Effects of heme degradation products on reactivation of latent HIV-1

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Summary. – Human immunodeficiency virus (HIV-1) infection can be currently controlled by combined antiretroviral therapy, but a sterilizing cure is not achievable as this therapy does not target persistent HIV-1 in latent reservoirs. Therefore, different latency reversal agents are intensively explored in various models. We have previously observed that heme arginate, a drug approved for human use, reveals a strong synergism with PKC inducers in reactivation of the latent provirus. Heme is physiologically decomposed by heme oxygenases into 3 degradation products: iron (Fe^{2+}), carbon monoxide (CO) and biliverdin which is further converted to bilirubin by biliverdin reductase. In this paper, we have studied the effects of individual heme-degradation products on latent HIV-1 reactivation in ACH-2 cells harboring integrated HIV-1 provirus and in H12 clone of Jurkat cells harboring HIV-minivirus expressing EGFP. We employed addition of ascorbate to generate Fe^{2+}, resulting in increased expression of both HIV-1 p24 Ag and EGFP in PMA-stimulated ACH-2 and H12 cells, respectively, as characterized on RNA and protein levels. On the other hand, addition of a CO-donor or bilirubin decreased the p24 expression. The reactivation of latent HIV-1 by iron or heme arginate was inhibited by antioxidant N-acetyl cysteine, or by an iron chelator desferrioxamine, suggesting that the effects were mediated by iron- or heme-induced redox stress. Finally, we demonstrated the stimulatory effects of heme arginate and PMA on HIV-1 expression in peripheral blood mononuclear cells of HIV-infected patients cultured *ex vivo*. These results may constitute a new direction in the latent HIV-1 reactivation and therapy.

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

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

Introduction

HIV/AIDS pathogenesis inherently involves increased generation of reactive oxygen and nitrogen species (ROS, RNS) mediated by pro-inflammatory cytokines. An increased redox stress with decreased levels of glutathione (GSH) were found already in early stages of HIV infection (Pace and Leaf, 1995). Redox stress regulates signal transduction by activating redox-sensitive transcription factors like NF-κB and NFAT, as well as chromatin remodeling, namely by inhibiting histone deacetylases (Kennedy *et al.*, 2012; Pan-

tano *et al.*, 2006; Rahman *et al.*, 2004). HIV-1 LTR includes multiple upstream DNA regulatory elements that serve as binding sites for cellular transcription initiation factors and facilitate NF-κB and NFAT binding. ROS are involved in the reactivation of HIV from latent reservoirs through post-translational activation of NF-κB (Pyo *et al.*, 2008). While activated, NF-κB facilitates HIV gene expression by directing recruitment of the histone acetyltransferases to the HIV-1 LTR (Gatignol, 2007). Iron and ascorbate-mediated redox stress affects methylation status of the promoters of genes involved in the redox stress response (Yara *et al.*, 2013).

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

Heme is an efficient inhibitor of reverse transcriptase (Argyris *et al.*, 2001; Levere *et al.*, 1991; Shankaran *et al.*, 2011), while its derivative hemin stimulates expression of various genes, especially HO-1 (Mense and Zhang, 2006). Free heme is toxic due to its ability to catalyze Fenton reaction generating hydroxyl radicals, highly reactive ROS (Shibahara, 2003). Heme from hemoglobin of aged erythrocytes is physiologically degraded by HO-1 in spleen, but the excess of heme and its derivatives stimulates HO-1 expression ubiquitously (Sheftel *et al.*, 2007; Soe-Lin *et al.*, 2008). HO-1 breaks down heme into iron (Fe^{2+}), carbon monoxide (CO), and biliverdin that is consequently reduced to bilirubin by the action of biliverdin reductase. Fe^{2+} might induce a short-term pro-oxidative state, while CO and especially the redox cycle bilirubin/biliverdin/biliverdin reductase act as antioxidants. In general, HO-1 is considered an antioxidant enzyme with immunomodulatory properties (Otterbein *et al.*, 2003).

