Biochemical and Biophysical Research Communications 464 (2015) 7–12

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Hemin activation of innate cellular response blocks human immunodeficiency virus type-1-induced osteoclastogenesis

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ARTICLE INFO

Article history:
Received 21 April 2015
Available online 19 May 2015

Keywords:
HIV-1
Hemin
Heme oxygenase-1
Osteoclasts

ABSTRACT

The normal skeletal developmental and homeostatic process termed osteoclastogenesis is exacerbated in numerous pathological conditions and causes excess bone loss. In cancer and HIV-1-infected patients, this disruption of homeostasis results in osteopenia and eventual osteoporesis. Counteracting the factors responsible for these metabolic disorders remains a challenge for preventing or minimizing this co-morbidity associated with these diseases. In this report, we demonstrate that a hemin-induced host protection mechanism not only suppresses HIV-1 associated osteoclastogenesis, but it also exhibits anti-osteoclastogenic activity for non-infected cells. Since the mode of action of hemin is both physiological and pharmacological through induction of heme oxygenase-1 (HO-1), an endogenous host protective response to an FDA-licensed therapeutic used to treat another disease, our study suggests an approach to developing novel, safe and effective therapeutic strategies for treating bone disorders, because hemin administration in humans has previously met required FDA safety standards.

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1. Introduction

Osteoclasts are the normal bone resorbing cells. These cells release calcium and growth factors from bone to maintain normal bodily functions and contribute to physiological bone remodeling, as well as pathological bone destruction in osteoporosis and rheumatoid arthritis; thus, they represent a pharmacological target for drug development [1–5]. Extensive evidence points convincingly towards increased activity of osteoclasts and impaired activity of osteoblasts in cancer and HIV-infected patients, leading to a significant increase in the prevalence of osteoporosis [6–16].

HIV-infected patients show bone loss and osteopenia/osteoporosis during the course of the disease [17–22]. The mechanisms underlying this degenerative process are largely unclear, and it has yet to be determined how bone dysfunction is linked to HIV-1-mediated direct and/or indirect effects on osteoblasts/osteoclasts and their cross-talk regulation. In addition, development of osteopenia and augmentation of osteoporosis are reported to be associated with antiretroviral treatment [23–30], although the mechanisms involved have also not yet been elucidated. Since osteoclasts, the large multinucleated cells responsible for resorption of bone, are the mediators of continuous bone loss, identification of host factors promoting or inhibiting osteoclastic activity will facilitate designing effective therapeutic strategies against osteoporosis induced by HIV and cancer.

In this report, we demonstrate that HIV-infected monocyte-derived macrophages (MDM) are morphologically and functionally related to osteoclasts, and they exhibit increased susceptibility to multinucleated giant cell formation by receptor activator of nuclear factor kappa-B ligand (RANKL), a pivotal factor for differentiation of pre-osteoclasts into osteoclasts. In addition, we show that pharmacologically relevant concentrations of hemin inhibit HIV-induced osteoclast formation, as well as blocking RANKL function to prevent osteoclastogenesis. This is the first report to our knowledge demonstrating that hemin can serve as a potentially novel biologic in mitigating the effects of both HIV and RANKL on osteoclastogenesis, thus suggesting new strategies for developing potentially safe therapeutic interventions for the treatment of osteopenia/osteoporosis associated with HIV or other medical conditions.

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http://dx.doi.org/10.1016/j.bbrc.2015.05.037
0006-291X/Published by Elsevier Inc.

2. Materials and methods

2.1. Reagents

The FDA-approved drug, Panhematin®, was purchased from Lundbeck (manufactured by APP Pharmaceuticals) and used to induce the cytoprotective enzyme heme oxygenase-1 (HO-1). Mouse-anti-human HO-1 antibody was purchased from Enzo Life Sciences (Farmingdale, NY). The HIV-1_{Ba-L} strain was purchased from Advanced Biotechnologies, Inc., Columbia, MD.

2.2. Isolation, culture, and infection of cultured monocytes

Human monocytes were isolated from peripheral blood mononuclear cells of donors seronegative for HIV-1 and hepatitis B after leukopheresis and were purified by countercurrent centrifugal elutriation [31]. Cell suspensions contained >95% monocytes based on cell morphology in Wright-stained cytosmears, granular peroxidase, and nonspecific esterase. The cells were cultured for 5 days in DMEM supplemented with 10% FBS, 20 μg/ml gentamicin, 1000 U/ml M-CSF, and then were infected with HIV-1_{Ba-L} as previously described [32]. Cell-free culture supernatants were assayed for HIV-1-p24 using an NEN/DuPont ELISA analysis kit (PerkinElmer) according to the manufacturer's instructions.

