Histone methyltransferase inhibitors induce HIV-1 recovery in resting CD4⁺ T cells from HIV-1-infected HAART-treated patients

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Objective: Reactivation of HIV-1 expression in persistent reservoirs together with an efficient HAART has been proposed as an adjuvant therapy aimed at reaching a functional cure for HIV. Previously, H3K9 methylation was shown to play a major role in chromatin-mediated repression of the HIV-1 promoter. Here, we evaluated the therapeutic potential of histone methyltransferase inhibitors (HMTIs) in reactivating HIV-1 from latency.

Design: We evaluated the reactivation potential of two specific HMTIs (chaetocin and BIX-01294, two specific inhibitors of Suv39H1 and G9a, respectively) in ex-vivo cultures of resting CD4⁺ T cells isolated from HIV-1-infected HAART-treated individuals.

Methods: We measured HIV-1 recovery in ex-vivo cultures treated with an HMTI alone or in combination with other HIV-1 inducers (in absence of IL-2 and of allogenic stimulation) of CD8⁺-depleted peripheral blood mononuclear cells (PBMCs) or of resting CD4⁺ T cells isolated from 67 HIV-infected, HAART-treated patients with undetectable viral load.

Results: We demonstrated, for the first time, that chaetocin induced HIV-1 recovery in 50% of CD8⁺-depleted PBMCs cultures and in 86% of resting CD4⁺ T-cell cultures isolated from HIV-1-infected, HAART-treated patients, whereas BIX-01294 reactivated HIV-1 expression in 80% of resting CD4⁺ T-cell cultures isolated from similar patients. Moreover, we showed that combinatorial treatments including one HMTI and either the histone deacetylase inhibitor suberoylanilide hydroxamic acid or the non-tumor-promoting NF-κB inducer prostratin had a higher reactivation potential than these compounds alone.

Conclusion: Our results constitute a proof-of-concept for the therapeutic potential of HMTIs in strategies aiming at reducing the pool of latent reservoirs in HIV-infected, HAART-treated patient.

Keywords: histone methyltransferase inhibitors, HIV-1, latency, reservoirs

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Introduction

Despite current effective and life-prolonging HAART, HIV-1 can still not be cured [1]. Indeed, the virus persists in HIV-infected, HAART-treated patients due to different mechanisms: residual ongoing viral replication, especially in cellular sanctuaries where drug penetration level is suboptimal, and/or reactivation of viral expression from latently infected cells [2]. A small population of latently infected resting CD4+ T cells, harboring transcriptionally silent but replication-competent HIV-1 proviruses [3], are insensitive to HAART and able to escape from the host immune response [4]. These HIV-1 cellular reservoirs are therefore a permanent source for virus reactivation and could be responsible for the rebound of plasma viral load observed after HAART interruption [1,2]. Several therapeutic approaches aiming at achieving either a sterilizing cure (elimination of HIV from the human body) or – more likely – a functional cure (long-term control of HIV in the absence of HAART) have been proposed [1]. In this context, activation of HIV gene expression in latently infected cells together with an efficient HAART could serve as an adjuvant therapy aimed at decreasing the pool of latent reservoirs.