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

Materials and Methods

Chemicals. All the media and growth supplements were purchased from Thermo Scientific (USA) or PAA Laboratories GmbH (Austria). Other chemicals used, including PMA, ferric nitrate, ascorbic acid, CORM-A1, bilirubin, desferrioxamine mesylate salt (DFO) and NAC were purchased from Sigma-Aldrich (Germany) unless otherwise specified. HA (Normosang) was purchased from Orphan Europe (France). The chemicals for RNA and DNA isolation, PCR and real-time PCR were purchased from Top-Bio (Czech Republic), for ddPCR from Bio-Rad (USA), primers and probes from IDT (Belgium) and Life Technologies (USA). TURBO DNA-free kit, Ambion, was from Life Technologies.

Cell lines, primary cells and their treatment. Human T-cell line ACH-2 harboring an integrated HIV-1 provirus (clone #4; Clouse *et al.*, 1989), their parental cell line A3.01, and the H12 clone of Jurkat cells latently infected with a "mini-virus" containing the HIV-1 LTR-Tat-IRES-EGFP-LTR (Blazkova *et al.*, 2009; Jordan *et al.*, 2003; Shankaran *et al.*, 2011), were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mmol/l glutamine, 12.5 mmol/l Hepes, and antibiotics (penicillin 1x10⁵ U/l, streptomycin 100 mg/l; 10% FBS-RPMI). The cells were plated at final concentration of 0.5 x 10⁶ cells/ml/well in 10% FBS-RPMI and then treated with iron (10 μmol/l) and ascorbate (0.25 mmol/l in ACH-2 cells; 0.25, 0.5, 1 and 2 mmol/l in A3.01 and H12 cells; Hermes-Lima *et al.*, 2000; Zhang *et al.*, 2008) or with heme arginate (2.5 μl/ml), followed by PMA (0.5 ng/ml); in certain experiments, the cells were pre-treated with additional compounds as specified. The cells were collected after 24 h (ACH-2 cells) or 48 h (A3.01 and H12 cells). Stock solutions of PMA and bilirubin were prepared in DMSO and freshly diluted in culture medium to 20x working solutions. Other working solutions were freshly prepared as follows: 100x Fe^{3+} (1 mmol/l FeNO₃ in 50 mmol/l Hepes pH = 8 and 20 mmol/l EDTA), 100x ascorbate (25 mmol/l ascorbic acid in H₂O), 100x CORM-A1 (5 mmol/l CORM-A1 in H₂O), 20x NAC (100 mmol/l in RPMI 1640), 100x DFO (12.5 mmol/l in H₂O). The compounds were added at final concentrations specified in each experiment. PBMC's of HIV-infected patients on cART with undetectable viremia (<50 copies/ml) were isolated by Ficoll gradient centrifugation (Histopaq, Sigma-Aldrich), differential blood count was determined by Advia 60 hematology system (Bayer Healthcare, USA) and PBMC's were resuspended at final concentration of 5 x 10⁶ lymphocytes/ml in 10% FBS-RPMI. PBMC's were plated at 1 ml/well in a 24-well plate and subjected to treatment with 2.5 μl of HA/ml and different concentrations of PMA for 18 h.

Western blot analysis. Cells were collected after 24 h of incubation, lysed in Laemmli reducing sample buffer, boiled and analyzed by SDS-PAGE and western blotting as previously described (Harlow, 1988; Laemmli, 1970; Shankaran *et al.*, 2011), using chemiluminescence (West Femto, Thermo Scientific). The cell lysates were resolved on a 12% SDS–PAGE and transferred to a PVDF membrane (Hybond, Bio-Rad). HIV-1 p24 was detected using a mouse monoclonal antibody ND-1 (dilution 1:500; Exbio, Czech Republic) and a peroxidase-conjugated goat anti-mouse IgG (dilution 1:20,000; Sigma Co., USA). β-actin was detected using a rabbit polyclonal antibody (dilution 1:10,000; Abcam, UK) and a peroxidase-conjugated goat anti-rabbit IgG (dilution 1:20,000, MP Biomedicals-Cappel,

USA). The chemiluminescence was recorded using ChemiDoc MP system (Bio-Rad) and the densitometry analysis was performed with ImageLab software version 5.0 (Bio-Rad).

