency can be established directly and early in both resting and activated primary T cells (Chavez et al., 2015). Many epigenetic mechanisms like histone protein modifications (eg. acetylation, methylation, phosphorylation, ubiquitination and sumoylation), DNA methylation, chromatin remodelling, or RNA interference are importnat factors determining the transcriptional silencing of the provirus. (Lewinski et al., 2005; Friedman, 2011; Trejbalova et al., 2016; Williams et al., 2006; Wang et al., 2015).

Although the 5'LTR of HIV-1 genome contains binding sites for several viral and cellular transcription factors, the transcription initiation of provirus is inhibited since the important cellular transcription factors, such as NF-κB, NFAT and positive transcription elongation factor (PTEFb) that play a key role in successful HIV-1 gene expression, are sequestered in the cytoplasm and inactive in the resting memory CD4+T cells (Colin and Van Lint, 2009).

2.3. Latency reversing agents

Combined antiretroviral therapy (cART) can successfully suppress the viraemia to undetectable levels. Nevertheless, it cannot completely cure the infection, due to therapy-resistant reservoirs of latent HIV-1 (Richman et al., 2009). Therefore, the elimination of viral reservoirs is essential for a sterilizing cure. Reactivation of the latent virus by stimulating the resting CD4+T cells, followed by a purge of the activated reservoir cells as a result of induced viral cytopathic effects, immune clearance by cytolytic T lymphocytes (CTL) or cell death is termed as "shock and kill" strategy (Deeks et al, 2012). The compounds that can reactivate the latent virus are termed as "latency reversing agents" (LRA). Blocking or activating factors involved in the establishment of latency, maintenance of transcriptional silence, reactivation, initiation and elongation of provirus transcription are the targets of LRA. Histone deacetylase inhibotrs (HDACI), histone methyltransferase inhibitor (HMTI), DNA methyltransferase inhibitors (DNMTI), protein kinase C (PKC) activators and other unclassified small molecules are being tested and developed as LRAs (Huber et al., 2011; Tripathy et al, 2015; Kauder et al., 2009; Gulakowski, 1997; Elliott et al., 2014, Wang et al., 2015).

2.4. Heme arginate and heme degradation products

Heme arginate (HA) is a product containing human hemin complexed with the amino acid L-arginine (Tenhunen et al., 1987). It is more stable and has lesser side effects on homeostasis than hematin (Volin et al., 1988). Heme is used in the treatment of acute hepatic porphyrias and it has also been shown to have many positive effects. Heme is degraded 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). HO-1 is inducible by heme and also by a variety of stresses, while HO-2 is constitutively expressed. Free iron (Fe^{2+}) produced from heme degradation is pro-oxidative and was shown to generate ROS. Iron and ROS are recognized 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. Anemia or iron deficiency (ID) is common in chronic HIV-1 infection (Redig and Berliner, 2013). Increased hepcidin (a hormone that regulates systemic iron homeostasis) and decreased plasma iron levels have been observed in HIV-1 acute infection, while hepcidin remained elevated in untreated or cART-treated persons with chronic HIV-1 infection (Armitage et al., 2014). Carbon monoxide is another product of heme catabolism. It is an odorless, colorless, tasteless gas. The major source of

endogenous CO in animals is heme degradation by HO-1, 2 (Abraham et al., 1983). CO is an antioxidant and 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). Biliverdin, the third product of heme catabolism by HO-1,2 is rapidly reduced to bilirubin by biliverdin reductase (BVR). Bilirubin is a major physiologic antioxidant and cytoprotectant (Baranano et al., 2002). Biliverdin 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).

2.5. HIV infection and oxidative stress

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). 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). 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). HIV-1 Tat has been shown to induce enterocyte apoptosis through a redox-mediated mechanism (Buccigrossi et al., 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, 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 as well as in HIV-1 transcription, is affected by the inhibitory/stimulatory effects of ROS. A high concentration of ROS may lead to tissue damage or cell death, while a low concentration of ROS would lead to a cytoprotective response such as activating the Nrf2-antioxidant response element signalling pathway (Ma, 2013).

3. 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.

4. Methodology

4.1. Cells and latency models

Human T-cell lines A3.01 and Jurkat (a clone with high expression of CD4) were used to study the effects of heme arginate on HIV-1 acute infection and to assess the cytoxicity of HA. ACH-2 cells harboring an integrated HIV-1 provirus, and A2 and H12 clones of Jurkat cells containing a latent ‘‘mini-virus’’ comprising the HIV-1 LTR-Tat-IRES-EGFP-LTR (Jordan et al., 2001) were used as HIV-1 latency models to study the effects of HA and heme degradation products on reactivation of provirus. Also, PBMCs isolated from HIV-1-infected cART-treated patients were used in ex vivo cell culture to study the effect of HA in latency reversal.