2.3. Detection of HO-1 expression by Western blot (WB) analysis

Total protein extracts were prepared from MDM in a proprietary formulation of lysis buffer containing SDS and protease and phosphatase inhibitors (Kendrick Labs, Inc., Madison, WI). Protein concentrations were determined using BCA protein assay kits (Pierce). After addition of loading buffer, equal protein amounts (2.5 μg) of each lysate preparation were subjected to electrophoresis using 10–20% polyacrylamide gradient gels (Invitrogen Life Technologies). Proteins were transferred to nitrocellulose by electroblotting, and nonspecific sites were blocked with 5% nonfat milk in PBS, pH 7.4, containing 0.1% Tween 20 (PBST) for 18 h at 4 °C. After washing three times with PBST, the blots were incubated for 1 h at room temperature with a cocktail of mouse or rabbit-anti-human HO-1 antibody and rabbit-anti-human actin antibody, and washed three times with PBST. Transferred proteins were incubated with the ECL Western blotting detection system (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions, followed by visualization with x-ray film.

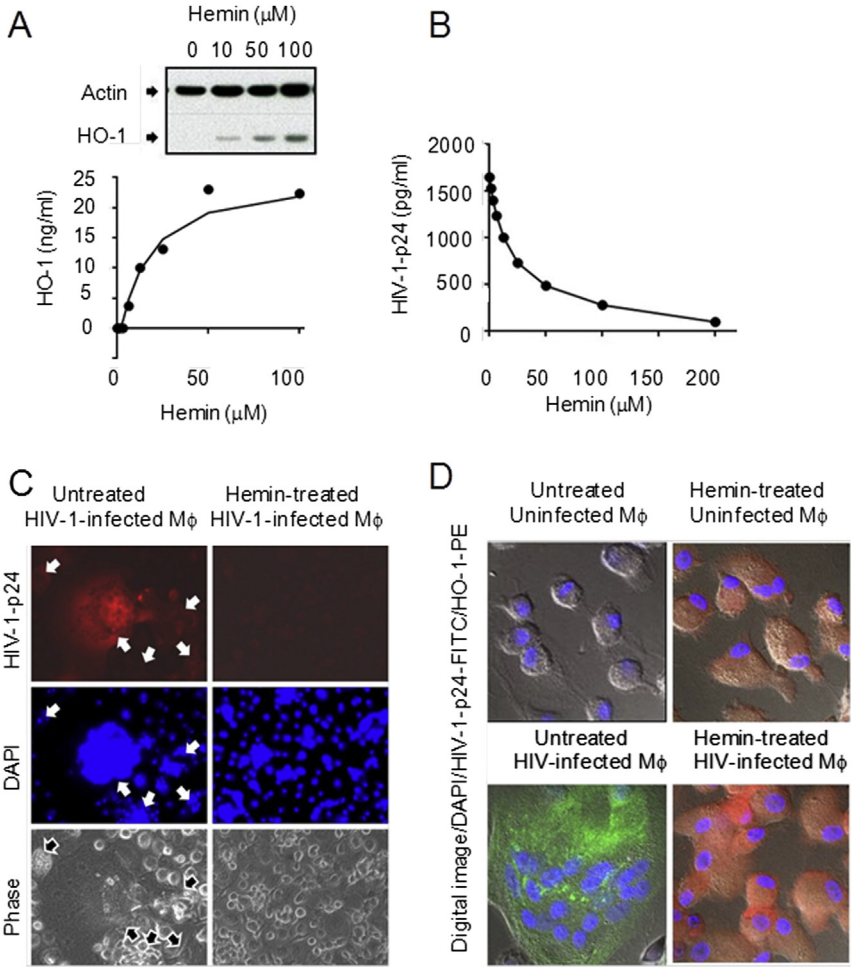

Fig. 1. HO-1 induction is inversely related to HIV infection of MDM. A. MDM treated with various concentrations of hemin and examined for cellular HO-1 induction by WB and ELISA. B. HIV-1-p24 levels in culture fluids from MDM infected with HIV in the absence or presence of various concentrations of hemin. C. HIV-1-p24 expression and nuclear staining by immunofluorescence microscopy and phase-contrast images of HIV-infected MDM cultured for 10 days in the absence or presence of 100 μM hemin. D. Confocal microscopy of uninfected and HIV-1-infected MDM cultured in the absence or presence of 100 μM hemin followed by simultaneous staining with FITC-conjugated mouse-anti-human HIV-1-p24 mAb and PE-conjugated mouse-anti-human HO-1 mAb. The data are representative of three independent experiments.