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

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

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

Quantification of HIV-1 RNA by real-time PCR. RNA isolated from cultured and stimulated PBMC's of HIV-infected patients treated with cART was used for detection of cell-associated HIV-1 RNA using semi-nested 2-step RT-qPCR adapted from previous papers (Kiselinova *et al.*, 2014; Pasternak *et al.*, 2008). Sixteen µl of DNase-treated RNA were used for reverse transcription in a final volume of 40 µl using random hexamer primers (Premium Reverse Transcriptase, Thermo Scientific). Then, 20 µl of cDNA were amplified in a total volume of 100 µl using GAG1 and SK431 primers (final concentration 250 nmol/l each; SK431 sequence: 5'-TGCTATGTCAGTTCCCCTTGGTTCTCT-3'; Pasternak *et al.*, 2008) and 0.05 U/µl of Blood Taq DNA polymerase (Top-Bio) in 1x PCR Blue buffer (Top-Bio) containing 2 mmol/l MgCl$_2$. The thermal cycling conditions were as follows: initial denaturation at 95ºC for 5 min, 15 cycles consisting of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s, and extension at 72ºC for 60 s, followed by terminal extension at 72ºC for 10 min using the Mastercycler gradient (Eppendorf AG). Finally, 10 µl of the first PCR product were used in duplicate for real-time PCR in a total volume of 50 µl containing GAG1 and GAG2 primers, GAG3 probe (final concentration 200 nmol/l each; GAG3 sequence: FAM-5'-ATTATCAGAAGGAGCCACCCCACAAGA-3'-TAMRA; Pasternak *et al.*, 2008), and 1x qPCR Blue buffer (Top-Bio) using the Applied Biosystems 7300 Real-time PCR system (Applied Biosystems, USA) and the universal cycling conditions. Four µl of the first PCR product were used for quantification of a reference gene in a 20 µl reaction also in duplicate. Four µl of DNase-treated RNA were used for a no-RT control and further processed in the way analogous to cDNA. The mean of technical duplicates was used for relative quantification of HIV Gag compared to GAPDH. Changes in expression of 10 additional house-keeping genes in healthy donors PBMCs after the individual treatments were determined using Real-time PCR kit for Human reference genes (Generi Biotech, Czech Republic).

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

Results

First, we aimed to determine the effects of iron-mediated redox stress on reactivation of the latent HIV-1 in ACH-2 cells harboring an integrated HIV-1 provirus. Since Fe^{2+} is quickly

(a)

