In Vitro Reactivation of Replication-Competent and Infectious HIV-1 by Histone Deacetylase Inhibitors

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ABSTRACT

The existence of long-lived HIV-1-infected resting memory CD4 T cells is thought to be the primary obstacle to HIV-1 eradication. In the search for novel therapeutic approaches that may reverse HIV-1 latency, inhibitors of histone deacytases (HDACis) have been tested to reactivate HIV-1 replication with the objective of rendering HIV-1-infected cells susceptible to elimination either by HIV-specific CD8 T cells or through virus-mediated cytopathicity. In the present study, we evaluated the efficiency of HDACis to reactivate HIV-1 replication from resting memory CD4 T cells isolated from aviremic long-term-treated HIV-1-infected subjects. We demonstrate that following prolonged/repeated treatment of resting memory CD4 T cells with HDACis, HIV-1 replication may be induced from primary resting memory CD4 T cells isolated from aviremic long-term-treated HIV-1-infected subjects. More importantly, we demonstrate that HIV-1 reactivated in the cell cultures was not only replication competent but also infectious. Interestingly, givinostat, an HDACi that has not been investigated in clinical trials, was more efficient than vorinostat, panobinostat, and romidepsin in reversing HIV-1 latency in vitro. Taken together, these results support further evaluation of givinostat as a latency-reversing agent (LRA) in aviremic long-term-treated HIV-1-infected subjects.

IMPORTANCE

The major barrier to HIV cure is the existence of long-lived latently HIV-1-infected resting memory CD4 T cells. Latently HIV-1-infected CD4 T cells are transcriptionally silent and are therefore not targeted by conventional antiretroviral therapy (ART) or the immune system. In this context, one strategy to target latently infected cells is based on pharmacological molecules that may force the virus to replicate and would therefore render HIV-1-infected cells susceptible to elimination either by HIV-specific CD8 T cells or through virus-mediated cytopathicity. In this context, we developed an experimental strategy that would allow the evaluation of latency-reversing agent (LRA) efficiency in vitro using primary CD4 T cells. In the present study, we demonstrate that HDACis are potent inducers of replication-competent and infectious HIV-1 in resting memory CD4 T cells of long-term ART-treated patients and identify givinostat as the most efficient LRA tested.

The relative lack of efficacy of LRAs to reactivate HIV-1 replication in vivo contrasts with the findings of clinical studies showing promising results on the ability of HDACis (via single-dose or multidose administration) such as vorinostat, romidepsin, and panobinostat to increase cell-associated RNA and more importantly to induce transient blips in viremia in otherwise aviremic antiretroviral therapy (ART)-treated subjects (19, 20, 31). In addition, a recent study from Dar et al. postulated that increasing baseline transcription noise can enhance the probability of successful viral release from HIV-infected cells (21). In this context, we hypothesized that repeated/prolonged treatment of resting memory CD4 T cells with HDACis in the presence of increased

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TABLE 1 Characteristics of the study group

| Subject no. | Age (yr) | Sex | Duration of HIV infection (yr) | CD4 cell count (cells/μl) | Viral load (copies/ml) | Time on HAART (yr) \(^a\) | HAART regimen \(^b\) | No. of: Integrated HIV-1 DNA copies/million resting memory CD4 T cells | No. of: HIV RNA copies/million resting memory CD4 T cells | No. of: Infectious units/million resting memory CD4 T cells \(^c\) |
|-------------|----------|-----|--------------------------------|--------------------------|-----------------------|-----------------------------|----------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|
| 1           | 47       | M   | 5                              | 760                      | <20                   | 5                           | ETR, FTC, TDF       | 33                                               | 4.17                                             | 0.35                                             |
| 5           | 41       | F   | 9                              | 1,370                    | <20                   | 6                           | TDF, FTC, ATV/r      | 5,951                                            | 29                                               | 0.77                                             |
| 6           | 51       | M   | 6                              | 391                      | <20                   | 6                           | FTC, TDF, ETV        | 7                                                 | 0.35                                             | <0.35                                            |
| 7           | 36       | M   | 6                              | 760                      | <20                   | 3                           | ABC, 3TC, ATV/r      | 1,765                                             | 2.05                                             | <0.35                                            |
| 8           | 41       | M   | 15                             | 323                      | <20                   | 6                           | ABC, 3TC, ETV        | 1,170                                             | 3.13                                             | <0.35                                            |
| 10          | 49       | M   | 8                              | 543                      | <20                   | 8                           | TDF, FTC, ETV        | 958                                               | 39.39                                            | 1.19                                             |
| 12          | 29       | F   | 8                              | 473                      | <20                   | 2                           | ABC, 3TC, DRV/r      | 1,179                                             | 4.28                                             | 0.35                                             |
| 14          | 37       | M   | 6                              | 1,267                    | <20                   | 2                           | TDF, FTC, ETV        | 972                                               | 29                                               | 0.77                                             |
| 16          | 49       | M   | 25                             | 549                      | <20                   | 2                           | ABC, 3TC, ETV        | 14,309                                             | 16.47                                            | 1.31                                             |
| 18          | 45       | M   | 3                              | 376                      | <20                   | 2                           | TDF, FTC, ETV        | 2,074                                             | 1.81                                             | <0.35                                            |

\(^a\) HAART, highly active antiretroviral therapy.
\(^b\) Designated “RUPM” in the text and selected figures.
\(^c\) Designated “IUPM” in the text and selected figures.

