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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).

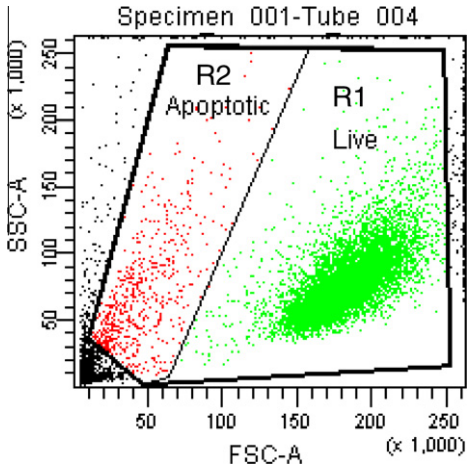


Fig. 1. Example of a flow cytometric analysis. Gating of the cell populations in dependence on their size and granularity. Cell populations in Regions 1 and 2 were used for further analysis; the two regions contained live and apoptotic cells, respectively. The samples were collected, incubated with individual 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.

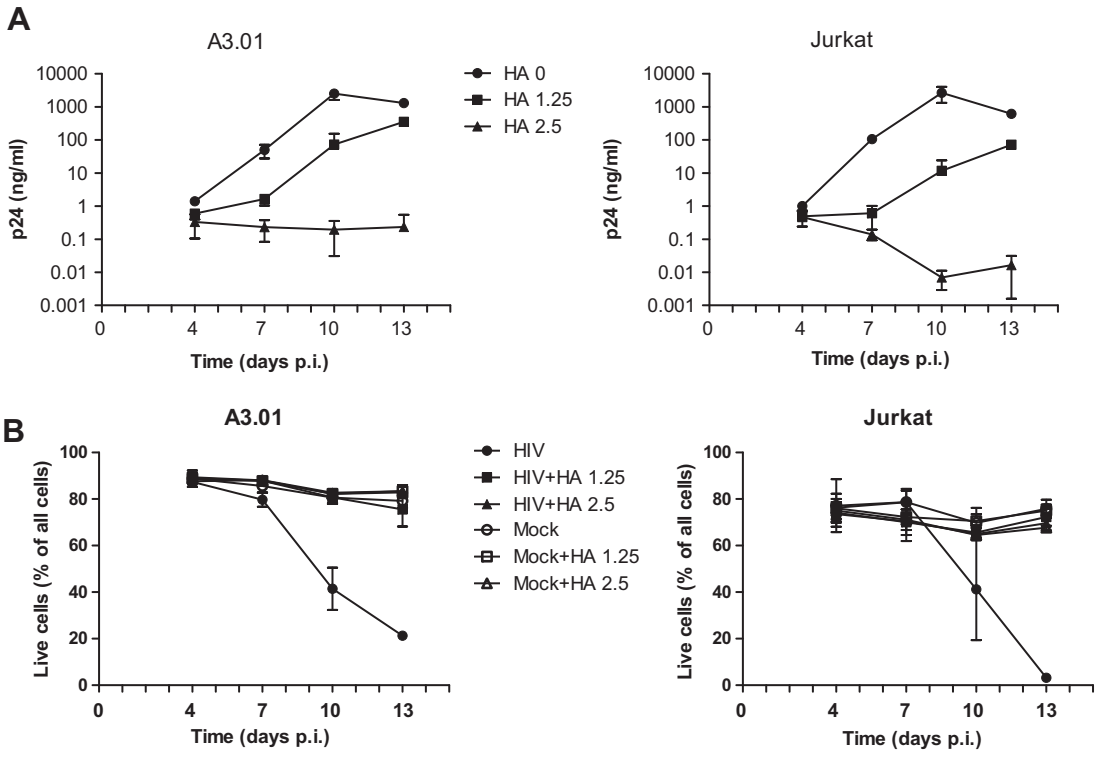
2.8. Statistical analysis

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,

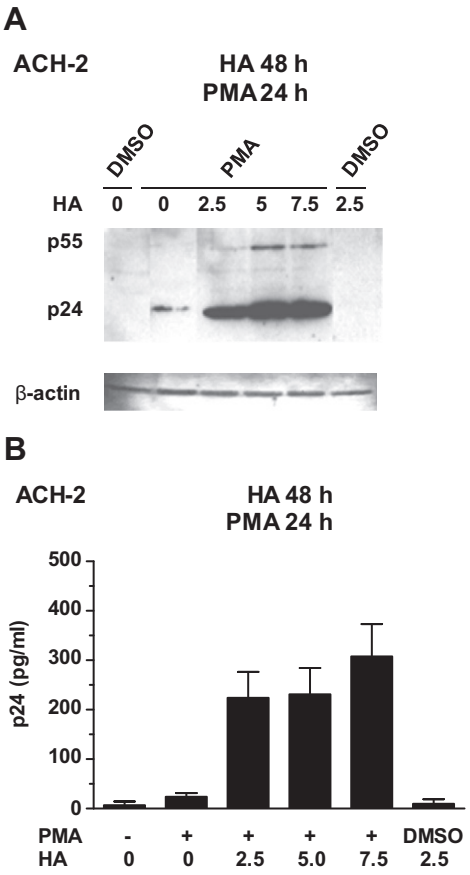