(b) **(c)**

(d)
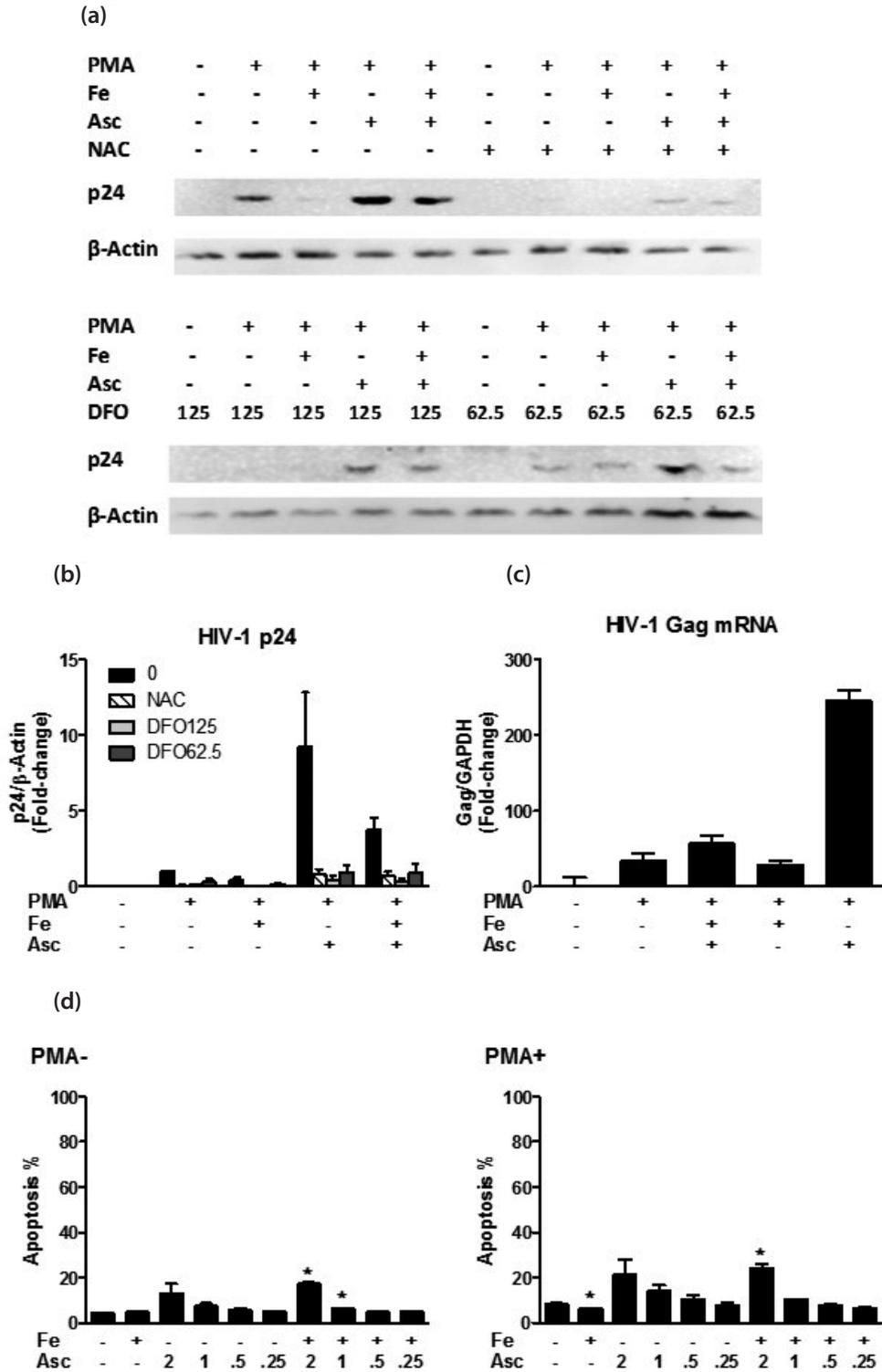
Fig. 1

Effect of iron and ascorbate on PMA-stimulated reactivation of the latent HIV-1

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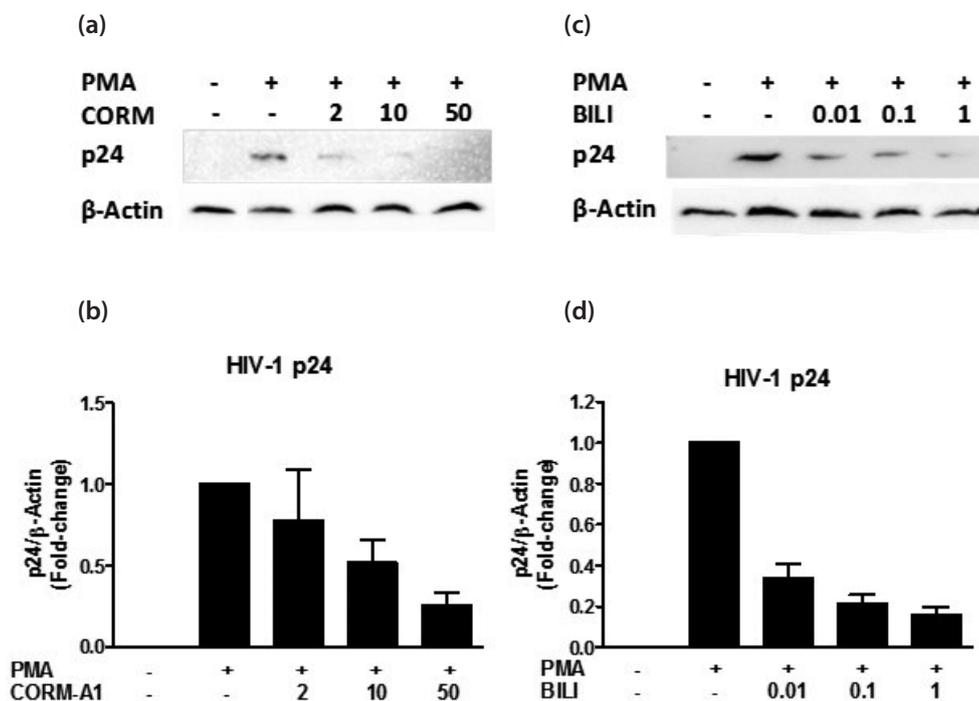
Fig. 2