We demonstrate that following prolonged/repeated treatment of resting memory CD4 T cells with HDACis, HIV-1 replication can be induced from primary resting memory CD4 T cells isolated from aviremic long-term-treated HIV-1-infected subjects.

Therefore, in the present study, we used a modified VOA that integrates a number of strategies that may potentiate the therapeutic effects of HDACis and create better experimental conditions for amplification of HIV-1 replication in vitro. These include (i) increased duration of treatment of resting memory CD4 T cells with HDACis to mimic the regimens associated with the better therapeutic effects of these drugs when administered to patients and (ii) use of allogeneic (irradiated or not) CD8-depleted blood mononuclear cells from HIV-1-uninfected subjects that may provide large numbers of novel CD4 T-cell targets to amplify HIV-1 replication in vitro and/or increase baseline transcription noise may reactivate HIV-1 replication from primary resting memory CD4 T cells isolated from aviremic long-term-treated HIV-1-infected subjects.

Reagents and cell culture. Vorinostat (Merck Research Laboratory, USA), romidepsin (Cellgene, USA), panobinostat (Novartis, Switzerland), givinostat (Italfarmaco; Italy), belinostat (Topotarget and Spectrum Pharmaceuticals, Denmark), and bryostatin were obtained from Hölzel Diagnostika (Germany) and resuspended in dimethyl sulfoxide (DMSO). Cells were cultured in RPMI (Gibco, Life Technologies) containing 10% heat-inactivated fetal bovine serum (FBS) (Institut de Biochemistry-Jacques Boy), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Bio Concept).

Antibodies. The following antibodies were used. Allophecoycanin (APC)-H7-conjugated anti-CD3 (clone SK7), peridinin chlorophyll protein (PerCP)-Cy5.5-conjugated anti-CD8 (clone SK1), Pacific Blue (PB)-, fluorescein isothiocyanate (FITC)-, or phycoerythrin (PE)-CF594-conjugated anti-CD4 (clone RPA-T4), V450-conjugated anti-HLA-DR (clone G46-6), PE-Cy7-conjugated anti-CD25 (clone M-A251), PerCP-Cy5.5-conjugated anti-CD69 (clone L78), purified coating anti-CD3 (clone UCHT1), and anti-CD28 (clone CD28.2) monoclonal antibodies (M Abs) were purchased from BD (Becton Dickinson, USA) or from Biologen (Switzerland). PE-conjugated anti-acetyl H3 and H4 were from Merck (Switzerland). PE-conjugated anti-acetyl H3 and H4 were from Merck Millipore (USA), and electron-coupled dye (ECD)-conjugated anti-CD45RA (clone 2H4) was from Beckman Coulter (USA).

Flow cytometry. Data were acquired on a 4-laser LSR Sora flow cytometer (405, 488, 532, and 633 nm) and were analyzed using FlowJo v9.4.11 (TreeStar, Inc., Ashland, OR). At least 100,000 events were acquired for each sample.

Sooring of resting memory CD4 T cells. Cryopreserved blood mononuclear cells were thawed, and CD4 T cells were enriched using EasySep human CD4 T-cell enrichment kit (StemCell Technologies, USA). CD4 T
cells were then stained with an Aqua LIVE/DEAD stain kit (4°C, 15 min) and then with anti-CD4–FITC, anti-CD45RA–ECD, anti-HLA-DR–PB, anti-CD25–PE-Cy7, and anti-CD69–PerCP-Cy5.5 (4°C, 25 min) and viable resting memory (CD4+ CD45RA– CD25– CD69– HLA-DR–) CD4 T-cell populations were sorted using a FACSArray fluorescence-activated cell sorter (Becton Dickinson). In all sorting experiments, the grade of purity of the sorted cell populations was >97%.

Integrated HIV-1 DNA quantification. Resting memory CD4 T cells were sorted as described above and lysed using lysis buffer (10 mM Tris-HCl, pH 8.0, 50 mM KCl, 400 µg/ml protease K [Invitrogen]), and integrated HIV-1 DNA and CD3 gene copy numbers were determined using a cross-clade ultrasensitive nested Alu PCR, as previously described (23). The frequency of HIV-1 integrated DNA per million cells was calculated as previously described (23).

