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The Suv39H1 methyltransferase inhibitor chaetocin causes induction of integrated HIV-1 without producing a T cell response

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ABSTRACT

Latent HIV-1 (human immunodeficiency virus-1) provirus is unaffected by current AIDS (acquired immunodeficiency syndrome) therapies. We show here that chaetocin, an SUV39H1 histone methyltransferase inhibitor, causes 25-fold induction of latent HIV-1 expression, while producing minimal toxicity and without causing T cell activation. Induction is associated with loss of histone H3 lysine 9 (H3K9) trimethylation at the long terminal repeat (LTR) promoter, and a corresponding increase in H3K9 acetylation. The effect of chaetocin is amplified synergistically in combination with histone deacetylase (HDAC) inhibitors. These results indicate that chaetocin may provide a therapy to purge cells of latent HIV-1, possibly in combination with other chromatin remodeling drugs.

1. Introduction

AIDS (acquired immunodeficiency syndrome) has become a manageable disease since the introduction of highly active antiretroviral therapy (HAART), which can suppress HIV loads to undetectable levels. During infection, a population of latently infected cells becomes established in unstimulated CD4+ T-helper cells, which are not affected by HAART [1]. Unfortunately, the latently infected population decays very slowly ($t_{1/2}$ = 6–44 months), making eradication of infection improbable [2]. A variety of strategies have been considered to force expression of latent HIV with the objective of "purging" this otherwise impenetrable infection [3], which include the use of histone deacetylase (HDAC) inhibitors to cause remodeling of nucleosomes at the LTR and force increased expression [4–7]. This approach shows some promise [6,7], but because latent HIV genomes are silenced by mechanisms associated with heterochromatin [8,9], it is unlikely that HDAC inhibitors alone will be capable of purging the entire population of latently infected cells.

Methylation at histone H3 lysine 9 (H3K9) is associated with repressive heterochromatin, which must be removed to allow transcription [10]. This can be encouraged by treatment with histone methyltransferase inhibitors. For example, the fungal metabolite chaetocin is an inhibitor of the lysine-specific histone methyltransferase SUV39H1 [11]. This compound was recently shown to have anti-cancer activities related to its ability to cause re-expression of tumor suppressor genes [12]. These findings prompted us to investigate whether chaetocin could also cause reactivation of latent HIV-1 (human immunodeficiency virus-1).

2. Materials and methods

2.1. Recombinant DNA molecules

The pTY-LAI-luc HIV-1 LTR mini-virus reporter was produced from the lentiviral vector pTY-EFeGFP (NIH AIDS Reagent Program). The luciferase ORF was cloned into the NsiI/SwaI sites, and a wild type 3′ LTR fragment, generated by PCR from the HIV-1 pLAI clone with oligos oMD027 (AAAGAATTCTTTAAGACCAATGAC TTACAAGGC) and oTM0238 (GGGGTACCCCCT TTATTGAGGCTTA

Abbreviations: HIV-1, human immunodeficiency virus-1; H3K9, histone 3 lysine 9; HDAC, histone deacetylase; SAHA, suberoylanilide hydroxamic acid; TSA, trichostatin A; AIDS, acquired immunodeficiency syndrome; HAART, highly active anti retroviral therapy

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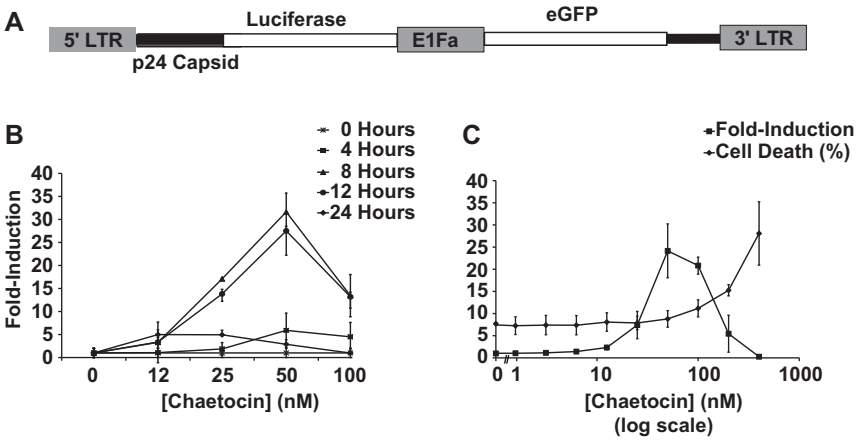

Fig. 1. Chaetocin activates an integrated HIV-1 minivirus. (A) Schematic representation of the pTY-LAI-luc reporter virus. (B) Jurkat cells with the integrated minivirus were left untreated (0 h) or stimulated with chaetocin at the indicated concentrations, and luciferase activity was measured after 4, 8, 12 or 24 h. (C) Cells were stimulated with chaetocin at the indicated concentrations; luciferase activity (fold induction) and % cell death were measured.