Carbon monoxide and bilirubin dose-dependently inhibit PMA-stimulated reactivation of the latent HIV-1

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

oxidized to Fe^{3+} in the aqueous environment, we employed ascorbate to generate Fe^{2+} in our system (Hermes-Lima *et al.*, 2000; Zhang *et al.*, 2008). We have treated unstimulated or PMA-stimulated ACH-2 cells with Fe^{3+}, ascorbate and with a combination of Fe^{3+} and ascorbate. In unstimulated cells, none of the treatments induced p24 expression as detected by western blot analysis and chemiluminescence, recorded and analyzed digitally by the ChemiDoc system. In the PMA-stimulated cells, Fe^{3+} alone decreased the PMA-stimulated p24 levels, while addition of ascorbate with Fe^{3+} reversed inhibition by Fe^{3+} due to generation of Fe^{2+} (Fig. 1 a,b); furthermore, ascorbate with PMA revealed the strongest stimulatory effects. All the stimulatory effects on p24 expression were inhibited by pretreatment of the cells with an antioxidant NAC, precursor of GSH, or with an iron chelator desferrioxamine. These results suggest involvement of free radicals and/or iron in the reactivation of the latent HIV-1 by these compounds, including ascorbate. Interestingly, the effect of Fe^{3+} on PMA- and ascorbate/PMA-stimulated p24 expression was inhibitory in a way similar to NAC. Thus, Fe^{3+} alone appears to reveal final antioxidant effects in this system.

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

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

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

(a)