4.2. HIV-1 virus and acute 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 used for infections in A3.01 and Jurkat cells.

4.3. Flow cytometry analysis

EGFP fluorescence of A2 and H12 cells was characterized by a flow cytometry analysis in FL1 (detecting at 515–545 nm). Detection of CD69 expression was performed using a mouse monoclonal antibody against human CD69 labelled with Alexa Fluor-700 and analysed in FL7 (detecting at 700–720 nm).

4.4. Cytotoxicity assays and determination of CC_{50}

Cytotoxicity of heme arginate was characterized by determination of induction of apoptosis using flow cytometry based on cell size and granulosity (FSC-A x SSC-A) and by the effects on cell viability and growth by MTT assay using a protocol adapted according to TOX-1 kit (Sigma Co., St. Louis, MO).

4.5. Western blot analysis

Determination of proteins such as HIV-1 p24, HO-1, EGFP, and β-Actin expressed as a result of treatment with different compounds was performed by western blotting and chemiluminescence detection. Specific primary antibodies against each protein and a peroxidase-conjugated secondary antibody were used to determine the proteins.

4.6. ELISA

HIV-1 p24 antigen level in the culture supernatant was quantified by using RETRO-TEK HIV-1 p24 Antigen ELISA kit (ZeptoMetrix Corp. Buffalo, NY) according to the manufacturer’s protocol.

4.7. PCR detection of HIV-1 reverse transcripts

Genomic DNA isolated from the cells was used to determine HIV-1 reverse transcripts by PCR using primers specific for LTR/gag (Schmidtmayerova et al., 1998) and for GAPDH.

4.8. Quantification of HIV-1 RNA expression

Total RNA was isolated from cells and treated with DNase. Quantification of HIV-1 Gag mRNA and house-keeping gene human GAPDH were quantified either by 1-step RT-ddPCR (QX-100, droplet digital PCR system, Bio-Rad) or semi-nested 2-step RT-qPCR with specific primer and probes adapted from (Kiselinova et al., 2014; Pasternak et al., 2008).

4.9. 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.

5. Results

5.1 Heme arginate inhibited 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.1).

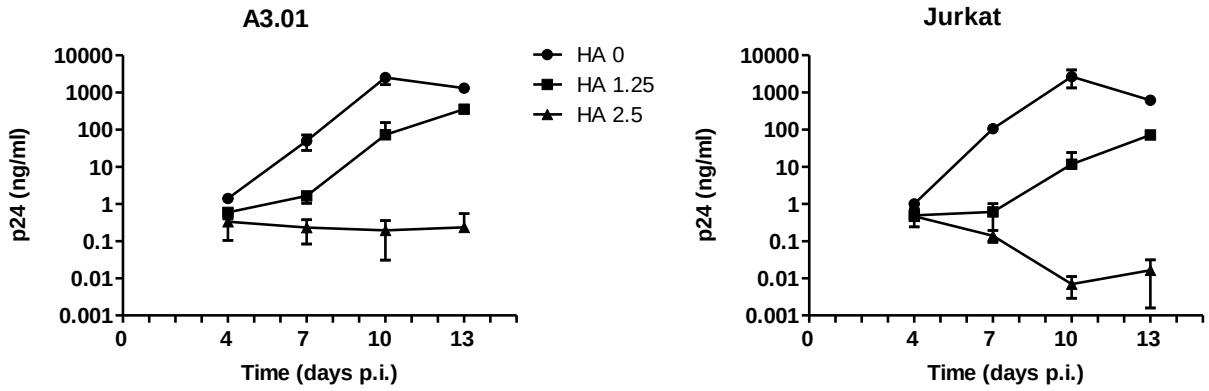


Fig. 1. Heme arginate inhibits HIV-1 acute infection. A3.01 and Jurkat cells were infected with HIV-1 and treated with HA 1.25 and 2.5 µl/ml. HIV-1 p24 levels in the culture supernatant were quantified by ELISA at indicated days after infection (p.i.). Graph represents the results of two independent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

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. 2).