Modified viral outgrowth assay. Different cell concentrations (5-fold limiting dilutions of 5 × 10^4, 2 × 10^4, and 4 × 10^3 cells) of sorted viable resting memory CD4 T cells (CD4+ CD45RA– CD25– CD69– HLA-DR–) from HIV-1-infected subjects (Table 1) were cultured (5 replicates per condition) in complete RPMI with allogeneic fresh CD8-depleted blood mononuclear cells (10^6 cells/ml) from HIV-infected subjects in the presence or absence (negative control) of an HDACi, i.e., vorinostat (400 nM), romidepsin (5 nM), panobinostat (15 nM), givinostat (400 nM), or belinostat (400 nM). Cells were then washed and stained (30 min, 4°C) with anti-CD3–APC-H7, anti-CD8–PerCP-Cy5.5, anti-CD4–PE, and anti-acetyl H3–PE or anti-acetyl H4–PE. Expression of acetyl histone H3 and H4 in CD4 T-cell population was assessed by flow cytometry.

Assessment of level of expression of activation marker and proliferation capacity of purified resting memory CD4 T cells exposed to allogeneic CD8-depleted mononuclear cells. Sorted viable resting memory CD4 T cells (CD4+ CD45RA– CD25– CD69– HLA-DR–) from HIV-1-uninfected subjects (n = 3) were labeled with CFSE and cultured with allogeneic fresh CD8-depleted blood mononuclear cells (10^6 cells/ml) from HIV-1-uninfected subjects or remained unexposed (negative control). As a positive control, cells were stimulated for 3 days with anti-CD3/CD28 MAb-coated plates (10 µg/ml) for 14 days. All cell cultures were conditioned in complete RPMI medium supplemented with IL-2 (50 U/ml) and IL-7 (10 ng/ml) for 14 days. Medium was replaced at day 5 and resupplemented with HDACi and cytokines. Supernatants were collected at day 14. The presence of P24 antigen was assessed by ECL Cobas HIV Ag (Roche; Switzerland). One enhanced chemiluminescence (ECL) unit corresponds to about 22 pg/ml. The presence of HIV-1 RNA was assessed by the Cobas AmpliPrep/Taqman HIV-1 test (Roche, Switzerland) or by the Abbott RealTime HIV-1 RNA Assay (Abbott, USA). The presence of infectious HIV-1 was assessed by ECL. In vitro HIV-1 infection assay. CD8-depleted blood mononuclear cells isolated from HIV-infected donors were activated for 48 h with anti-CD3/CD28 microbeads (Miltenyi) in complete RPMI medium supplemented with IL-2 (50 U/ml). Activated allogeneic CD8-depleted blood mononuclear cells (10^6 cells/ml) from HIV-1-infected donors were washed and exposed (6 h, 37°C, 5% CO2) for 100 µl PB4+ supernatants collected at day 14 following various modified VOA conditions (anti-CD3/CD28 MAb or HDACi treatments). Following 6 h of exposure, cells were washed twice with complete medium and cultured for an additional 10 days in complete RPMI medium. The presence of infectious HIV-1 particles was determined in the culture supernatants at days 0, 3, and 10 postincubation as assessed by P24 production assay using ECL.

Assessment of CD4 T-cell viability. CD4 T cells isolated from 3 HIV-uninfected individuals were exposed or not to vorinostat (400 nM), romidepsin (5 nM), panobinostat (15 nM), givinostat (400 nM), or belinostat (400 nM) in the presence of allogeneic CD8-depleted PBMCs isolated from HIV-uninfected subjects. After 24, 48, 96, and 144 h, cells were washed with the Aqua LIVE/DEAD stain kit (4°C, 15 min), washed, and stained with anti-CD3 APC–H7, anti-CD4–FITC, and annexin V–APC (4°C, 20 min). Cells were washed with annexin buffer, and the percentages of CD3+CD4+ Aqua– and CD3+CD4+ annexin+ cells were assessed by flow cytometry.

Proliferation inhibition assay. Blood mononuclear cells were stained with 0.25 µM 5,6-carboxyfluorescein succinimidyl ester (CFSE [Molecular Probes, USA]) as previously described (22) and stimulated for 6 days with anti-CD3/CD28 MAb-coated plates (10 µg/ml), or left unstimulated, in the presence or in the absence of HDACi: vorinostat (400 nM), romidepsin (5 nM), panobinostat (15 nM), givinostat (400 nM), or belinostat (400 nM). Cells were then washed and stained (30 min, 4°C) with anti-CD3–APC-H7 and anti-CD4–PE-CF594, and the percentage of viable cells was assessed using the Vivid LIVE/DEAD stain kit (Invitrogen). Proliferating CD4 T cells were defined as viable CD3+ CD4+ CFSElow cells.