2.4. Osteoclasts

Osteoclast precursor cells and growth media were purchased from Lonza (Walkersville, MD), and differentiated into matured osteoclasts according to the supplier's instructions. Cells were cultured at 37 °C for 10 days in the absence or presence of 100 μM hemin.

2.5. Determination of tartrate-resistant acidic phosphatase (TRAP) activity and cell fusion

TRAP cytochemistry was performed using the leukocyte acid phosphatase assay kit (Sigma, St. Louis, MO) following the procedures described by the manufacturer. TRAP-stained cells were scored under a light microscope. For cell fusion analyses, TRAP-stained cells were counter-stained with hematoxylin solution. The cell fusion index was determined as the number of nuclei contained in multinucleated (>5 nuclei per cell) TRAP-positive cells.

2.6. Confocal microscopy

Human monocytes (1.5×10^4) were cultured in 8-well chambered slide systems (Nunc, Rochester NY) then treated with HIV and/or hemin as described above. Cells were fixed with 4% paraformaldehyde and stained with primary antibodies for HIV-1-p24 and HO-1, followed by secondary antibodies conjugated with FITC or PE. Nuclear counterstaining was performed using DAPI. The cells were imaged with a 63× objective lens on a Zeiss Cell Observer Spinning Disk Confocal Microsope system (Carl Zeiss, Thornwood, NY). Excitation wavelengths of 405, 488, and 561 nm laser lines were used for UV, FITC, and PE channels, respectively. The emission filter for UV (DAPI) was 450/50, for FITC was 525/50, and for PE was 629/62. Zeiss AxioVision software (ver. 4.8.2) was used for image acquisition. The image data were stored in zvi format for further analysis.

2.7. Statistics

The statistical values were calculated by one-way ANOVA with Dunnett's multiple comparison post-test.

3. Results and discussion

Excessive bone loss is a skeletal dysfunction frequently associated with breast and prostate cancer, but is also a hallmark bone disorder in HIV-infected patients treated with antiretroviral drugs. Clinical and epidemiological investigations have documented a high prevalence of reduced bone mineral density due to osteoclastogenesis in cancer and AIDS patients during the course of the disease. These are major health concerns requiring complicated, multiple clinical interventions. In this study, we describe a unique approach that uses a hemin-induced host protection mechanism that not only resists factors promoting osteoclastogenesis while inhibiting HIV infection, but also exhibits anti-osteoclastogenic activity when tested on non-HIV-infected cells.

Hemin is a critical component of hemoglobin and the active ingredient of Panhematin®, the first FDA-approved formulation of hemin for clinical use in the United States (reviewed in Ref. [33]). Intravenous Panhematin® has been used clinically to treat patients with acute porphyrias for nearly four decades. We have demonstrated that hemin induction of an endogenous host factor, HO-1, enhances cellular defense against viral transcription and productive replication [32]. We cultured primary MDM in the absence or presence of hemin, infected with HIV-1, and then examined for HO-1 induction, HIV replication, multinucleated giant cell formation, and expression of HIV-1-p24 antigen 10 days after infection. Consistent with previous reports on the role of HO-1 in host defense against several pathogenic infections [34–38], hemin treatment induced HO-1 expression in a dose-dependent manner in MDM (Fig. 1, panel A) and was directly related to decreased virus replication in HIV-infected MDM (Fig. 1, panel B).

Immunostaining showed high expression of intracellular HIV-1-p24 antigen in the HIV-infected MDM, confirming productive infection (Fig. 1, panel C). Simultaneous staining with DAPI and phase-contrast microscopy revealed large clusters of cells containing multiple nuclei, and many had more than 100 nuclei in a single infected cell. This phenomenon is a typical characteristic of the extensive cytopathic effects seen in HIV-infected MDM. Cells infected with HIV and cultured in the presence of hemin were negative for the expression of HIV-1-p24 with fewer nuclei per multinucleated cell. The absence of HIV-1 antigen and multinucleation in hemin-treated HO-1 expressing cells was further confirmed by confocal microscopy (Fig. 1, panel D).