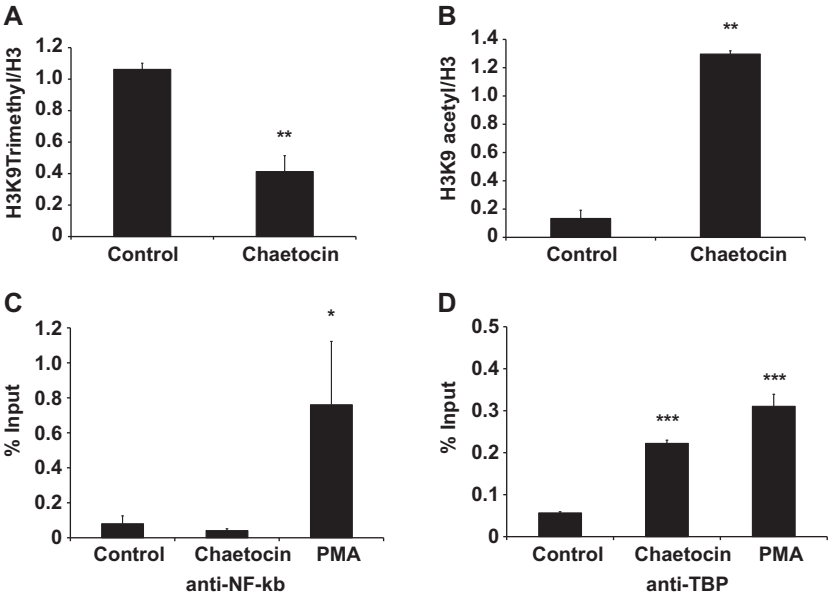

Fig. 2. Chaetocin affects histone modification and transcription factor binding on the HIV-1 LTR. Cells were left untreated or induced with 100 nM chaetocin for 8 h. ChIP was performed with anti-trimethyl-H3K9 (Panel A), anti-acetylated H3K9 (Panel B), anti-p65 NFκB (Panel C) or anti-TBP (Panel D). Error bars represent the standard deviation, * represents *P* values <0.05, ** represents *P* values <0.005 and *** represents *P* values <0.0005.

AGCAGT), inserted into the EcoRI/KpnI sites to produce pTY-LAI-luc (Fig. 1A). pGL3-Basic and pGL3-HIV-1 were used for transient transfections [13].

2.2. Cell lines, transfections and cell culture

VSV pseudotyped pTY-LAI-luc virus was produced by co-transfection of Human embryonic kidney (HEK) 293T cells (ATCC) as previously described [14]. Infected Jurkat-tat (NIH-AIDS) cells were monitored for eGFP expression and clones isolated by FACS. Jurkat cells were transiently transfected using an Amaxa Nucleofector (Lonza). For luciferase assays, cells were plated at 1×10^6 cells/mL, and induced with PMA (Sigma), chaetocin (Sigma) and/or SAHA (suberoylanilide hydroxamic acid) (Toronto Res. Chemicals) for the indicated times. Reporter expression was determined using the Bright-Glo System (Promega). Cell viability was determined by staining with Hoechst (0.5 μg/mL), and propidium iodide (PI, 0.25 μg/mL) in 96 well plates. After 30 min at 37 °C the plates were scanned in a Cellomics™ Arrayscan fluorescence imager; Hoechst and TRITC channels were used to determine total cell numbers and PI positive (dead) cells, respectively. To determine CD69 expression, cells were first blocked with 2.4G2 antibody, and then stained on ice for 30 min with 5 μL of anti-human CD69 antibody conjugated to R-phycoerythrin (PE) or PE conjugated mouse IgG1 isotype control antibody (BD Biosciences). The cells were then washed and stained with 25 ng/mL 4′,6-diamidino-2-phenylindole (DAPI) to distinguish live cells before analysis on an BD Biosciences flow cytometer. Data were analyzed using FlowJo analysis software (Tree Star). To measure IL-2 production, culture supernatants were collected and assayed by ELISA using a human IL-2 ELISA System (eBioscience).