Evaluation of HDACi-induced histone H3 and H4 acetylation. Freshly isolated blood mononuclear cells were exposed or not to HDACi (i.e., vorinostat [400 nM], romidepsin [5 nM], panobinostat [15 nM], givinostat [400 nM], or belinostat [400 nM]) for 24 h, and histone H3 and H4 acetylation was assessed as previously described (26). Briefly, cells were stained with the Aqua LIVE/DEAD stain kit (4°C, 20 min), permeabilized (4°C, 1 h) with a Foxp3 fixation/permeabilization kit (eBioscience), blocked (4°C, 10 min) with PBS, and washed and stained (4°C, 25 min) with anti-CD3–APC-H7, anti-CD8–PerCP-Cy5.5, anti-CD4–PE, and anti-acetyl H3–PE or anti-acetyl H4–PE. Expression of acetyl histone H3 and H4 in CD4 T-cell population was assessed by flow cytometry.

Statistical analyses. Statistical significance (P values) was obtained either using a two-tailed chi-square analysis for comparison of positive proportions or by one-way analysis of variance (ANOVA) (Kruskal-Wallis test) followed by a Wilcoxon matched-pair signed rank test for multiple comparisons. When required, Bonferroni’s correction was applied for multiple comparisons. Finally, Spearman’s rank test was used for correlations.

RESULTS

Experimental strategy to evaluate the efficiency of HDACis to reverse HIV-1 latency in resting memory CD4 T cells isolated from long-term-treated HIV-1-infected individuals. The modified VOA is schematically outlined in Fig. 1A. As a positive control, resting memory CD4 T cells were stimulated with anti-CD3/CD28 MAbs for 3 days in the presence of IL-2 and IL-7. The presence of HIV-1 RNA and P24 in the culture supernatants were measured using validated diagnostic assays (Fig. 1A). It is important to underscore that the concentrations of HDACis used in the modified VOA assay corresponded to the clinical doses used in patients. The proportion of responders, proportion of positive wells, and HIV-1 RNA and P24 levels were generated using the 5 replicates of the lowest dilution of cells (5 × 10^5 cells/condition) in the modified VOA (Fig. 1A), while the limiting dilution format was used to evaluate the frequencies of inducible replication-com-
petent virus from latently HIV-1-infected cells. The frequencies of cells containing replication-competent virus were assessed by (i) the detection of HIV RNA in VOA supernatants, expressed as replication-competent RNA units per million (RUPM) (24), and by (ii) the detection of HIV P24 in VOA supernatants, expressed as infectious units per million (IUPM) (15) (Fig. 1A).

In preliminary experiments, it was determined whether all the HDACis tested, including vorinostat, romidepsin, belinostat, FIG 1

HDACis efficiently reactivate HIV-1 replication from latently infected resting memory CD4 T cells isolated from long-term-treated HIV-1-infected subjects. (A) Schematic representation of the modified VOA. (B) Proportion of responders to HDACis treatment based on the detection of HIV-1 RNA (n = 15; 5 replicates per condition). Individuals having at least one replicate with detectable HIV-1 RNA (≥200 HIV-1 RNA copies/ml) are indicated as “responders” for the condition tested. (C) Proportion of responders to HDACi treatment based on the detection of P24 (n = 15; 5 replicates per condition). Individuals having at least one replicate with detectable P24 (≥1 ECL unit/ml) are indicated as “responders” for the condition tested. (D) Proportion of HIV-1 RNA-positive wells induced following HDACis treatment (n = 10; 5 replicates per condition). Wells with detectable HIV-1 RNA (≥200 HIV-1 RNA copies/ml) are indicated as HIV-1 RNA positive for the condition tested. (E) Proportion of P24-positive wells induced following HDACis treatment (n = 10; 5 replicates per condition). Wells with detectable P24 (≥1 ECL unit/ml) are indicated as P24 positive for the condition tested. (F) Levels of HIV-1 RNA copies per milliliter induced following HDACis treatment (n = 10; 5 replicates per condition). (G) Levels of P24 (ECL units per milliliter) induced following HDACis treatment (n = 10; 5 replicates per condition). (H and I) Frequencies of inducible replication-competent virus as measured by replication-competent (RNA) units per million (RUPM) (H) or as measured by infectious units per million (IUPM) (I). (J) Correlation between P24 and HIV-1 RNA levels (n = 350; 10 subjects, 7 conditions, 5 replicates per condition). Panels B to G and J were generated using the 5 replicates of the lowest dilution of cells (5 x 10^5 cells) of all conditions by modified VOA. Subjects were color coded, and each color corresponds to a subject (F to J). Histograms correspond to the mean (B to I), and red error bars correspond to the standard error of the mean (SEM) (F to I). Blue lines correspond to the median (F to I). Red asterisks indicate statistical significance compared to unstimulated or unexposed (US) (P < 0.05). Green asterisks indicate statistical significance compared to vorinostat (P < 0.05). romi, romidepsin; pano, panobinostat; givi, givinostat; beli, belinostat. Statistical significance (P values) was obtained using two-tailed chi-square analysis for comparison of positive proportions (B to E), by one-way ANOVA (Kruskal-Wallis test) followed by Wilcoxon matched-pair two-tailed signed rank test (F to I), or by using Spearman’s rank correlations (J). Bonferroni’s correction was applied for multiple comparisons.
panobinostat, and givinostat, were functionally active. The results obtained showed that all HDACis tested significantly increased acetyl histone H3 and/or H4 mean fluorescent intensity (MFI) \((P < 0.05)\) \((\text{Fig. 2A and B})\) and significantly inhibited T-cell receptor (TCR)-induced CD4 T-cell proliferation \((P < 0.05)\) \((\text{Fig. 2C})\), thus indicating that all HDACis tested were functionally active. In addition, the viability of CD4 T cells exposed to clinically relevant concentrations of HDACis was also assessed by a flow cytometry-based assay. The results obtained showed that using the aforementioned VOA experimental strategy, all HDACis tested did not significantly influence CD4 T-cell viability compared to that of the unstimulated cells \((\text{Fig. 2D and E})\).