Osteoclasts are bone-resorbing cells that are characterized by intracellular TRAP activity [39]. We stained HIV-infected MDM with TRAP reagent to assess TRAP activity. HIV-infected MDM were cytologically positive for TRAP activity, typically characterized by insoluble intracellular red-brown precipitates in all multinucleated cells (Fig. 2A, panel a), indicating that these cells are of

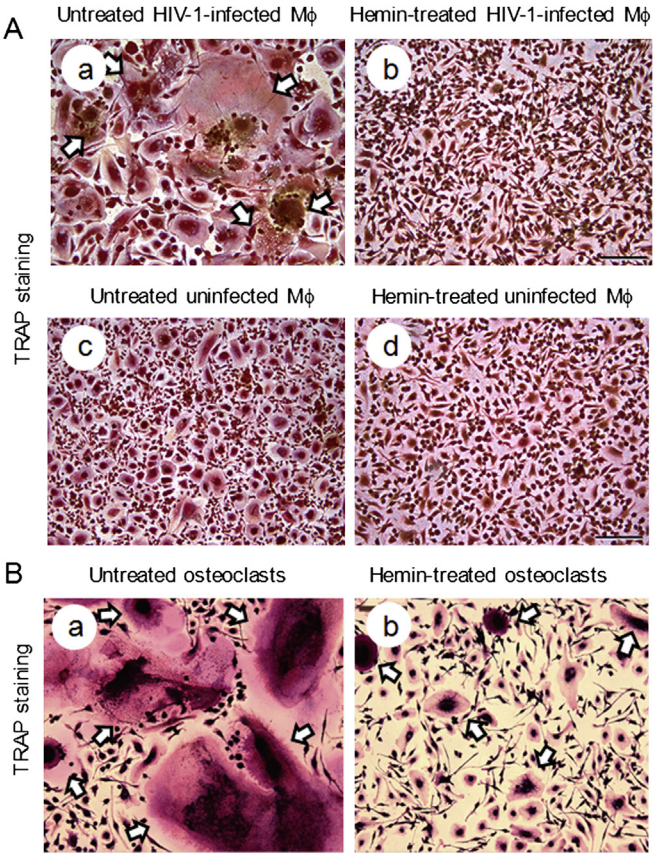

Fig. 2. TRAP activity in HIV-infected MDM and normal osteoclasts. Uninfected or HIV-infected MDM (Mϕ) (Panel A) and normal osteoclasts (Panel B) were cultured for 10 days in the absence or presence of hemin, stained with TRAP reagent, and examined for TRAP enzymatic activity by microscopy. Red-brown color in multinucleated giant cells represents TRAP activity (shown by arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

osteoclast lineage. Importantly, however, hemin-treated HIV-infected MDM both contained much fewer nuclei per multinucleated cell and exhibited little or no TRAP activity (Fig. 2A, panel b). Similarly, untreated uninfected MDM and hemin-treated uninfected MDM exhibited fewer than 3 nuclei per cell with little or no TRAP activity (Fig. 2A, panels c and d, respectively, respectively).

To examine the effect of hemin on HIV-1-independent osteoclastic activity, uninfected osteoclast precursor cells were cultured at 37 °C in the absence or presence of 100 μM hemin, and osteoclastogenesis was identified by TRAP staining. The number of TRAP-positive multinucleated cells per well were scored 10 days after incubation. Data shown in Fig. 2B demonstrate that hemin treatment significantly reduced the number of TRAP-positive multinucleated giant cells formed (panel b) as compared to cells cultured in the absence of hemin (panel a). The latter results are consistent with a previous report [40] and those observed in this study in HIV-1-infected MDM, suggesting that hemin could be a beneficial biologic for the treatment of pathological bone loss.

Since osteoclasts, the primary cells responsible for bone resorption, are derived from hematopoietic precursors of the monocyte-macrophage lineage, we addressed in further depth the questions: Does HIV infection of MDM lead to the development of osteoclasts? If so, does induction of a host HO-1 response inhibit osteoclastogenesis in HIV-infected MDM? Because cellular immunity and bone metabolism are intimately connected in the osteoimmune network, addressing these specific questions could provide useful clues for understanding the pathophysiology and for developing effective therapeutic strategies to treat the bone disorders in HIV-infected individuals. CD4+ T lymphocytes exposed to HIV-1 envelope glycoprotein gp120 produce RANKL, the primary cytokine in osteoclast differentiation and bone resorption [41,42]. In addition, HIV-Tat is a RANKL inducer [43]. Therefore, our culture system containing MCSF could provide a favorable environment for generation of osteoclasts from HIV-infected MDM.