HIV-1 transcriptional repression is crucial for the establishment and maintenance of postintegration latency [5,6]. The chromatin organization and the epigenetic control of the HIV-1 promoter are key elements in this transcriptional silencing. The repressive nucleosome nucleolus 1, located immediately downstream of the transcription start site, is maintained hypoacetylated by histone deacetylases (HDACs) in latent conditions [7,8]. In addition, histone H3 lysine 9 (H3K9) methylation was shown to play a major role in chromatin-mediated repression of HIV-1 expression [9,10]. The histone methyltransferases (HMTs) Suv39H1 [9], which is primarily involved in H3K9 trimethylation (H3K9me3), and G9a [10], which is responsible for H3K9 dimethylation (H3K9me2), have been demonstrated to play a role in HIV-1 transcriptional silencing. Indeed, Benkirane’s group [9] has shown that Suv39H1, HP1γ (heterochromatin protein 1) and H3K9me3 mediate HIV-1 repression in different cellular models including peripheral blood mononuclear cells (PBMCs) from HIV-infected patients. Moreover, we have previously reported a similar mechanism in HIV-1-infected microglial cells, where Suv39H1 is recruited to the HIV-1 promoter via the transcriptional corepressor CTIP2 (COUP-TF interacting protein 2) [11,12]. In addition, G9a-mediated H3K9me2 can also recruit HP1 and therefore participate in the maintenance of HIV-1 silencing [10]. Consequently, histone methyltransferase inhibitors (HMTIs) represent new candidate drugs for reducing the pool of latent reservoirs.

Two specific HMTIs have been described so far: chaetocin and BIX-01294. Chaetocin, a fungal mycotoxin from Chaetomium minutum [13], acts as a specific inhibitor of Suv39H1 in a S-adenosylmethionine-competitive manner [14]. Chaetocin belongs to 3–6-epi-dithio-diketopiperazines, which have been previously reported to have biological effects including immunosuppressive [15], anti-inflammatory [16] and/or antiviral [17] activities. BIX-01294, a diazepin-quinazolin-amine derivative, functions as a specific inhibitor of G9a [18] in an uncompetitive manner with S-adenosylmethionine by binding the G9a SET catalytic domain [18,19].

Here, we demonstrated for the first time that the HMTIs chaetocin and BIX-01294, alone or in combination with other HIV-1 inducers, cause HIV-1 recovery in CD8+ depleted PBMCs and in resting CD4+ T cells from 1-infected, HAART-treated patients with undetectable viral load. Although chaetocin and BIX-01294 cannot be safely administered to humans, these results highlight the proof-of-concept for the therapeutic potential of HMTIs in strategies aimed at reducing the HIV-1 latent reservoirs pool in HAART-treated patients.

Methods

Reagents

Chaetocin (C9492), BIX-01294 (B9311), Prostratin (PE187-0001) and suberoylanilide hydroxamic acid (SAHA) (Vorinostat; 270-288-M001) were purchased from Sigma Aldrich (Bornem, Belgium) (HMTIs), Enzo Life Sciences (Antwerp, Belgium) and Alexis Biochemicals (Antwerp, Belgium), respectively.

Cell lines and cell culture

The T-lymphoid cell lines Jurkat and J-Lat 15.4 were grown as previously reported [20].

Transient transfection assays

Jurkat cells were transiently transfected with the pLTR_HIV-1-luc episomal vector (pLTR [1–789]-luc [21]) using the jetPEI method. At 24-h posttransfection, cells were treated as indicated. At 24-h posttreatment, cells were lysed and assayed for luciferase activity (Promega, Leiden, The Netherlands). Luciferase activities were normalized with respect to protein concentrations.

Virus production assays

HIV-1 production was measured in cell supernatant by CA-p24 ELISA (Innogenetics, Ghent, Belgium) and cellular viability was evaluated by the colorimetric test WST-1 (Roche, Vilvoorde, Belgium).

Study subjects

We selected 55 HIV-infected volunteer patients at the St-Pierre Hospital (Belgium) and 12 patients at the Bicêtre Hospital (France) with undetectable viral load (<50 HIV-1
RNA copies/ml of plasma since at least 1 year), under HAART (for at least 1 year) and with a CD4+ T-cell level superior to 300 cells/μl of blood. Characteristics of these patients were well documented. Ethical approval was granted by the Human Subject Ethics Committees of both hospitals. All patients enrolled in the study provided written informed consent for donating blood.