We then evaluated RUPM and IUPM frequencies in 10 aviremic long-term-treated patients (duration of treatment, 2 to 8 years; average, 4.2 years) using the VOA after stimulation with anti-CD3/CD28 MAbs. The cumulative data shown in \text{Fig. 1B through I and Table 1} showed that anti-CD3/CD28 MAb treatment consistently induced HIV-1 replication, as assessed by HIV-1 RNA and P24 production in the culture supernatants, while all replicates (except one at the limit of detection) of the unstimulated condition remained negative for both HIV-1 RNA and P24, demonstrating that the use of allogeneic CD8-depleted blood mononuclear cells from HIV-uninfected subjects in the presence of IL-2 and IL-7 was not sufficient to induce HIV-1 replication and confirmed the results obtained by Bosque et al. \((27)\).

In addition, the inducible RUPM and IUPM frequencies measured in the 10 aviremic long-term-treated HIV-1-infected subjects studied were consistent with the frequencies reported in previous studies \((15, 24)\). Of note, the use of anti-CD3/CD28 MAb to stimulate HIV-1 replication did not underestimate the frequencies of inducible replication-competent virus compared to phytohemagglutinin (PHA) stimulation (data not shown).

**FIG 2** Assessment of functional activity and toxicity of the HDACis tested. (A and B) Mean fluorescence intensity (MFI) of acetyl histone H3 (A) or histone H4 (B). Blood mononuclear cells isolated from 4 HIV-seronegative individuals were exposed or not (unexposed control [US]) to vorinostat (400 nM), romidepsin (Romi [5 nM]), panobinostat (Pano [15 nM]), givinostat (Givi [400 nM]), or belinostat (Beli [400 nM]) for 24 h, and acetyl histone H3 or H4 MFI was analyzed on CD4 T cells by flow cytometry. (C) Percentage of CD4 T-cell proliferation upon HDACi treatment. CFSE-labeled blood mononuclear cells isolated from 3 HIV-seronegative individuals were exposed or not (US) to vorinostat (400 nM), romidepsin (5 nM), panobinostat (15 nM), givinostat (400 nM), or belinostat (400 nM) for 24 h and stimulated for 6 days with anti-CD3/CD28 MAbs, and the percentage of proliferating CD4 T-cell proliferation (CFSE low) was assessed by flow cytometry. The percentage of annexin V-positive (D) or Aqua-positive (E) CD4 T cells was assessed at 24, 48, 96, and 144 h. CD4 T cells isolated from 3 HIV-uninfected individuals were exposed or not to vorinostat (400 nM), romidepsin (5 nM), panobinostat (15 nM), givinostat (400 nM), and belinostat (400 nM) in the presence of allogeneic CD8-depleted PBMCs isolated from HIV-uninfected subjects. Red horizontal lines or histograms correspond to the mean, and red error bars correspond to the SEM. Red asterisks indicate statistical significance \((P < 0.05)\) compared to unexposed conditions. Statistical significance \((P\) values) was obtained using a one-way ANOVA (Kruskal-Wallis test) followed by a paired \(t\) test (A to C).
use of the Roche TaqMan assay did not overestimate the quantity of HIV-1 RNA present in the culture supernatants, as indicated by the cross-validation test using the Abbott RealTime HIV-1 assay, which avoids the potential detection of HIV-1 DNA (28) (Fig. 3). Indeed, no significant differences ($P > 0.05$) were observed under any condition tested (HDACi treatment and anti-CD3/CD28 MAbs) between the quantification of HIV-1 RNA using the Roche TaqMan assay or the Abbott RealTime HIV-1 assay, demonstrating that the Roche TaqMan assay used in the present study did not overestimate the quantity of HIV-1 RNA present in the culture supernatants under the experimental conditions used.