While multiple factors, including RANKL, have been identified which are produced by tumor and HIV-infected cells in promoting bone destruction, the etiology of this metabolic disorder in HIV infection is unclear. It has been linked to multiple causes including the interplay of viral–host interactions and, in addition, to treatment of HIV-infected patients with antiretroviral therapy (ART). We have defined hemin activation of innate HO-1 cellular response as an alternative approach to induce host cell responses that might serve as a substitute for ART or be used concurrently could help alleviate some of these HIV-related metabolic complications.

We tested the role of RANKL in promoting further osteoclastogenesis in HIV-infected MDM. Culturing HIV-infected MDM in

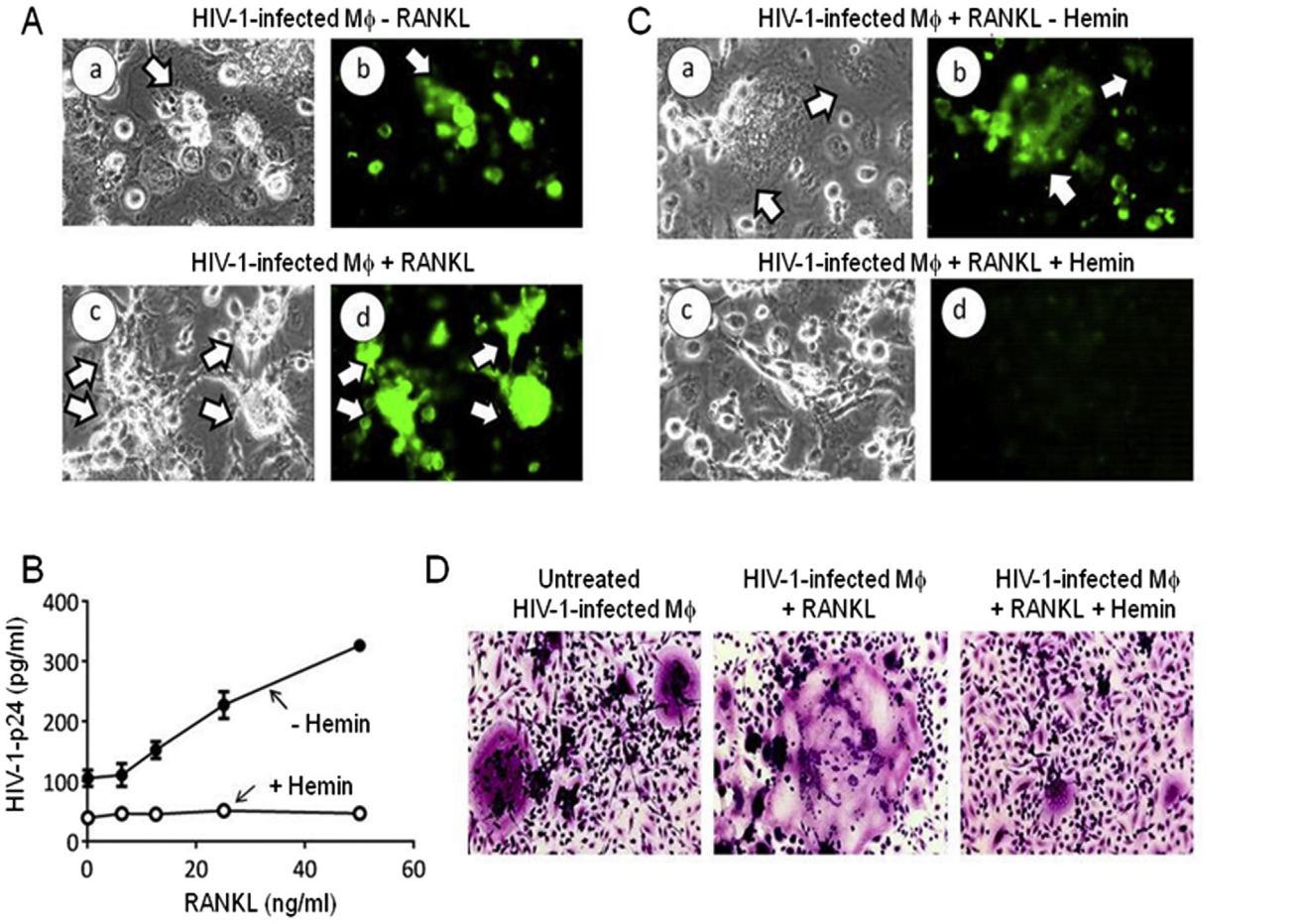

Fig. 3. Hemin treatment inhibits RANKL-induced osteoclastogenesis. A. RANKL-induced enhancement of HIV-induced multinucleated giant cell formation in HIV-infected MDM (shown by arrows and intracellular staining using FITC-conjugated mouse anti-human HIV-1-p24 monoclonal antibody). Panel a: phase-contrast image and panel b: fluorescence image of MDM cultured in the absence of RANKL. Panel c: phase-contrast image and panel d: fluorescence image of MDM cultured in the presence of RANKL. B. Cell-free HIV-1-p24 levels in supernatants from RANKL-treated MDM cultured in the absence or presence or 100 μM hemin. C. The hemin-induced HO-1 expression in HIV-1-infected MDM correlates with inhibition of RANKL-enhanced cytopathicity (arrows). Panel a: phase-contrast image and panel b: fluorescence image of MDM cultured with RANKL in the absence of hemin. Panel c: phase-contrast image and panel d: fluorescence image of MDM cultured with RANKL in the presence of hemin. D. Effect of hemin treatment on RANKL-induced cytopathicity in HIV-infected MDM. HIV-infected MDM were Wright-stained 10 days after infection, and HIV-induced multinucleated giant cells were scored by light microscopy. The data are representative of three independent experiments.

the presence of RANKL significantly increased the percentage and number of nuclei in HIV-1-antigen-positive multinucleated cells (Fig. 3, panel A, arrows). In addition, we found that hemin treatment not only suppressed the RANKL-induced increase in virus replication (Fig. 3, panel B), but also reduced multinucleated cell cytopathicity in HIV-infected MDM (Fig. 3, panels C and D).