**Isolation of CD8+–depleted peripheral blood mononuclear cells and resting CD4+ T cells**

CD8−-depleted PBMCs used in reactivation assays and HLA DR+CD4+ T cells for limiting-dilution assays were isolated from fresh whole blood of HIV-infected patients as previously described [20,22]. Resting CD4+ T cells (HLA DR−CD69−CD25−CD4+) were isolated using a negative selection by Magnetic Cell Sorting (Miltenyi Biotec, Leiden, The Netherlands). PBMCs isolated by density centrifugation on a Ficoll-Hypaque gradient (Pharmacia, Capelle Aan Den IJssel, The Netherlands) were washed with NaCl 0.9%, and incubated with biotin-labeled antibodies directed against all cells other than CD4+ T lymphocytes (CD4+ T-cell Biotin Antibody Cocktail) and against markers of activated T cells (CD25 and CD69). A second incubation step with magnetic beads coated with anti–HLA DR and antibody antibodies allowed HLA DR−CD69−CD25−CD4+ T cells isolation. CD8−-depleted PBMCs or resting T cells were seeded at 2 × 10^6 cells/ml and 0.8 × 10^6 cells/ml, respectively, in culture medium [20]. One day after isolation, cells were mock-treated or treated with anti-CD2+anti-CD28 antibodies as a positive control for global T-cell activation [23,24] or by the indicated compounds.

**Quantitative assessment of HIV-1 RNA**

Six days after treatment, culture supernatants were tested for quantitative HIV-1 RNA levels using the COBAS AmpliPrep/COBAS AMPLICOR HIV-1 MONITOR Test for CD8−-depleted PBMCs and HLA DR−CD4+ T cells according to the manufacturer’s instructions (Roche Diagnostics, Vilvoorde, Belgium) or the Abbott Real-time HIV-1 assays for HLA DR−CD69−CD25−CD4+ T cells (detection limits: 40 copies HIV-1 RNA/ml).

**Quantification of total HIV-1 DNA**

Total cellular DNA was extracted from patient cells using the DNA blood test, the QIAampDNA Micro or QIAampDNA blood mini kit (Qiagen, Venlo, The Netherlands). Total HIV-1 DNA was then quantified by the Generic HIV DNA cell kit (Biocentric, Bandol, France [25]).

**Results**

Chaetocin induces HIV-1 recovery in CD8−-depleted PBMCs and in HLA DR−CD4+ T cells from HIV-1-infected patients with undetectable viral load. We and Benkirane’s group have previously shown that the HMT Suv39H1 is recruited to the HIV-1 promoter in latent condition in different cell types [9,12], suggesting that chaetocin, a specific inhibitor of this HMT, represents a promising therapeutic drug in strategies aiming at purging HIV-1 latent reservoirs. Here, we investigated the reactivation potential of chaetocin in ex-vivo cultures of cells isolated from HIV-1-infected, HAART-treated patients with undetectable viral load.

We first evaluated the effect of increasing doses of chaetocin in Jurkat cells transfected with an LTR–luc episomal reporter construct (Fig. 1a) and in the latently infected J-Lat 15.4 cell line (Fig. 1b). We observed that chaetocin induced HIV-1 promoter transcriptional activity and viral production in a dose-dependent manner (peaking at 90 nmol/l), in agreement with previous studies [26,27]. Moreover, cellular viability was not affected below 100 nmol/l (Fig. 1c). To assess these results in patient cells, we purified CD8−-depleted PBMCs from seven selected patients (Fig. 1d). After a 1-day culture, cells were mock-treated or treated with chaetocin (30, 60 or 90 nmol/l) or with a positive control [23,24]. Importantly, in all our experiments, purified cells were cultured in the absence of both IL-2 and allogenic stimulation in order to avoid nonspecific global T-cell activation and proliferation, which might have caused an increase in genomic viral RNA level. Six days after treatment, we measured HIV-1 genomic RNA concentrations in culture supernatants.