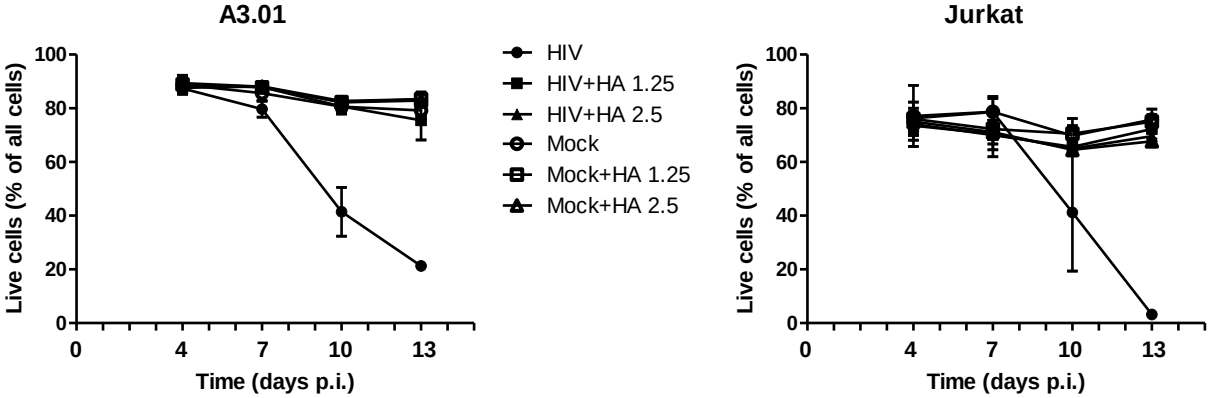


Fig. 2. Effect of HA on T cell viability. (A) Viability of HIV-1-infected, untreated and HA-treated A3.01 and Jurkat cells as characterized by flow cytometry. Graphs represent the results of two independent experiments performed in duplicate.

The CC_{50} 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.

5.2. Heme arginate inhibited reverse transcription of HIV-1

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. 3). 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.

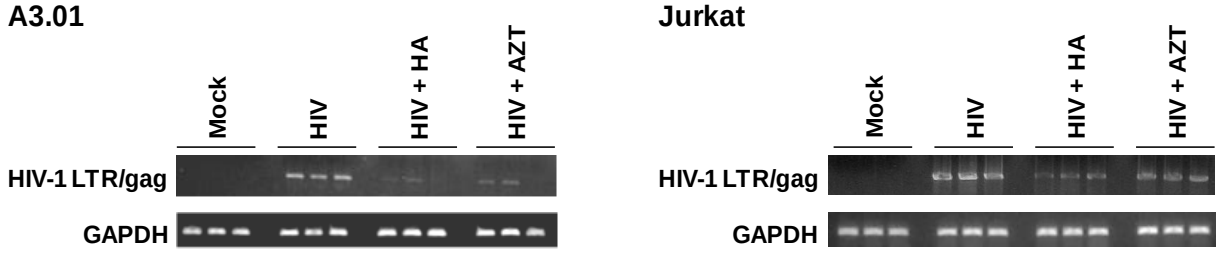


Fig. 3. HA inhibits reverse transcription of HIV-1. HIV-1 LTR/Gag specific PCR in DNA isolated from HIV-1 infected, HA2.5 µl/ml treated A3.01 and Jurkat cells demonstrated the inhibitory activity of heme arginate on HIV-1 RT. Representative results of two independent experiments performed in triplicates.

5.3. 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 analyzed 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. 4A), and culture supernatants (Fig. 4B). 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).