P24 production was also measured in the cell cultures treated...
with HDACis compared to HDACi-untreated cell cultures (Fig. 1C and E). Interestingly, anti-CD3/CD28 and givinostat were able to induce P24 in a higher proportion of subjects (i.e., 60 and 50%, respectively) compared to the other HDACis, which induced P24 production in 10 to 30% of subjects (Fig. 1C). Compared to the untreated cultures from the 10 subjects, the proportion of subjects with positive P24 was significantly different (P < 0.05) only for anti-CD3/CD28- and givinostat-treated cultures (Fig. 1C). Along the same line, the proportion of wells positive for P24 was significantly higher in anti-CD3/CD28 MAb- and givinostat-treated cultures compared to the other HDACi-treated and -untreated cultures (P < 0.05) (Fig. 1E).

With regard to the levels of HIV-1 RNA and P24 measured in the presence of the different HDACis, vorinostat and romidepsin were less effective at inducing HIV-1 RNA and P24 production than panobinostat, givinostat, and belinostat, which induced levels comparable to anti-CD3/CD28 MAb stimulation (Fig. 1F and G). However, the proportions of P24 responders and P24-positive wells were lower in panobinostat- versus givinostat-treated cultures (Fig. 1C and E). All of the HDACis induced significantly higher levels of HIV-1 RNA and P24 than untreated cell cultures (P < 0.05) (Fig. 1F and G).

Finally, the RUPM and IUPM frequencies were then calculated for all conditions. The results indicate that RUPM frequencies induced by all HDACis tested (i.e., vorinostat, romidepsin, panobinostat, givinostat, and belinostat) were significantly increased compared to the frequencies under the unexposed conditions (P < 0.05) (Fig. 1H). However, the IUPM frequencies induced by all HDACis tested (i.e., vorinostat, romidepsin, panobinostat, givinostat, and belinostat) were not significantly increased (P > 0.05) (Fig. 1I).

We then determined the relationship between HIV-1 RNA and P24. These analyses indicated that the levels of P24 production correlated with HIV-1 RNA levels (r = 0.59 and P < 0.0001) (Fig. 1J). To determine whether HDACis could have differential effect on HIV-1 RNA and P24 production, the levels of HIV-1 RNA and P24 were plotted per treatment condition (Fig. 4A to F). The results obtained showed a direct correlation between P24 and HIV-1 RNA levels in all HDACis tested except following romidepsin exposure (vorinostat, r = 0.4293, P = 0.0021; romidepsin, r = 0.1844, P = 0.1998; panobinostat, r = 0.7327, P < 0.0001; givinostat, r = 0.8024, P < 0.0001; belinostat, r = 0.4845, P = 0.0004), suggesting that P24 production induced following HDACi treatments was strongly associated with HIV-1 RNA levels.

**Estimation of the proportion of provirus induced following anti-CD3/CD28 MAb and HDACi treatments.** Characterization of full-length proviruses has illustrated that only 10 to 12% of provirus was inducible, while only a fraction of the provirus was induced following the VOA (29). To estimate the efficiency of HDACis to reverse HIV-1 latency, the proportion of provirus induced by HDACis among total provirus (Fig. 5A and B) or among the proportion of provirus induced by anti-CD3/CD28 MAb was then estimated (Fig. 5C and D). The results indicate that only a small fraction (~2.6%) of HIV-1 proviruses were reactivated to produce virions, as assessed by HIV-1 RNA detection following anti-CD3/CD28 MAb and HDACis (Fig. 5A), supporting the recent results of Cillo et al. (16). However, the fraction of HIV-1 proviruses induced by givinostat represented about 57% of that induced by anti-CD3/CD28 MAb treatment and was significantly higher than the fraction of HIV-1 proviruses induced by vorinostat or romidepsin (Fig. 5B). The fraction of HIV-1 proviruses reactivated to produce virions as assessed by HIV-1 P24 detection following treatment with anti-CD3/CD28 MAb and HDACis was even lower and represented about 0.13% (Fig. 5C). However, the fraction of HIV-1 proviruses induced by givinostat as assessed by HIV-1 P24 detection represented about 74% of that induced by anti-CD3/CD28 MAb treatment and was higher than the fraction of HIV-1 proviruses induced by any of the other HDACis tested (Fig. 5D), suggesting that givinostat is the most efficient HDACi tested for reversing HIV-1 latency in vitro.