Several reports, including those from our laboratory, have demonstrated extensive HIV-associated cytopathic effects in MDM characterized by the formation of multinucleated giant cells [32,44,45]. The molecular and cell biology of these giant cells has been studied, but approaches are needed to characterize them further and to target these cells to block or reduce cytopathicity. Moreover, despite their close morphological resemblance to osteoclasts, whether the HIV-infected multinucleated MDM are actually osteoclasts or of the osteoclast lineage had not previously been established. In addition, whether bone changes are caused by increased osteoclastic activity or by inhibited osteoblastic activity has remained unknown. Our study demonstrates that HIV-infected MDM are morphologically and functionally osteoclastic, and that this alteration can be blocked by hemin treatment. Interestingly, RANKL can enhance not only more osteoclast formation, but also HIV-1 virus production; both effects are also blocked by hemin treatment.

Given that a hemin formulation has already met FDA safety requirements for use in humans to treat patients with acute porphyrias for nearly four decades, our studies could provide a basis for potential clinical use of hemin in treating HIV-1-associated metabolic disorders, especially osteopenia/osteoporosis, for which safe and effective ART therapeutic interventions are quite challenging. A first-in-human report describing safe and effective HO-1 induction by hemin in healthy volunteers was published in 2010, thus further ruling out other possible regulatory hurdles [46].

The prevention and management of bone involvement in cancer and HIV-infected patients is critical for quality-of-life. There are various clinical modalities for treating bone loss; for example, denosumab, a monoclonal antibody against RANKL, odanacatib (a cathepsin K inhibitor), anti-sclerostin antibodies (romozumab, blosozumab), and bisphosphonates are currently being used to treat bone resorption [47–49]. Although clinical significance of these drugs has been reported, their mechanism of action is entirely distinct from our observations. Our approach of using a hemin-induced cellular general protection mechanism against the formation of osteoclasts is novel and, therefore, may be potentially beneficial in treating osteopenia and osteoporosis, regardless of the medical condition such as cancer and AIDS, as well as potentially in combination with other therapies.

Author contributions

S.D.: conceptualized, designed, performed, analyzed experiments, and wrote the paper; K.T. performed confocal microscopy and analyzed experiments; R.A. performed TRAP activity experiments; K.M.Y. analyzed experiments. We thank Dr. Zu-Xi Yu, National Heart, Lung and Blood Institute, NIH, for his expertise in microscopy. The findings and conclusions in this paper have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any Agency determination or policy. This work was supported by FDA and NIDCR Intramural Research Programs.

Conflict of interest

None.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.037.

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