2.3. ChIP assays

Cells were induced with 500 nM PMA or 100 nM chaetocin, and crosslinked with 1% formaldehyde for 10 min at room temperature.

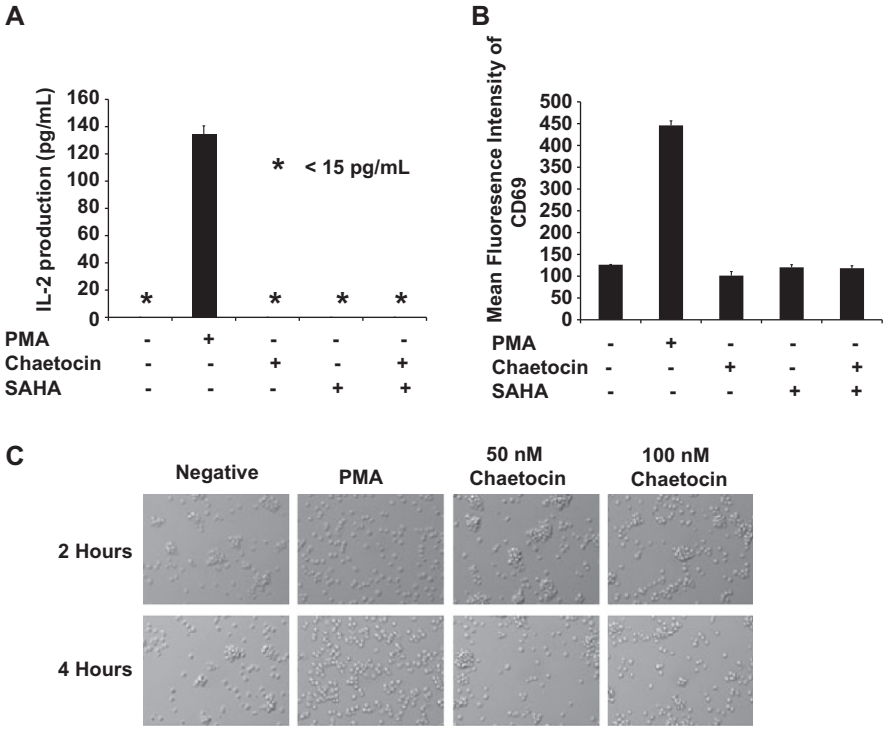

Fig. 3. Chaetocin does not cause activation of T-cells. Cells were induced with PMA, chaetocin and SAHA for 4 h. (A) IL-2 expression in the supernatant was determined. (B) Cell surface expression of CD69 was measured with CD69 antibodies and FLOW cytometry. (C) Photographs were taken after 2 and 4 h treatment.

ChIP assays were performed with antibodies from Abcam according to the suppliers instructions, and assayed by qPCR using oligos specific for the HIV-1 LTR core promoter (P1: AGTGGCGAGCCCTCAGAT, and P2: AGAGCTCCCAGGCTCAAATC). Results for NFκB p65 and TBP are normalized to controls using oligos specific for GAPDH [14], and for reactions with anti-H3K9trimethylation and acetylation to controls for total histone H3.

3. Results

3.1. Chaetocin activates the HIV-1 LTR in Jurkat T cells

To examine whether chaetocin was capable of causing activation of latent HIV-1, we used a Jurkat T-cell line bearing an integrated HIV-1 minivirus where luciferase is produced from the 5′ LTR as an in-frame fusion with $p24^{gag}$ (Fig. 1A). We observed some induction as early as 4 h post-treatment, and a peak induction of 25-fold at 8 h was sustained for up to 12 h treatment over a concentration range of 12–100 nM (Fig. 1B). Importantly, at this concentration, we observed minimal cell toxicity as measured by PI staining, and chaetocin does not have a significant effect on viability at concentrations as high as 100 nM (Fig. 1C).