Fig. 4. HA potentiates PMA-stimulated reactivation of the HIV-1 provirus in ACH-2 cells. 0.5×10^6/ml of ACH-2 cells harboring an integrated HIV-1 provirus were pretreated with increasing concentrations of HA for 24 h, and then stimulated with phorbol myristate acetate (PMA; final concentration 0.5 ng/ml) 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

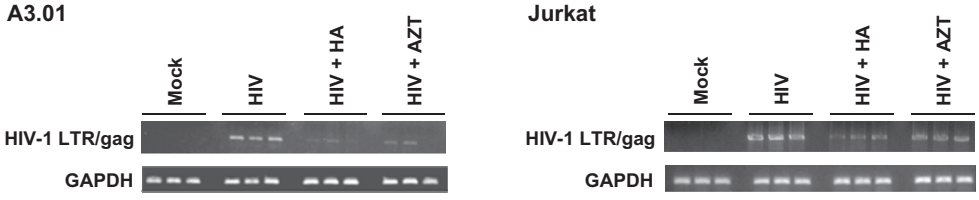


Fig. 3. HA inhibits reverse transcription of HIV-1. 0.5×10^6/ml of A3.01 or Jurkat cells were mock-infected or infected with a DNase-treated inoculum of HIV-1, and grown in 24-well plates in the presence of 2.5 μl/ml of HA or 10 μM AZT. 48 h after infection, the cells were collected and total DNA was 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.