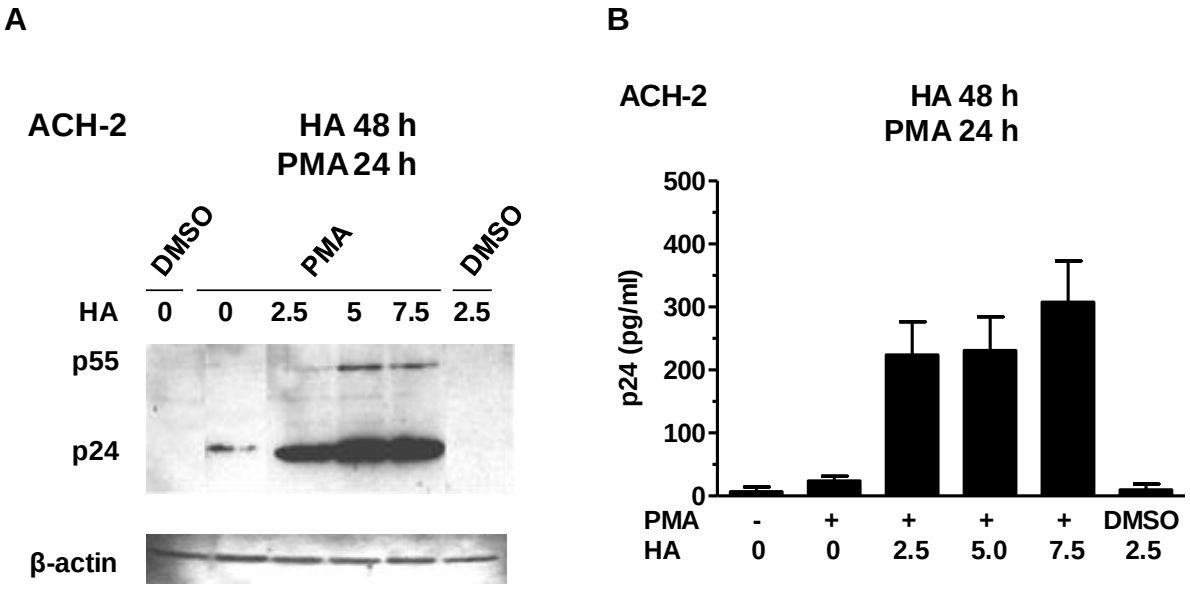


Fig. 4. Effects of HA on reactivation of the latent HIV-1 provirus in ACH-2 cells. ACH-2 cells were pretreated with increasing concentrations of HA for 24 h, and then stimulated with (PMA; final concentration 0.5 ng/ml) for 24 h. **(A)** Western blot analysis of p24 antigen in the cells. Representative 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.

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

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 blotting 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. 5) and the result was in agreement with the results obtained in ACH-2 cells. Interestingly, EGFP expression was stimulated also by HA alone, but the effect was weaker than that in combination with PMA (Fig. 5). 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 stimulatory effects of HA-alone and in combination with PMA on the reactivation of HIV-1 mini-virus were comparable in western blot (data not included here) and flow cytometry analysis.

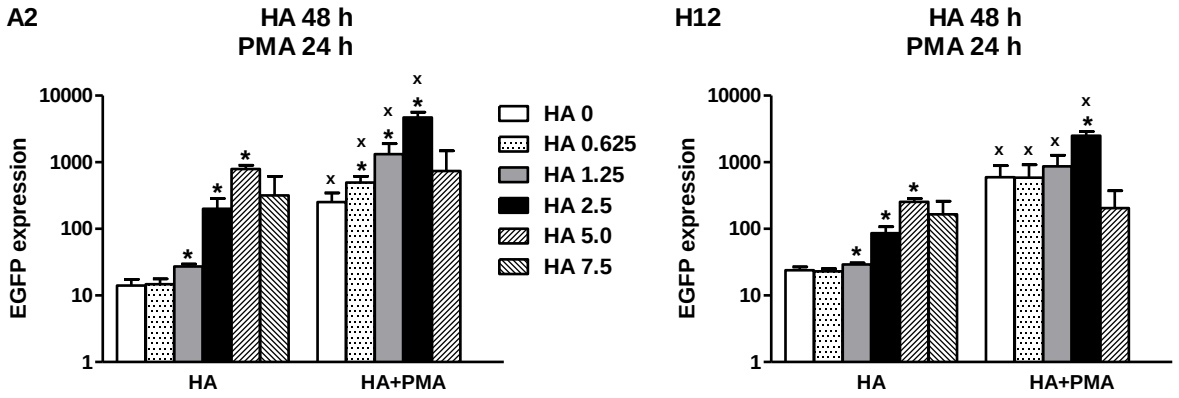


Fig. 5. Effect of HA on reactivation of the latent HIV-1 ‘‘mini-virus’’. Pretreatment with increasing concentrations of HA alone or in combination with PMA (final concentration 0.5 ng/ml) reactivated latent HIV-1 “mini-virus”. FACS analysis of EGFP from $0.1x10^6$/200 μl cells cultured. The graphs show 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 ($p < 0.05$).

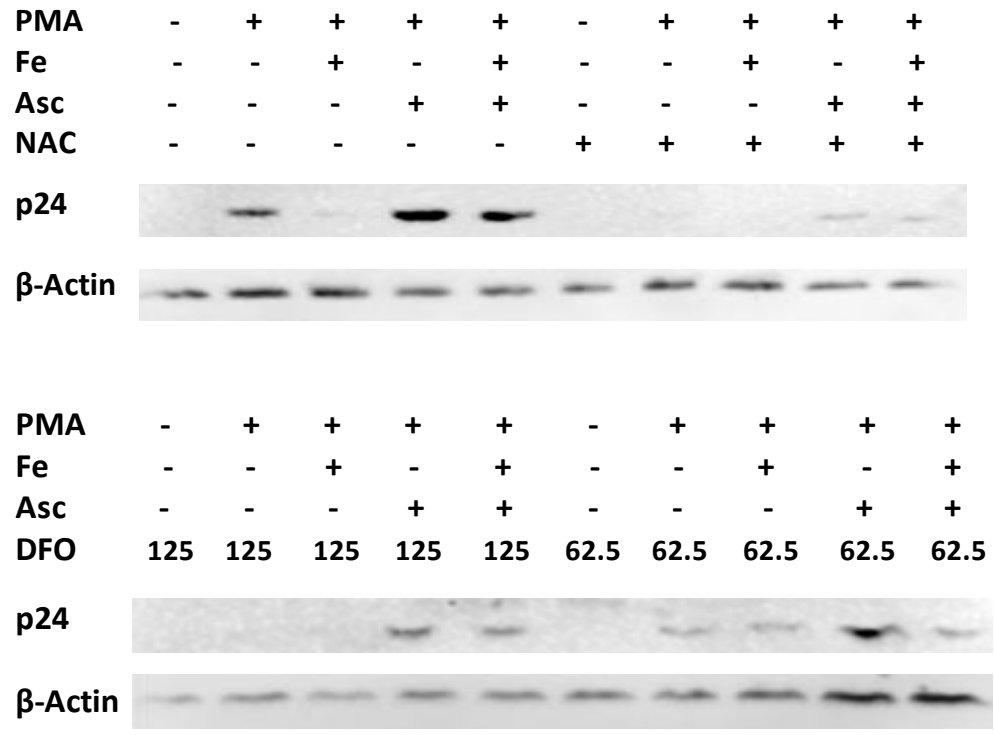
5.5. Antioxidant N-Acetyl cysteine and iron chelator desferrioxamine decreased PMA- and ascorbate-stimulated HIV-1 provirus reactivation