For one out of seven patients, we did not detect viral RNA in the positive control condition and this patient was therefore removed from our study. As shown in Fig. 1d, we observed HIV-1 recovery in cell cultures from one patient with 30 nmol/l, from three patients with 60 nmol/l and from four patients with 90 nmol/l of chaetocin. Moreover, chaetocin treatment induced weak toxicity in some healthy donors CD8−-depleted PBMCs cultures (data not shown). Consequently, our results demonstrated that chaetocin 90 nmol/l was the optimal concentration to induce HIV-1 recovery while maintaining acceptable cell viability.

We next tested the reactivation potential of chaetocin (90 nmol/l) on CD8−-depleted PBMCs from a larger number of patients (31 patients). After purification, total DNA was extracted from 5 × 10^6 cells in order to quantify total viral DNA. For three patients, we detected viral RNA in the mock-treated culture supernatant and, for 10 patients, HIV RNA production was not reactivated by the positive control. Therefore, these patients were removed from our study. Among the 18 remaining patients, we detected HIV-1 recovery after chaetocin exposure in nine cell cultures (Fig. 2a). Of note, we did not observe any significant correlation between the patient characteristics or total HIV-1 DNA and the potencies of chaetocin to induce HIV-1 recovery (Fig. 3).
Fig. 1. Chaetocin induces HIV-1 recovery in a dose-dependent manner. (a) Chaetocin increases the transcripational activity of the HIV-1 5'LTR in Jurkat cells. The Jurkat cell line was transiently transfected with the PLTRhu-luc episomal reporter construct. At 24-h posttransfection, cells were mock-treated or treated with chaetocin as indicated. At 24-h postinduction, cells were lysed and assayed for luciferase activity. Luciferase activities were normalized with respect to protein concentrations. The result obtained with the mock-treated cells was arbitrarily set at a value of 1. (b, c) Chaetocin increases HIV-1 production in the latently infected J-Lat 15.4 cell line. The J-Lat 15.4 cell line was mock-treated or treated with chaetocin as indicated. p24 production in cell supernatants (b) or cellular viability (c) were measured. The result obtained with mock-treated cells was arbitrarily set at a value of 1 or 100%, respectively. (d) Chaetocin induces HIV-1 recovery in a dose-dependent manner in CD8^-depleted PBMCs isolated from HIV-1-infected HAART-treated patients with undetectable viral load. Cultures of CD8^-depleted PBMCs were mock-treated or treated with chaetocin (30, 60 or 90 nmol/l) or with the positive control. Six days after treatment, the concentration of viral RNA in culture supernatants was determined (in copies/ml; ‘I’ indicates below the threshold).

Among the cell types present in CD8^-depleted PBMCs, latently infected resting CD4^+ T cells represent the primary long-lived source of HAART persistent HIV-1 [28–30]. These resting cells carrying a nonproductive HIV-1 infection derive from infected CD4^+ lymphoblasts that have reverted back to a resting memory state and show a specific pattern of surface markers (including CD4^+, CD25^+, CD69^+ and HLA DR^-) [28]. In order to confirm in resting T cells the results obtained using CD8^-depleted PBMCs, we performed limiting-dilution cultures of purified HLA DR^-CD4^+ T cells from 12 HIV-infected HAART-treated patients. After isolation, cells were mock-treated or treated with chaetocin (45 or 90 nmol/l) or with positive control. In two patient cultures, viral RNA was detected down to a cell density of 10^3 cells in three cultures. Virus production after treatment with chaetocin 90 nmol/l was detected down to a cell density of 10^3 cells in three cultures (Fig. 2b; P4, P6, P7) and of 5 x 10^3 cells in one culture (Fig. 2b; P5). These results suggested the presence of at least one HIV-specific cell harboring replication-competent and chaetocin-inducible provirus among these 10^3 or 5 x 10^3 HLA DR^-CD4^+ T cells. Chaetocin 45 nmol/l was less efficient in reactivating HIV-1 than chaetocin 90 nmol/l, confirming our previous results (Fig. 1). Importantly, the reactivation level, corresponding to the quantity of viral RNA copies per milliliter of cell supernatant, observed after chaetocin treatment in a cell population. Therefore, the important data are the last cell density where virus production is still detected after chaetocin treatment. As shown in Fig. 2b, we confirmed the important reactivation potential of chaetocin as HIV-1 recovery occurred in six out of the seven cultures. 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treatment was similar to that obtained with the positive control, thereby further supporting the important reactivation potential of this HMTI.