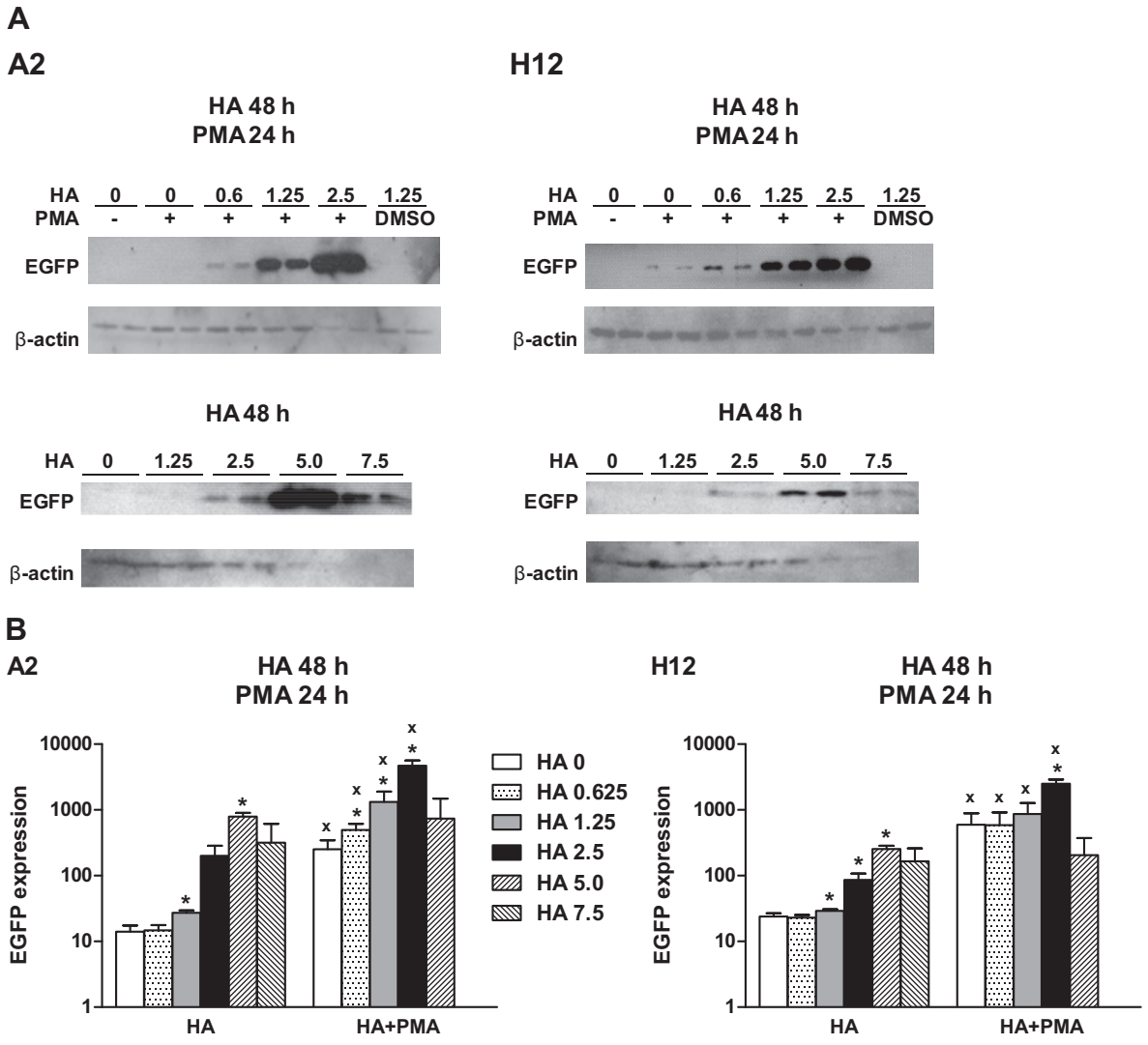


Fig. 5. HA reactivates the latent HIV-1 'mini-virus' and expression of EGFP. 0.5×10^6/ml (A) or 0.1×10^6/200 μl (B) of Jurkat cell clones A2 and H12 latently infected with a "mini-virus" containing the HIV-1 LTR-Tat-IRES-EGFP-LTR were pre-treated with increasing concentrations of HA for 24 h, and then 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

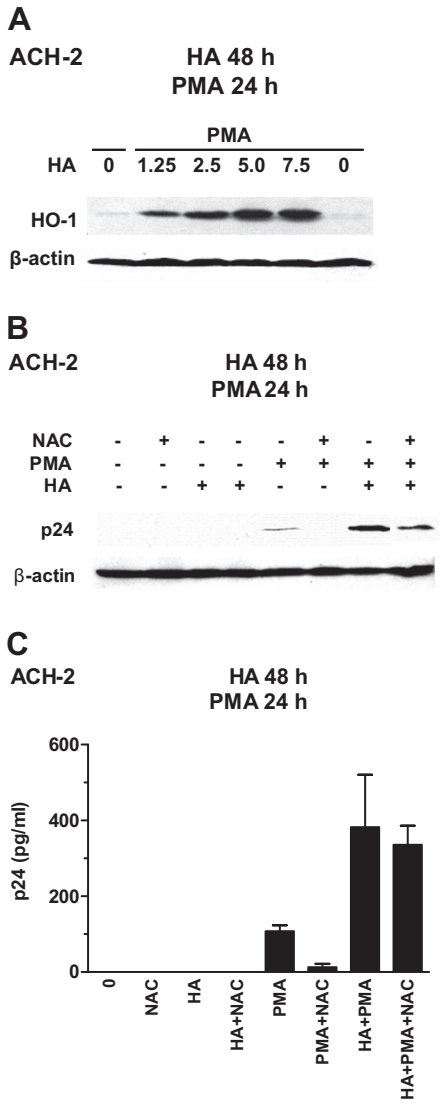


Fig. 6. (A) HA with PMA stimulate expression of heme oxygenase-1 in ACH-2 cells. 0.5×10^6/ml of ACH-2 cells harboring an integrated HIV-1 provirus were pretreated with increasing concentrations of HA for 24 h, and then stimulated with PMA (final concentration 0.5 ng/ml) in the presence of HA. 24 h after 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 pretreated 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.