3.2. Activation of the HIV-1 LTR by chaetocin is accompanied by loss of H3K9 tri-methylation and increased binding of TBP, but not NFκB

To examine whether induction of the LTR by chaetocin involves chromatin modification, we used chromatin immunopreciptiation (ChIP) to examine modification of histone H3K9. We found that the ratio of trimethylation at H3K9, relative to total histone H3, was decreased by 70% in cells that were induced with chaetocin for 8 h (Fig. 2A). In contrast, histones associated with the LTR in unstimulated cells are hypoacetylated, but acetylation at H3K9 increases by 90% after 8 h treatment (Fig. 2B). The finding that chaetocin decreases H3K9 trimethylation, which is accompanied by an increase in acetylation, suggests that this compound causes reorganization of chromatin at the LTR.

The activator NFκB plays a significant role in induction of genes downstream of the T-cell receptor (TCR) [15]. In T cells, PMA causes translocation of NFκB into the nucleus where it binds TCR-responsive gene promoters, including the LTR in HIV-1 infected cells [15]. Consequently, we wondered whether NFκB is involved in induction of the LTR in chaetocin-treated cells. Using ChIP, we found that the amount of NFκB present on the LTR increased by 70% in cells treated with PMA, but NFκB p65 did not become associated with the LTR in cells treated with chaetocin (Fig. 2C). In contrast to NFκB p65, we did observe recruitment of TATA-binding protein (TBP) to the LTR in cells treated with either chaetocin or PMA (Fig. 2D). TBP as part of TFIID binds to the TATA-box, and is required for assembly of the general transcription factors (GTFs) at the core HIV-1 promoter [16]. These results indicate that chaetocin must produce an environment on the LTR which allows recruitment of the GTFs and activation of transcription by mechanism(s) that bypass stimulation of NFκB. This is consistent with the finding that chaetocin causes loss of H3K9 tri-methylation and accumulation of H3K9 acetylation, indicating significant chromatin remodeling at the LTR.

3.3. Chaetocin does not cause activation of Jurkat T-cells

One problem with potential therapies pursuing the goal of activating latent virus is that general T-cell activation produces cytotoxic effects [17]. Activated T-cells secrete cytokines such as IL-2, which plays a pivotal role in immune response. T-cell activation is also characterized by increased surface expression of CD69 which acts in T-cell proliferation and signaling [18], and accordingly we observe induction of IL-2 and CD69 in Jurkat cells treated with PMA (Fig. 3A and B). In contrast, treatment of cells with chaetocin for 8 h did not cause a change in CD69 or IL-2 expression, indicating that chaetocin is capable of inducing HIV-1 expression

without causing global T-cell activation. Activated T-cells undergo a variety of morphological changes including decreased aggregation and adherence to the substratum [18]. This was observed in Jurkat cells treated with PMA, where after 2 h treatment the cells become dispersed, flatten out and attach to the plate (Fig. 3C). However, we do not observe these effects in cells treated with chaetocin (Fig. 3C).

3.4. Chaetocin acts synergistically with TSA and SAHA

Because chaetocin seems to activate HIV-1 expression by a mechanism involving chromatin remodeling, we examined whether it produced synergistic effects with other chromatin remodeling drugs. Histone acetylation is a marker of active chromatin and is increased on the activated HIV LTR [8,9] (Fig. 2B). The HDAC inhibitors TSA (trichostatin A) and SAHA were shown to cause some induction of integrated HIV-1 LTRs [9], and consistent with this we found that TSA on its own increased expression from the pTY-LAI-luc minivirus by only 10-fold at a concentration of 130 nM (Fig. 4A). The effect of TSA was significantly enhanced in combination with 50 and 100 nM chaetocin, where we observe 40- and 90-fold induction, respectively (Fig. 4A). Similarly, SAHA on its own caused 14-fold induction at a concentration of 25 μM, but this effect is significantly enhanced by chaetocin, such that we observe 80-fold induction with 100 nM chaetocin in combination with 6.25 μM of SAHA (Fig. 4B). Importantly, chaetocin in combination with the HDAC inhibitors caused minimal toxicity at concentrations that produce peak induction (Fig. 4C and D). Higher concentrations of SAHA of 100 μM causes 20% cell death, but this effect is not exaggerated by chaetocin (Fig. 4D). Induction by chaetocin in combination with TSA and SAHA appears to be synergistic, as 100 nM chaetocin in combination with 0.5 μM TSA or 6.25 μM SAHA produces 80-fold induction, whereas these compounds alone cause less than 20-fold stimulation (Fig. 4E and F).