In conclusion, these results show that chaetocin, a specific inhibitor of the HMT Suv39H1, can induce HIV-1 recovery in 50% of CD8^+^-depleted PBMCs cultures and in 86% of HLA DR^−^CD4^+^ T cells isolated from HIV-1-infected, HAART-treated patients with undetectable viral load.

The combinations chaetocin+SAHA and chaetocin+prostratin have higher potentials than the compounds alone in reactivating HIV-1 in resting memory CD4^+^ T cells from HIV-1-infected patients.

Our laboratory has previously demonstrated that the combination SAHA+prostratin (an HDACI already in clinical use for the treatment of cutaneous T-cell lymphoma [31] and a non-tumor-promoting NF-κB inducer) allow HIV-1 reactivation in a higher number of CD8^+^-depleted PBMCs cultures from HIV-infected HAART-treated patients as well as to a higher extent compared to these activators individually [20]. To evaluate the potential of such combinatory treatments including chaetocin, we purified resting memory CD4^+^ T cells from blood of 24 HIV-infected patients based on their surface marker properties (HLA DR^−^CD25^−^CD69^−^CD4^+^ T cells) [28]. Indeed, among the HLA DR^-^CD4^+^ T cells, some still present T-cell activation markers including CD69 and CD25. After purification, total DNA from at least 1 × 10^6 cells was extracted to quantify total viral DNA. After a 1-day culture, cells were treated either with SAHA, prostratin or chaetocin alone or with combinations of these drugs. Six days after treatment, viral RNA was quantified in culture supernatants.

We removed from our study the data concerning eight patients for which we detected viral RNA in culture supernatants.

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### Table 1: Reactivation of HIV-1 in CD8^−^-depleted PBMCs and in HLA DR^−^CD4^+^ T cells

| Patients | Mock | Chaetocin C^+^ | HIV DNA (copies/10^6^ cells) | Log HIV DNA |
|----------|------|---------------|-------------------------------|------------|
| H1       | I    | I             | 467 / /                      | /          |
| H2       | I    | I             | 1825 / /                     | /          |
| H3       | I    | I             | 354 / /                      | /          |
| H4       | I    | I             | 2057 / /                     | /          |
| H5       | I    | I             | 219 / /                      | /          |
| H6       | I    | I             | 2371 / /                     | /          |
| H7       | I    | I             | 306 / 1230                   | 3.09       |
| H8       | I    | I             | 1480 / 2373                  | 3.38       |
| H9       | I    | I             | 1566 / 1920                  | 3.14       |
| H10      | I    | I             | 8405 / 1527                  | 3.18       |
| H11      | I    | I             | 797 / 19958                  | 3.05       |
| H12      | I    | I             | 477 / 4132                   | 3.52       |
| H13      | I    | I             | 272 / 2111                   | 3.32       |
| H14      | I    | I             | 2388 / 387                   | 3.38       |
| H15      | I    | I             | 2691 / 995                   | 3.00       |
| H16      | I    | I             | 467 / 7458                   | 3.54       |
| H17      | I    | I             | 2562 / 187                   | 2.27       |
| H18      | I    | I             | 3695 / 3796                  | 3.58       |

**Reactivated patients:** 9 / 18

**% of reactivation:** 50 / 100

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### Table 2: Chaetocin induces HIV-1 recovery in CD8^−^-depleted PBMCs and in HLA DR^-^CD4^+^ T cells from HIV-1-infected, HAART-treated patients with undetectable viral load.