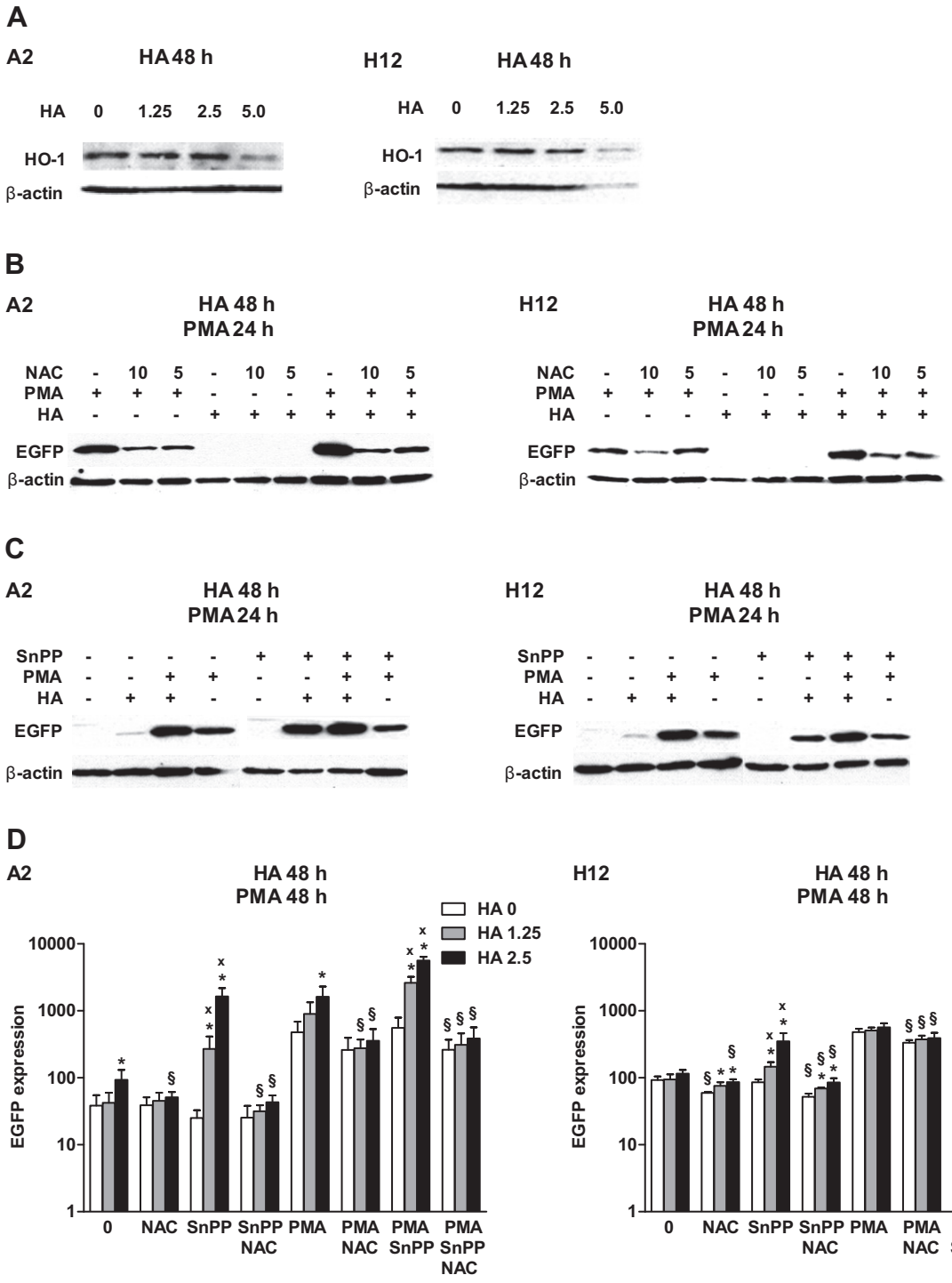


Fig. 7. (A) Expression of heme oxygenase-1 in A2 and H12 cells. 0.5×10^6/ml of Jurkat cell clones A2 and H12 latently infected with a "mini-virus" containing the HIV-1 LTR-Tat-IRES-EGFP-LTR were treated with increasing concentrations of HA for 48 h. The cells were collected, lysed in Laemmli reducing 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 ($p < 0.05$).