3.5. Chaetocin causes a slower and weaker response of transient LTR templates

In contrast to the above results, we found that an HIV-1 LTR reporter gene introduced into Jurkat T-cells by transient transfection was induced more slowly and to less significant levels, such that we only observe 2-fold induction after 72 h treatment (Fig. 5). It is well documented that transiently transfected reporters do not become properly assembled into chromatin [19], and consequently this result supports the view that chaetocin causes activation of the LTR by mechanism(s) which promote formation of transcriptionally permissive chromatin on the integrated virus.

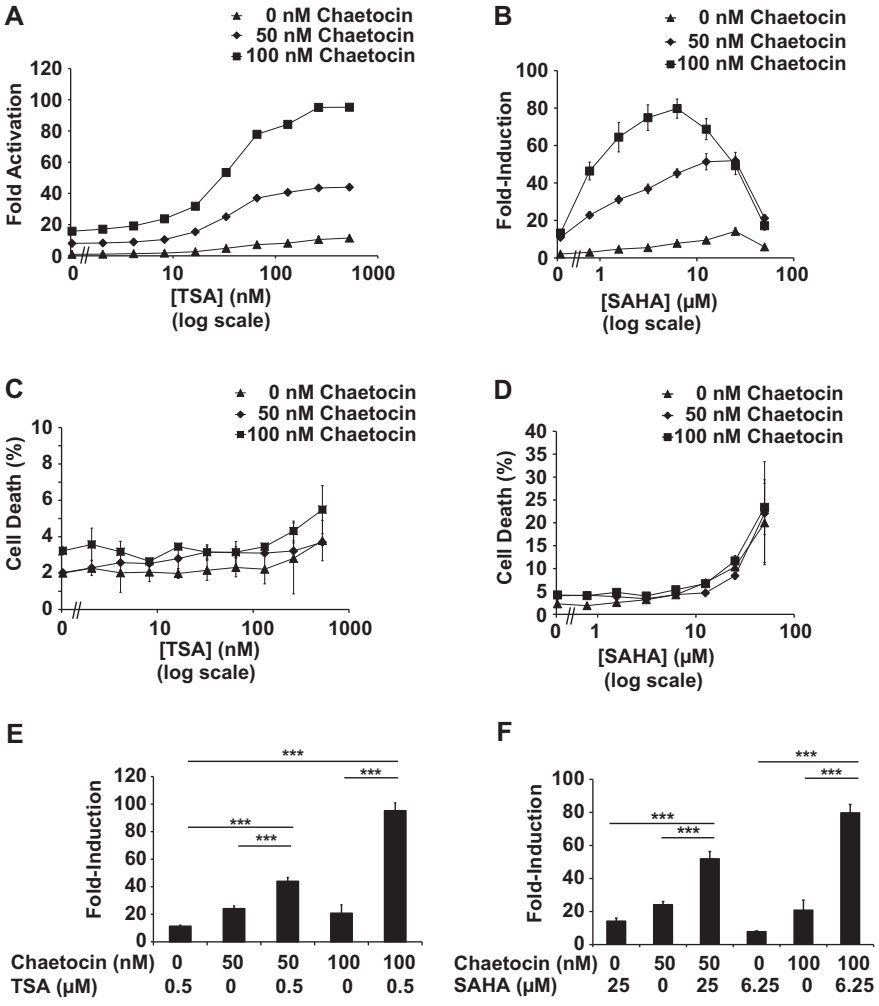

Fig. 4. Chaetocin causes synergistic activation of the HIV-1 LTR with SAHA and TSA. Fold luciferase expression, relative to untreated controls, was measured in cells treated with SAHA (B) or TSA (A) at the indicated concentrations, alone (0 nM) and in combination with 50 or 100 nM chaetocin. Cell death caused by TSA (C) or SAHA (D) alone or in combination with chaetocin was determined. Luciferase expression was measured in cells treated with TSA (E) or SAHA (F) in combination with the indicated concentrations of chaetocin. ** represents *P* values <0.005, and *** represents *P* values <0.0005.