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/ml) either alone or in combinations. 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 treatments, namely Fe^{3+} alone, ascorbate

alone or Fe^{3+} and ascorbate together, revealed any effect on p24 expression in ACH-2 cells (data not shown). 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. 6A and B).

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 antioxidant N-Acetyl cysteine (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/ml) 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. 6A 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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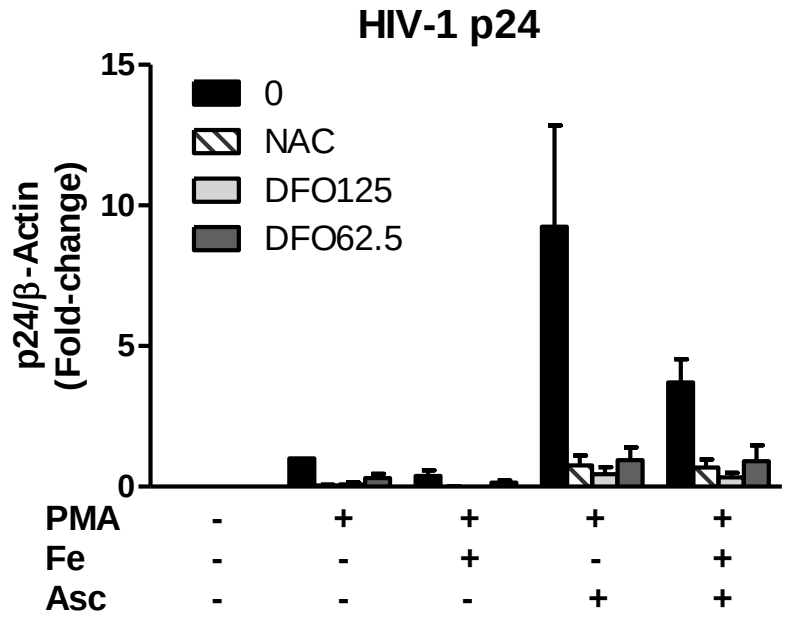

Fig. 6. NAC and iron chelator DFO decreased PMA and ascorbate stimulated HIV-1 provirus reactivation. ACH-2 cells pre-treated with either NAC (5 mM) or DFO (125 or 62.5 µM) for 4 h and treated with ferric nitrate (Fe; 10 µM), ascorbate (Asc; 0.25mM) and PMA either alone or in combination for 24 h. **(A)** 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.

5.6. 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 act as 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 analyzed 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. 7A and B) as well as in PMA-stimulated ACH-2 cells treated with iron and ascorbate (Fig. 7C and D). Biliverdin also showed the same pattern of dose-dependent decrease in p24 levels in PMA-treated ACH-2 cells (Fig. 7E 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).