| Patients | Mock | Dose of chaetocin | 1.5 × 10^6^ cells | 10^6^ cells | 10^5^ cells | 10^4^ cells | 5 × 10^4^ cells | C^+^ |
|----------|------|-------------------|-------------------|------------|------------|------------|----------------|------|
| P1       | I    | 45 nM / 284       | 179 / I           | /          | /          | /          | /              | 308  |
| P2       | I    | 45 nM / /         | 245 / 83          | I          | /          | /          | /              | 111  |
| P3       | I    | 45 nM / /         | 118 / 51          | I          | /          | /          | /              | 210  |
| P4       | I    | 45 nM / /         | 94 / I            | /          | /          | /          | /              | 120  |
| P5       | I    | 45 nM / /         | 178 / 68          | 50 / 76    | /          | /          | /              | 880  |
| P6       | I    | 45 nM / 288       | 97 / /            | /          | /          | /          | /              | 1451309 |
| P7       | I    | 45 nM / 831       | 702 / 769         | 238 / I    | /          | /          | /              | 1021 |

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**Fig. 2. Chaetocin induces HIV-1 recovery in CD8^-^-depleted PBMCs and in HLA DR^-^CD4^+^ T cells from HIV-1-infected, HAART-treated patients with undetectable viral load.**

(a) Cultures of CD8^-^-depleted PBMCs were mock-treated or treated with chaetocin (90 nmol/l). Six days after treatment, the concentration of viral RNA in culture supernatants was determined. Total HIV-1 DNA is expressed as HIV-1 DNA copies/10^6^ cells or as log HIV-1 DNA copies/10^6^ cells ("/' indicates not-tested condition).

(b) Limiting-dilution cultures of HLA DR^-^CD4^+^ T cells were mock-treated or treated with chaetocin (45 or 90 nmol/l). The concentration of viral RNA in culture supernatants was determined. The last positive dilution culture indicates the presence of at least one cell carrying replication-competent and chaetocin-responsive virus.
supernatant in the absence of any treatment or for which HIV-1 expression was not reactivated by the positive control. Effects of prostratin and SAHA in combination showed that the combined treatment chaetocin strongly benefited from the combinatory treatment since chaetocin had an as high reactivation potential as SAHA (Fig. 3). Moreover, we observed that SAHA reactivated HIV-1 expression in cell cultures from seven out of 10 patients, while prostratin alone allowed HIV-1 reactivation when the drugs alone did not. In conclusion, we showed that combinatorial strategies may be beneficial for some patients either by increasing the viral production induced by chaetocin or by allowing viral reactivation when the drugs alone did not.

In addition, we observed that SAHA reactivated HIV-1 expression in 12 out of the 16 resting memory CD4+ T-cell cultures tested and that chaetocin had an as strong reactivation potential as SAHA (Fig. 3). Moreover, we showed that the combined treatment chaetocin + SAHA favored a higher level of viral production in cultures from five patients (Fig. 4b1) and induced HIV-1 recovery in a synergistic manner in cultures from three patients (Fig. 4b2). Consequently, our results demonstrated that the HDACI SAHA increased the reactivation potential of the HMTI chaetocin.

In contrast, we showed that combinatorial strategies may be beneficial for some patients either by increasing the viral production induced by chaetocin or by allowing viral reactivation when the drugs alone did not.