**Infectivity of HIV-1 reactivated by HDACi treatment.** The presence of infectious virus in the culture supernatants of P24-positive cell cultures was then assessed by analyzing the ability of culture supernatants to transmit HIV-1 infection in vitro. Activated allogeneic CD8-depleted blood mononuclear cells from HIV-1-uninfected donors were inoculated with supernatants collected from P24-positive cell cultures (term modified VOA supernatants) of HIV-1-infected subjects for 6 h, and P24 production was determined in the culture supernatants at days 3 and 10 postinoculation by ECL. Of note, P24 was never detected in all culture supernatants at day 0. The cumulative data showed that both the proportion of P24-positive wells and the levels of P24 significantly increased between days 3 and 10 (P < 0.05) postinoculation, thus demonstrating that HIV isolated from the modified VOA cell cultures was infectious (Fig. 6A and B). Interestingly, the P24 levels detected in the cell culture supernatants from the new in vitro infection correlated with the P24 levels (r = 0.8417, P < 0.0001) measured in the modified VOA supernatants (Fig. 6C).

**Assessment of potential synergistic effect between givinostat treatment and TCR stimulation or PKC agonist on the reactivation of HIV-1 replication.** To evaluate whether the HDACis and TCR stimulation may synergize in the reactivation of HIV-1 replication, resting memory CD4 T cells isolated from aviremic long-term-treated HIV-1-infected subjects known to be P24 positive following givinostat treatment were exposed to givinostat or anti-CD3/CD28 MAb alone or in combination. The cumulative data showed that neither the proportion of HIV-1 RNA/P24-positive wells (Fig. 7A and B) nor the levels of HIV-1 RNA/P24 (Fig. 7C and D) significantly increased upon the combined anti-CD3/CD28 MAb-givinostat treatment (P > 0.05). These results suggested that both HDACis and TCR signals may target similar populations of latently HIV-1-infected resting memory CD4 T cells.

The study by Laird et al. has recently demonstrated that combination of mechanistically distinct LRAs may synergize to induce HIV-1 transcription (18). To address whether givinostat may synergize with bryostatin (a protein kinase C [PKC] agonist), resting memory CD4 T cells were isolated from 3 long-term-treated aviremic HIV-infected subjects and cultured in the presence of allogeneic CD8-depleted PBMCs. Cells were then exposed to givinostat or bryostatin alone or in combination for 18 h. As an internal control, cells were also exposed to givinostat for 14 days. HIV replication was assessed at day 14 of the VOA by the detection of HIV-1 RNA and P24 in the culture supernatants. The cumulative data showed that under all conditions tested viral replication was significantly reactivated compared to under unstimulated culture conditions, as assessed by the proportion of HIV-1 RNA/P24-positive wells or by the levels of HIV-1 RNA/P24 (P < 0.05) (Fig. 7E to H). Interestingly, the combined givinostat-bryostatin treatment significantly increased the levels of HIV-1 RNA/P24 com-

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pared to cells exposed to givinostat alone for 18 h. However, the levels of HIV RNA/P24 produced in the combined givinostat-bryostatin treatment were not significantly higher than the levels of HIV RNA/P24 produced in the culture supernatants of cells exposed to bryostatin alone or those with prolonged/repeated exposure to givinostat ($P > 0.05$) (Fig. 7E to H). Taken together, these results suggest that givinostat may synergize with mechanistically different LRAs such as bryostatin. However, further investigations may be required.

**Prolonged/repeated treatment of resting memory CD4 T cells to HDACis is the primary mechanism responsible for efficient induction of HIV-1 replication by HDACi.** Finally, we evaluated (i) the contribution of prolonged/repeated treatment (i.e., 14 days) of resting memory CD4 T cells to HDACis and (ii) the role of allogeneic CD8-depleted blood mononuclear cells in the modified VOA as a source of potential target cells for HIV-1 and/or as the provider of an activation signal.

To address the first issue, resting memory CD4 T cells isolated from aviremic long-term-treated HIV-1-infected subjects were treated with givinostat for 18 h or 14 days in the presence of autologous irradiated PBMCs. The use of autologous irradiated CD8-depleted PBMCs prevented the increase of transcriptional noise induced by mixed leukocyte reaction (MLR), and the fact that the cells were irradiated prevented the amplification of the virus through viral spreading. The results clearly indicate that the treatment with givinostat for 14 days induced significantly higher...

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**FIG 4** Correlation between P24 and HIV-1 RNA levels detected in culture supernatants following the viral outgrowth assay. Correlation between P24 and HIV-1 RNA levels ($n = 50$; 10 subjects, 5 replicates per condition) was obtained following anti-CD3/CD28 MAb treatment (A) or treatment with vorinostat (B), romidepsin (C), panobinostat (D), givinostat (E), or belinostat (F). Each circle corresponds to one replicate, and each color corresponds to one HIV-1-infected subject. Dotted lines correspond to the limit of detections. Statistical significance ($P$ values) was obtained using Spearman’s rank correlations.
levels of HIV-1 RNA and P24 in the culture supernatants than in the culture treated for 18 h ($P < 0.05$) (Fig. 8A). Therefore, prolonged treatment with HDACis is a powerful strategy to break HIV-1 latency in resting memory CD4 T cells from long-term aviremic ART-treated patients in vitro.