(b)
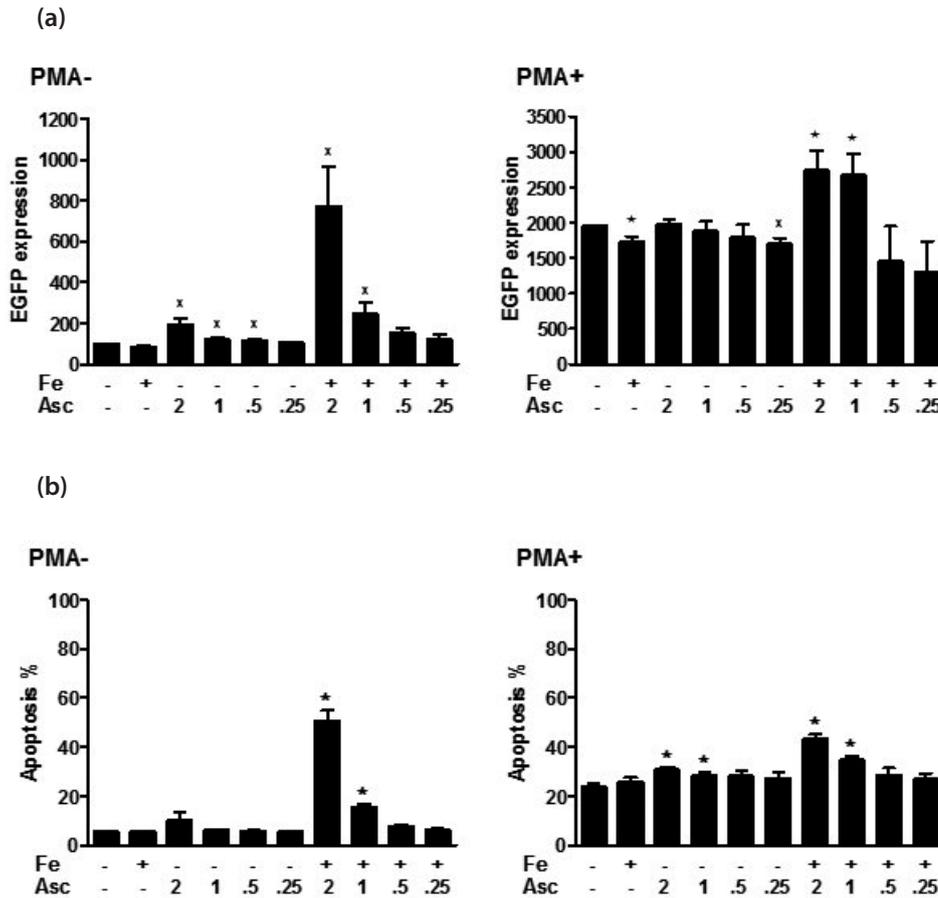
Fig. 3

Flow cytometric analysis of EGFP expression
H12 cells treated with 10 μmol/l ferric nitrate (Fe), 2, 1, 0.5 or 0.25 mmol/l ascorbate (Asc), and 0.5 ng/ml PMA for 48 h. **(a)** Flow cytometric analysis of EGFP expression. **(b)** Percentage of apoptosis. ˣ,*Increase is statistically significant when compared to control in each group (p <0.1, 0.05, respectively).

Additionally, we used the H12 clone of Jurkat cells harboring a latent HIV-1 "mini-virus" expressing EGFP under control of HIV-1 LTR (Shankaran *et al.*, 2011; Jordan *et al.*, 2003). This clone was previously shown to possess an increased methylation of the HIV-1 LTR (Blazkova *et al.*, 2009) and revealed similar responses to HA administration as ACH-2 cells, except that HA alone, in the absence of PMA, induced EGFP expression also (Shankaran *et al.*, 2011). The combination of iron and ascorbate stimulated EGFP expression both with and without PMA, as determined by flow cytometry, while higher concentrations of ascorbate alone induced EGFP expression also in the unstimulated H12 cells (Fig. 3a). In other concentrations, including 0.25 mmol/l that was used in ACH-2 cells, ascorbate did not induce any reactivation. Further, we have determined the percentage of apoptosis induced by the individual treatments in these cells (Fig. 3b). PMA treatment increased the background apoptosis about 5-times, while percentage of apoptosis

induced by the individual treatments seemed to reveal a similar pattern as EGFP expression. Then, we tested the effects of CORM-A1 and bilirubin in H12 cells. CORM-A1 increased the levels of EGFP expression in PMA-stimulated H12 cells while pretreatment with bilirubin did not seem to affect EGFP expression (data not shown). On the other hand, percentage of apoptosis was not affected either by CORM-A1 or bilirubin (around 5 and 25% in unstimulated and PMA-stimulated cells, respectively).

Previously, we have demonstrated a synergistic effect of heme arginate and PMA on reactivation of the latent HIV-1 in ACH-2 cells that was inhibited by the antioxidant NAC (Shankaran *et al.*, 2011). In order to determine the role of iron in the HA-mediated effects, we have now pre-treated the ACH-2 cells with DFO before addition of HA and PMA. As shown in Fig. 4 (a,b) DFO decreased HA- and PMA-mediated reactivation of latent HIV-1 as characterized by levels of p24 antigen and western blot analysis. Of note, DFO

(a)