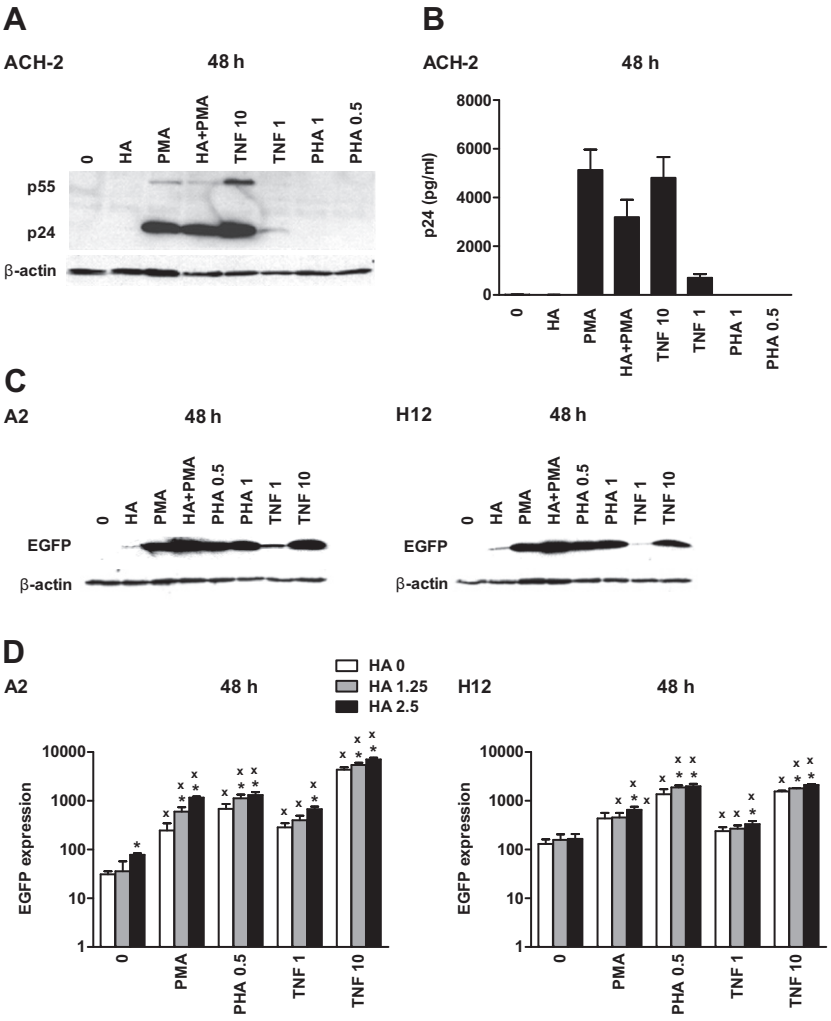


Fig. 8. Effects of TNF-α and phytohemagglutinin. (A and B) ACH-2 cells. 0.5×10^6/ml of ACH-2 cells harboring an integrated HIV-1 provirus were treated with 5 μl/ml of HA, PMA (final concentration 0.5 ng/ml), HA and PMA, TNF-α (final concentration 1 and 10 U/ml) or phytohemagglutinin (PHA; final concentration 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

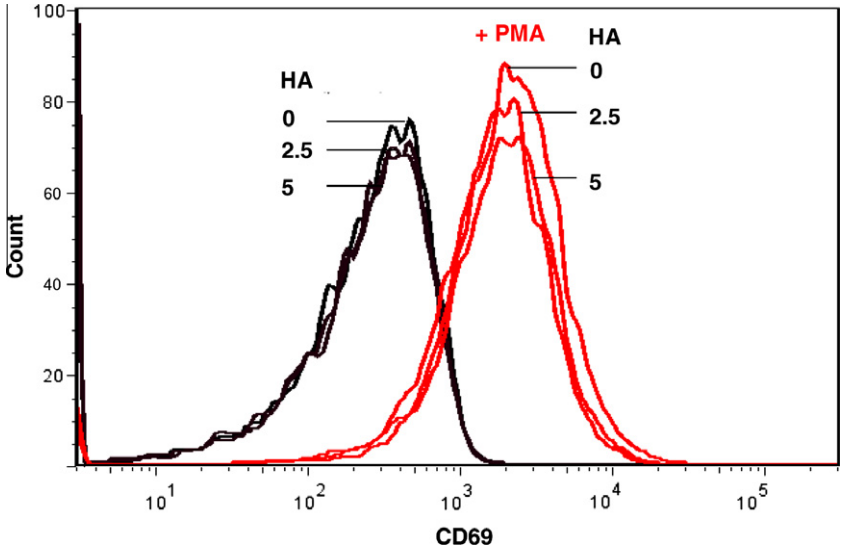


Fig. 9. Heme arginate does not affect expression of CD69 in A3.01 cells. 0.5×10^6/ml of A3.01 cells were treated with increasing concentrations of HA in the presence or absence of PMA (final concentration 0.5 ng/ml). At 24 h after stimulation, the cells were collected and stained with a mouse monoclonal 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.