![Fig. 3. Patient's characteristics and reactivation status of ex-vivo cultures of patients' cells. Cultures of patient cells were mock-treated or treated with indicated compounds. Six days after treatment, the concentration of viral RNA in culture supernatants was determined (in copies/ml; I means below the threshold and '/' indicates an untested condition). Total HIV-1 DNA is expressed as HIV-1 DNA copies/10^6 cells. The cultures indicated in gray showed a higher viral production for 2 patients (H23 and H33; Figs 3 and 4a, in black). Moreover, this combined treatment allowed a higher level of viral production for 2 patients (H23 and H33; Figs 3 and 4a, in gray). Of note, we could not observe any correlation between the patients characteristics or the reactivation of HIV-1 expression and the total viral DNA level in the patients tested (Fig. 3), except that patient H27, which did show HIV-1 recovery only in the positive control but not after treatment with other drugs, presented the lowest number of HIV-1 DNA copies per 10^6 cells.

![Table of patient characteristics and reactivation status of ex-vivo cultures of patients' cells.](attachment://table.png)
Another HMTI BIX-01294 induces HIV-1 recovery in resting memory CD4\(^+\) T cells from HIV-1-infected, patients with undetectable viral load. Okamoto’s group [10] has previously shown that BIX-01294, a specific inhibitor of the HMT G9a, causes HIV-1 reactivation in latently infected cell lines. Here, we tested the BIX-01294 reactivation potential in HLA DR\(^-\) CD25\(^-\) CD69\(^-\) CD4\(^+\) T-cell cultures from 10 HIV-1-infected, HAART-treated patients. We observed that BIX-01294 induced HIV-1 recovery in 80% of the cell cultures (Fig. 5a). In addition, we tested the combination BIX-01294 + SAHA in five patient cultures in order to confirm the importance of combining different drugs to increase the level of reactivation and/or the number of responding cells. Among the three reactivated cultures out of the five tested cultures (Fig. 3), we observed a synergistic reactivation with the combination BIX-01294 + SAHA in two patient cultures (Fig. 5b). Interestingly, HIV-1 recovery was detected after the combinatory treatment BIX-01294 + SAHA, but not after treatment with BIX-01294 alone, in cell cultures from patient H28 (Fig. 5b).

In conclusion, the HMTI BIX-01294 acted as a strong HIV-1 inducer and its reactivation properties were potentiated when used in combination with the HDACI SAHA in the context of resting memory CD4\(^+\) T cells isolated from HIV-1-infected, patients with undetectable viral load. The results we obtained with chaetocin and...
BIX-01294 strongly support the high reactivation potential of HMTIs, which therefore represent promising compounds in strategies to reduce the pool of HIV-1 latent reservoirs.

Discussion

Activation of HIV gene expression in latently infected cells together with an efficient HAART has been proposed as an adjuvant therapy aimed at reducing the pool of latent reservoirs in HIV-infected, HAART-treated patients [1]. In this context, our laboratory has previously reported a proof-of-concept study for the therapeutic potential of coadministration of two kinds of HIV-1 inducers (an NF-κB inducer prostratin and an HDACI SAHA) in reactivating HIV from latency [20]. However, HIV-1 recovery was only observed in 60% of the CD8\(^+\) -depleted PBMCs cultures (isolated from blood of HIV-1-infected, HAART-treated patients) tested in this study, suggesting that a stronger molecular silencing of some integrated proviruses may hinder efficient viral transcriptional reactivation in the unresponsive patient cell cultures.

In this context, histone methylation marks (including H3K9me2 catalyzed by G9a, H3K9me3 catalyzed by Suv39h1 as well as H3K27me3 catalyzed by EZH2) have been shown to play an important role in the establishment and maintenance of HIV-1 transcriptional silencing in different postintegration latency models [9,10,12,26,27]. Our results and several recent studies demonstrate that the specific HMTIs chaetocin and BIX-01294 as well as the broad spectrum HMTI DZNep reactivate latent HIV-1 in different latently infected cell lines (Fig. 1b, [10,26,27]). However, we noted divergences between these studies concerning different parameters, probably relying on the fact that different latently infected cell lines have been used. In contrast with the previous studies, we used a latently infected cell line that carries a full-length HIV-1 provirus [32] integrated into the intron of a transcriptionally active cellular gene [33], what was previously reported to occur in patient cells [34,35]. Moreover, we also evaluated the reactivation potential of HMTIs in the more physiological context of CD8\(^+\) -depleted PBMCs and resting memory CD4\(^+\) T cells isolated from blood of HIV-1-infected, HAART-treated patients with undetectable viral load. Importantly, in contrast to all the other HIV-1 reactivation assays performed in patient cells, we did culture purified cells in the absence of both IL-2 and allogenic stimulation to avoid extensive nonspecific T-cell activation and proliferation, and thus resulting in no amplification of the viral RNA level. Therefore, our system constitutes today the gold standard tool for screening and evaluating antilatency drug candidates.