PMA	-	-	+	+	-	-	+	+	-	-	+	+
HA	-	+	-	+	-	+	-	+	-	+	-	+
DFO	-	-	-	-	125	125	125	125	62.5	62.5	62.5	62.5

p24

β-Actin

(b) HIV-1 p24

(c) HIV-1 Gag mRNA
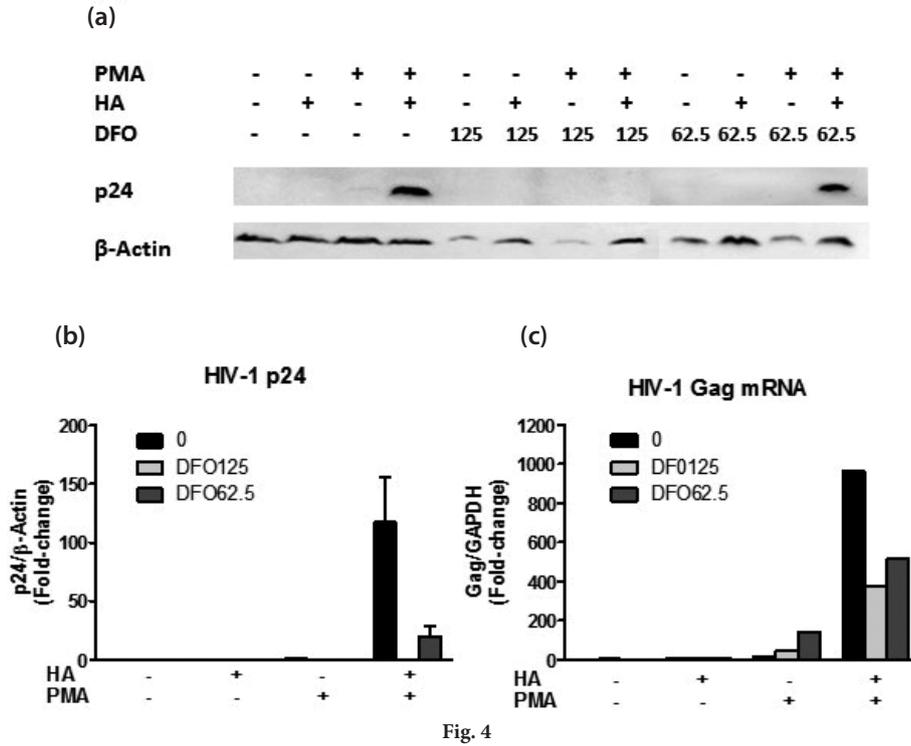
Fig. 4

Iron chelator prevents hem arginate- and PMA-stimulated reactivation of the latent HIV-1

ACH-2 cells pre-treated with 125 and 62.5 μmol/l DFO for 4 h, and treated with 2.5 μl of heme arginate/ml and 0.5 ng/ml PMA for 24 h **(a)** Western blot analysis of HIV-1 p24 antigen. **(b)** Quantification of the western blots presented in panel (a). **(c)** ddPCR analysis of HIV-1 RNA.
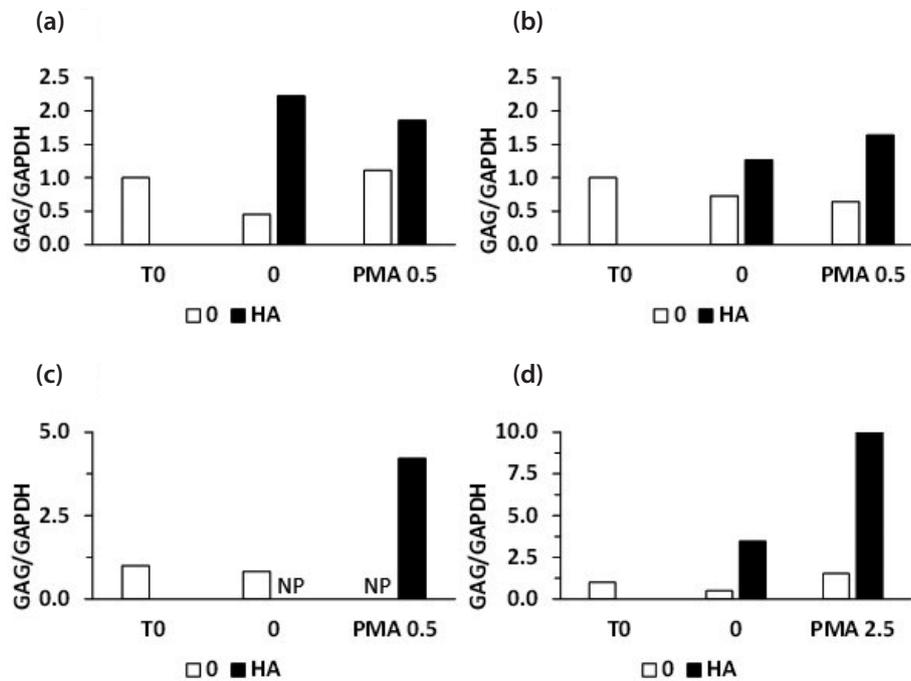
Fig. 5

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

HIV+ human PBMC's treated with 2.5 μl of heme arginate/ml and 0.5 or 2.5 ng/ml PMA for 18 h. **(a,b,c,d)** Fold-changes in Gag/GAPDH mRNA levels in PBMC's of 4 aviremic HIV+ patients on cART. T0 = time 0, 0 = no treatment, HA = heme arginate 2.5 μl/ml, PMA 0.5 or 2.5 ng/ml, NP = not performed.

decreased also the levels of control β-actin in the absence of HA in these experiments. Further, we have determined the levels of HIV-1 mRNA using 1-step RT-ddPCR (Fig. 4c). As expected, DFO also decreased the levels of HA- and PMA-stimulated levels of HIV-1 mRNA, indicating the role of iron in PMA- and HA-mediated effects.