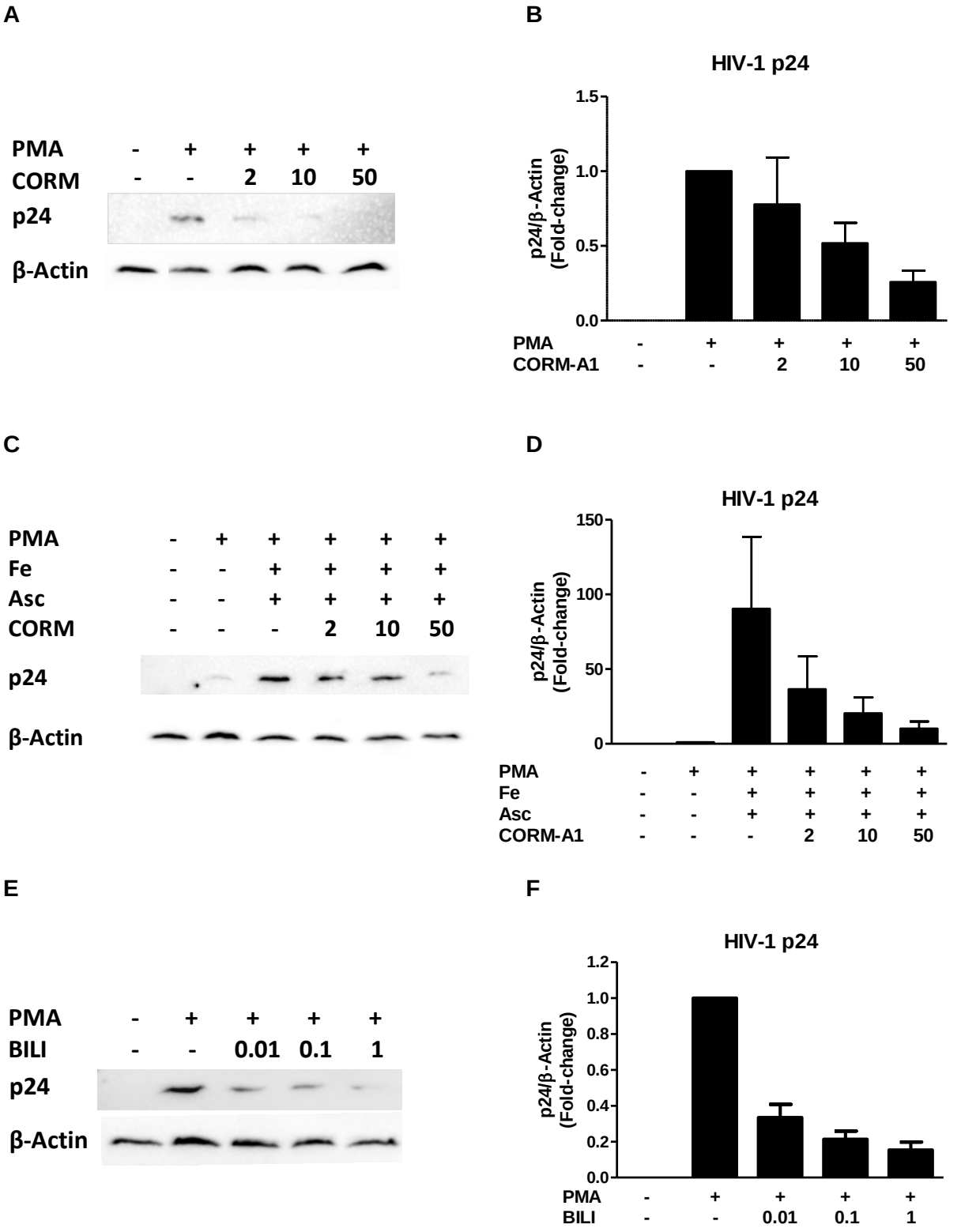

Fig. 7. Effect of CO and bilirubin on PMA-stimulated reactivation of the latent HIV-1. ACH-2 cells pre-treated with CORM-A1 (2, 10 and 50 μM) for 5 min or with bilirubin (0.01, 0.1 and 1 μM) for 6 h, and treated with 0.5 ng/ml PMA for 24 h. (ferric nitrate (Fe; 10 μM) and ascorbate (0.25 μM) were added 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.

5.7. 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. 8A and B) and Gag mRNA expression levels (Fig. 8C) are dose-dependently decreased by DFO in HA- and PMA-stimulated HIV-1 provirus reactivation in ACH-2 cells. The results confirmed the 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.