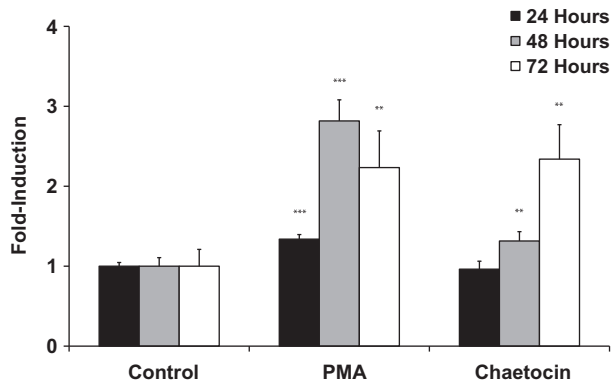

Fig. 5. Chaetocin produces a weaker and slower response of a transiently transfected HIV-1 reporter. Jurkat cells were transfected with an HIV-1 LTR-luciferase reporter, and cells were left untreated or treated with PMA (500 nM) or chaetocin (100 nM) for 24, 48 or 72 h. Fold induction of luciferase is indicated; ** represents *P* values <0.005, and *** represents *P* values <0.0005.

4. Discussion

In this study we show that chaetocin, an inhibitor of SUV39H1, causes induction of an integrated HIV-1 mini-virus. The effect is observed within 8 h post-treatment, occurs without producing global T-cell activation, and produces a synergistic effect on HIV-1 expression in combination with HDAC inhibitors. Induction is accompanied by loss of tri-methylation at H3K9 and a corresponding increase in H3K9 acetylation. These results are similar to a previous study that shows chaetocin is capable of reactivating epigenetically silenced tumor suppressor genes through inhibition of SUV39H1 to produce a decrease in H3K9 tri-methylation [12]. We also note that a previous study did not observe a significant effect of chaetocin on latent HIV-1 [20], but we believe the difference in our experiments is that peak induction occurs after 8 h, and is lost by 24 h (Fig. 1B), whereas in contrast this previous study only examined expression after 16 h. Additionally, an shRNA directed against SUV39H1 caused only small induction of the LTR within a comparable time frame [20].

T-cells normally become activated by T-cell receptor engagement during antigen presentation [18]. This can be mimicked using agonists such as PMA, which stimulates T-cell responses including enhanced cell adhesion and increased expression of IL-2 and CD69 [18]. Cells stimulated with chaetocin and/or the HDAC inhibitor SAHA do not exhibit these phenotypes.. Stimulation of Jurkat cells with PMA causes activation of the HIV-1 LTR through NFκB [21]. Consistent with the finding that chaetocin does not cause global T-cell activation, we find that NFκB p65 does not bind the LTR in chaetocin-treated cells. Chaetocin was shown to cause oxidative stress in myeloma cells [22], and importantly oxidative stress of Jurkat cells induces expression of HIV-1, apparently through activation of NFκB [23]. Because we do not observe an effect of chaetocin on association of NFκB p65 with the LTR, it is possible that this compound does not produce a comparable stress response in the Jurkat cell line. TSA and SAHA were also shown to stimulate HIV-1 expression without NFκB activation [5,6]. The HIV-1 LTR is bound by numerous sequence-specific transcription factors in vitro that are present in unstimulated T-cells [24]. It is well established that latent HIV-1 provirus is produced by repressive chromatin, which would normally preclude activation by these factors [8,9]. Consequently, we propose that chaetocin initiates extensive remodeling of chromatin at the LTR promoter to produce an environment that is permissive for binding of multiple activators.

Effective treatment regimes typically involve combinations of drugs because of synergy, where lower concentrations can be used to decrease side effects. We show here that chaetocin produces synergistic effects on HIV-1 expression in combination with the HDAC inhibitors TSA and SAHA. Several other histone methyltransferase inhibitors, including BIX1294 specific for G9a, and the broad-spectrum inhibitor DZNep, were also shown to act synergistically with SAHA to induce HIV-1 [20,25]. Also, SUV39H1 shRNA was shown to cause a small increase of HIV-1 expression, and this effect was activated synergistically with shRNA directed against the EZH2 methyltransferase [20]. These studies, in combination with the results shown here, support a view that combinations of histone methyltransferase inhibitors, including chaetocin, and HDAC inhibitors may represent potential safe and effective therapies for purging latent HIV-1 reservoirs.

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