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

Discussion

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

Previously published results indicated that a longer pretreatment with ascorbate suppressed the induction of HIV reactivation in latently infected T-cell lines (Harakeh and Jariwalla, 1997). 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

HEME ARGINATE ACTION
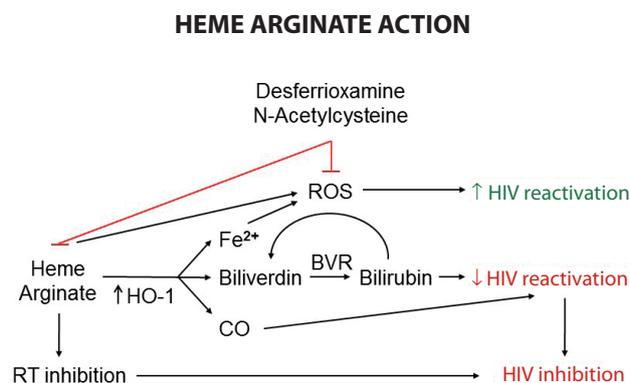
Fig. 6

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

attributed to a double faced character of ascorbate that can act as an anti- or pro-oxidant, depending on its concentration (Best, 2014). Further, the stimulatory effects of ascorbate but also those of PMA were inhibited by DFO, suggesting involvement of iron in these processes. In addition to direct effects of DFO on iron chelation, the inhibition by DFO might be explained also by previously described inhibitory effects of DFO on activation of NF-κB, the redox-sensitive transcription factor involved in both PMA signaling and HIV-1 reactivation (Holden *et al.*, 2008; 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 this paper were comparable or lower than effective concentrations of heme arginate. On the other hand, effective concentrations of heme arginate are achievable *in vivo* (Tokola *et al.*, 1986).

In ACH-2 cells, the effect of ascorbate or iron with ascorbate on induction of latent HIV expression was relatively lower at both RNA and protein levels than the effect of HA. Indeed, HA serves as a source of both iron and hemin. Heme similarly to iron generates ROS by Fenton reaction (Shibahara, 2003), while hemin is known to regulate expression of various genes (Furuyama *et al.*, 2007; Mense and Zhang, 2006). On the other hand, pretreatment with a CO-donor or

bilirubin dose-dependently decreased p24 levels in ACH-2 cells, while the CO donor increased EGFP expression and bilirubin was ineffective in H12 cells. These differences may be attributable to a different intracellular redox milieu, possibly affected by a constitutive expression of HO-1 in H12 cells (Shankaran *et al*., 2011). These results also suggest that the contribution of the individual heme-degradation products may vary in different cell types *in vivo*. In the first approach to verify the stimulatory effects of heme arginate we have intentionally used PBMC's as they are closer to the situation *in vivo*. Nevertheless, additional experiments on individual cell types, namely on isolated total and resting CD4+ cells, will better assess the magnitude of HA-stimulatory effects and help to delineate the underlying mechanisms.

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

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

In our short-term experimental conditions, the stimulatory effects of heme arginate on HIV-expression clearly prevailed in ACH-2 and H12 cells as well as in HIV-infected PBMC's (Shankaran *et al*., 2011). On the other hand, heme arginate inhibits the acute HIV infection at the level of reverse transcription (Levere *et al*., 1991; Shankaran *et al*., 2011). Thus, the ability of heme arginate to stimulate a short-term reactivation of the latent HIV while inhibiting reverse transcription and further replication of the new virus progeny could make it a useful and safe agent in help to eliminate the HIV-1 latent pool in the presence of antiretroviral drugs.

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

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