In the present work, we demonstrated that the HMTI chaetocin induces HIV-1 recovery in 50% of the CD8\(^+\) -depleted PBMCs cultures tested and in 86% of the resting CD4\(^+\) T-cell cultures from HIV-1-infected, HAART-treated patients with undetectable viral load. We next confirmed the high reactivation potential of HMTIs using BIX-01294, which induced HIV-1 recovery in 80% of the patient resting memory CD4\(^+\) T-cell cultures tested. Moreover, we showed that the combinations chaetocin + prostratin and chaetocin + SAHA caused a
higher HIV-1 reactivation than these compounds used alone in most cases. Indeed, a combination of two different HIV-1 inducers can act on different mechanisms of latency. We next confirmed our results using the combination BIX-01294 + SAHA in a smaller group of patient cultures.

We could not find any correlation between patient’s characteristics or between the number of HIV-1 DNA copies in patients cells and the reactivation level observed after the different treatments tested (Fig. 3). However, we observed that patient H27, for which no tested compound could reactivate HIV-1 in resting memory CD4+ T-cell cultures, presented the lowest number of total HIV-1 DNA copies per 10⁶ cells. This measure quantifies unintegrated and integrated forms of HIV-1 DNA, including defective viruses. In the case of HAART-treated patients, it has been demonstrated that total HIV DNA copies and integrated HIV DNA level are similar [36]. Consequently, this measure is a good representative marker of the amount of latently infected cells in the patient [37]. This low number of total HIV-1 DNA copies, representative of a small pool of latently infected cells, may remain unresponsive to reactivation strategies either because a high proportion of remaining proviruses are defective or because these particular proviruses are extremely difficult to reactivate.

Interestingly, we also observed that HIV-1 expression in cell cultures from patient H34 was not reactivated by chaetocin, SAHA, prostratin or chaetocin + SAHA, but was reactivated by chaetocin + prostratin or BIX01294 (Fig. 3). In addition, patient H28 was unresponsive to BIX-01294, whereas HIV-1 recovery was detected after treatment with chaetocin alone or in combination (Fig. 3). These results underline the importance of studying different epigenetic drugs targeting different mechanisms responsible for HIV-1 silencing in latent reservoirs as well as different combinations of these drugs in order to reactivate viral expression in a high proportion of patients. Indeed, we showed that the stimuli effective to induce HIV reactivation in CD4+ T cells are not necessarily the same for all patients in agreement with previous studies [20,38]. Different forms of latency could coexist in a single patient varying according to the cell type and/or the activation status of the reservoir cell or differ from one patient to the other.

In conclusion, we showed here for the first time that HMTIs used alone or in combination with other HIV-1 inducers cause HIV-1 recovery in resting memory CD4+ T cells from HAART-treated patients. Although chaetocin and BIX-01294 cannot be safely administered to humans, our results constitute a proof-of-concept for the use of HMTIs in strategies aimed at reducing the pool of HIV-1 latent reservoirs. Since HMTIs also represent promising compounds in anticancer therapies [39–41], other safer HMTIs should be synthesized soon and evaluated for their reactivation potential in cells from HIV-1-infected, HAART-treated individuals.

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**Conflicts of interest**

There are no conflicts of interest.

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