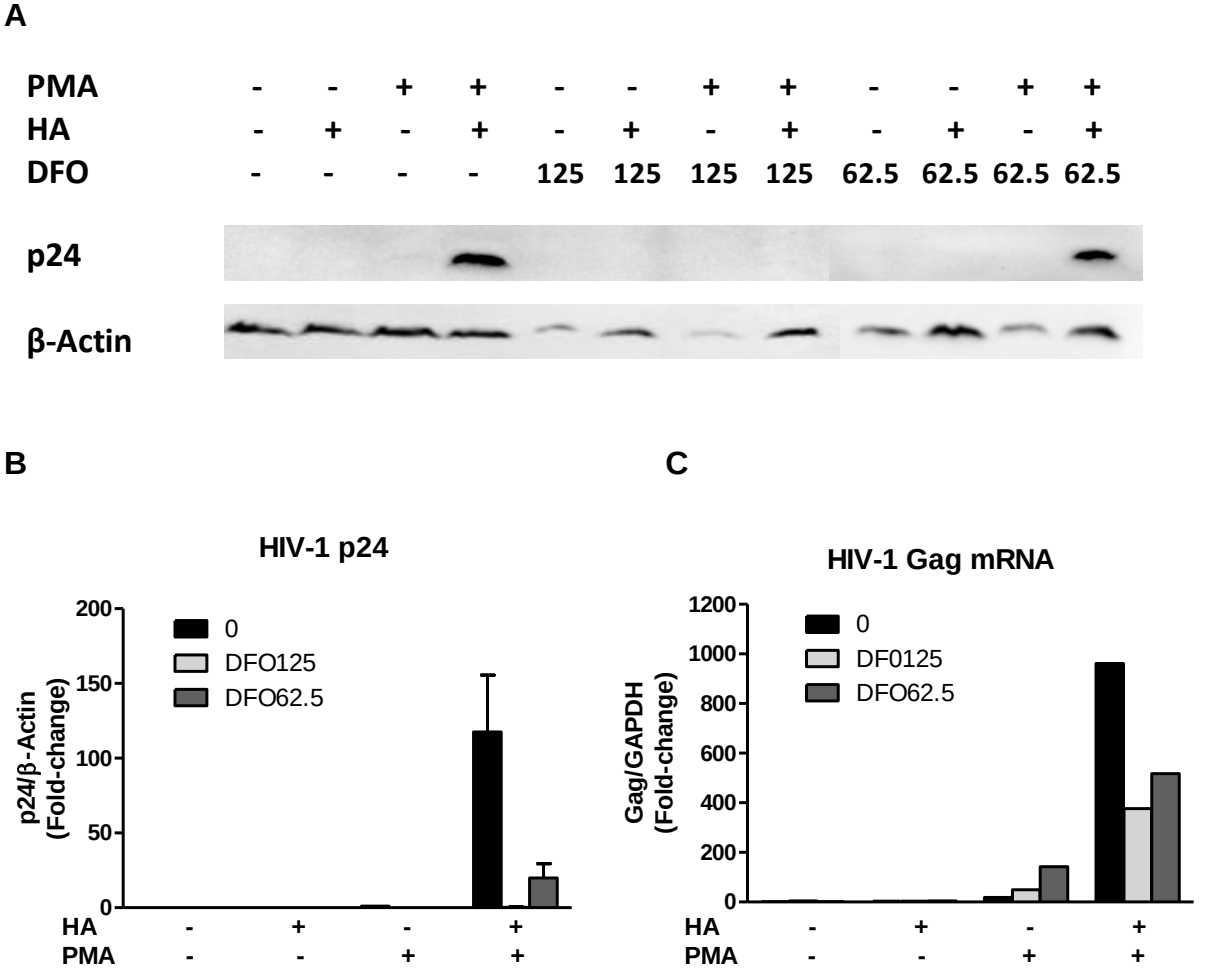

Fig. 8. Role of iron in HA-mediated and PMA-stimulated reactivation of the latent HIV-1. ACH-2 cells pre-treated with 125 and 62.5 µM DFO for 4 h, and treated with 2.5 ul of heme arginate/ml for 24 h and additional 24 h with PMA (0.5 ng/ml) **(A)** Western blot analysis of HIV-1 p24 antigen. Representative 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.

5.8. HA 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 is 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. 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 standardized to human GAPDH mRNA. As shown in Fig. 9, 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.

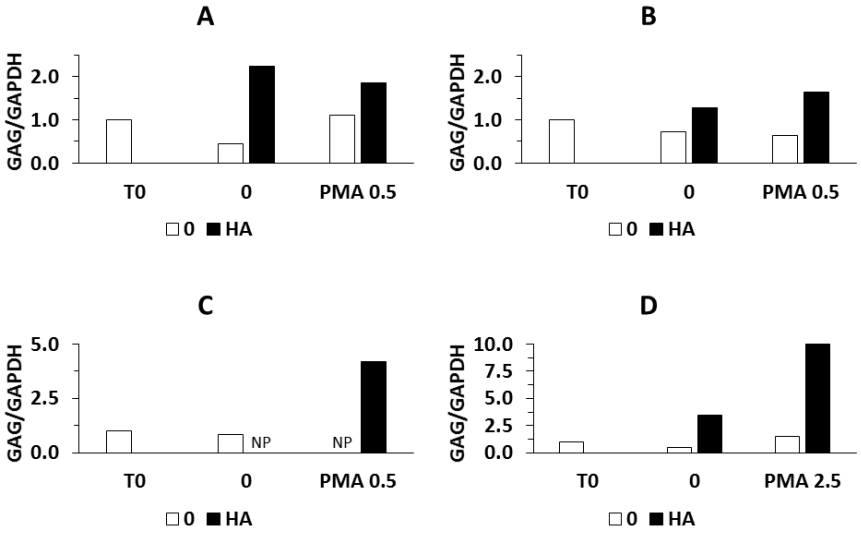

Fig. 9. Heme arginate stimulates HIV-1 expression in human HIV+ PBMC's *ex vivo.* 5 x 10^6/ml of human PBMC's were treated with HA 2.5 µl/ml with or without addition of 0.5 or 2.5 ng/ml PMA for 18 h. HIV-1 Gag RNA was analysed using semi-nested 2-step RT-qPCR and standardized to GAPDH mRNA. Results of 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.

5.9. 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. 10). Importantly, HA alone did not activate the T cells when used alone.

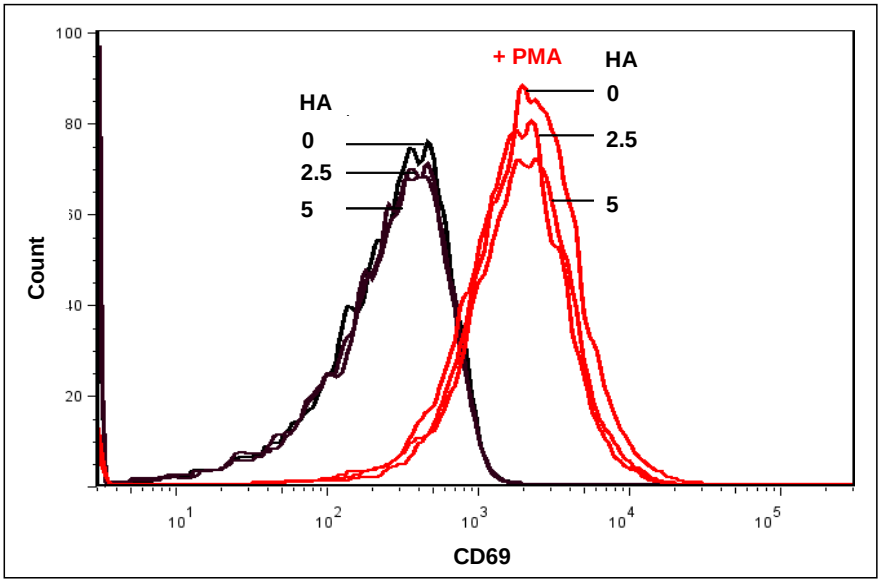

Fig. 10. HA neither stimulated nor interfered with activation of T cells. A3.01 cells were treated with increasing concentrations of HA in the presence or absence of PMA (final concentration 0.5 ng/ml) for 24 h. Flow cytometry detection of T cell activation marker CD69. Representative results of two independent experiments performed in duplicates.

6. Discussion

The introduction of 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 eradicate the virus, which 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 reservoirs becomes crucial. Latently infected $CD4^+$ T cells constitute one reservoir of replication-competent HIV that needs to be eliminated to completely purge virus from antiretroviral drug-treated patients. Since increased redox stress plays an important role in HIV-1 replication (Pace and Leaf, 1995) our aim was to harness the effects of redox stress through redox modulators and to reactivate the latent HIV-1.

Heme arginate is a compound containg human hemin and 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. Also heme arginate is more stable and has lower or no side effects on homeostasis (Volin et al., 1988) when compared to its equivalent hematin. 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 showed 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. In the time-course experiments (Fig. 1), HA inhibited HIV-1 replication characterized by levels of p24 Ag. In similar time-course experiments (Fig. 2), 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-alone or in combination with HA. 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 different inducers (data not included) 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 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 Ca2+ 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. 4A and B). 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 Fe2+, increased redox stress and activation of the redox-sensitive transcription factor NF-κB can be suggested (Devadas and Dhawan, 2006; 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.

With these findings on 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 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.

Addition of anti-oxidant N-acetyl cysteine (NAC) decreased the reactivation of provirus by HA-and PMA-stimulated and also PMA- and ascorbate-stimulated ACH-2 and Jurkat clones, suggesting generation of ROS by these treatments. 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.

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 (data not shown). 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/AIDS, similarly to other chronic infections, has been reported to lead to immune-mediated anemia of chronic disease, iron deficiency anemia or their combination (Minchella et al., 2015). The concentrations of HA used throughout this research work are thus very close to the levels achieved in clinics. 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.

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 proinflammatory cytokines should be avoided.

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 from 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 potentiation. 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 protection. In fact, very recently an approach towards using a combination of compounds to reactivate HIV-1 provirus and reservoir clearance has been also 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-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.

7. Conclusions

Despite the successful suppression of 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 work aimed at exploring the effect of heme arginate on HIV-1 acute infection and reactivation of latent virus. The key findings of our research could be summarise and concluded 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*.

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9. 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.

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

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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

Patent applications

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