With regard to the second issue, resting memory CD4 T cells isolated from aviremic long-term-treated HIV-1-infected subjects were treated with givinostat for 14 days in the presence of allogeneic irradiated or allogeneic nonirradiated CD8-depleted PBMCs, and levels of HIV-1 RNA and P24 were compared to those found in the cultures containing autologous irradiated CD8-depleted PBMCs. In this context, the use of allogeneic irradiated CD8-depleted PBMCs allowed the evaluation of the impact of the increase of transcriptional noise induced by MLR and prevented the amplification of the virus through viral spreading. Finally, the use of allogeneic nonirradiated CD8-depleted PBMCs allowed the evaluation of both the increase of transcriptional noise induced by MLR and the signal amplification through viral spreading. No significant differences ($P > 0.05$) were observed in the levels of HIV-1 RNA and P24 in the culture supernatants of cultures treated with givinostat for 14 days in the presence of autologous or allogeneic irradiated CD8-depleted PBMCs (Fig. 8A and B). These results further confirm the importance of the prolonged treatment of HDACis as the primary mechanism responsible for the induction of HIV-1 replication in resting memory CD4 T cells. However, a slight significant increase ($P < 0.05$) in the levels of HIV-1 RNA and P24 was found in the culture supernatants of the allogeneic CD8-depleted nonirradiated PBMCs compared to autologous irradiated CD8-depleted PBMC cultures (Fig. 8A and B). The results suggested that using allogeneic CD8-depleted nonirradiated PBMCs may be associated with some degree of cell activation that can influence the amplification of HIV in the cell cultures. In this regard, it was observed that the use of allogeneic nonirradiated CD8-depleted PBMCs was associated with a moderate increase in the percentage of activated and proliferating CD4 T cells, as assessed by CD25 and HLA-DR expression (5.1% versus 0.8% and 3.5% versus 0.4% at day 3 in the allogeneic nonirradiated and allogeneic irradiated cell cultures, respectively) (Fig. 9B) and by CFSE-based assay (5.1% versus 0.8% at day 6; $P > 0.05$) (Fig. 9D).

**DISCUSSION**

Systematic preclinical evaluation of potential LRAs in primary CD4 T cells isolated from HIV-infected individuals on ART represents a fundamental step to advance toward clinical trials. Evidence that LRAs reactivate HIV-1 replication from primary resting memory CD4 T cells isolated from aviremic long-term-treated HIV-1-infected subjects is limited and remains challenging, while recent clinical trials based on vorinostat, romidepsin, and panobinostat performed in HIV-1 patients under ART showed modest
but encouraging effects (20, 26; O. S. Sogaard, presented at AIDS 2014: 20th International AIDS Conference, Melbourne, Australia [www.aids2014.org]). In the present study, we hypothesized that increasing the duration of exposure of resting memory CD4 T cells to HDACis may result in efficient induction and amplification of HIV-1 replication in resting memory CD4 T cells of aviremic long-term-treated HIV-1-infected subjects in vitro. The bulk of the data presented in Fig. 1, 3, 4, and 5 were generated using a 14-day treatment of cultures with the different HDACis in combination with allogeneic nonirradiated CD8-depleted PBMCs. The individual contributions of the modifications implemented in the newly developed VOA were also assessed.

The presence of HIV-1 RNA and P24 was measured in VOA culture supernatants using validated diagnostic assays and allowed the assessment of (i) the proportion of responders, (ii) the proportion of positive wells, (iii) the levels of HIV-1 RNA and P24, and (iv) the RUPM and IUPM frequencies, as well as (v) determination of the proportion of provirus induced following anti-CD3/CD28 MAb and HDACi treatments. The results first indicated that the signal induced by allogeneic stimulation was not sufficient to induce HIV-1 replication, as assessed by HIV-1 RNA and P24 detection in the culture supernatants, since all replicates (except one at the limit of detection) of the unstimulated condition remained negative for both HIV-1 RNA and P24. Interestingly, the results indicated that vorinostat and romidepsin were less efficient at inducing HIV-1 RNA and P24 production than panobinostat, givinostat, and belinostat. Of note, the results obtained with panobinostat mirror the results obtained in vivo by Rasmussen et al., which showed that repeated exposure to panobinostat effectively disrupts HIV-1 latency in vivo (20). Interestingly, givinostat was more efficient at inducing P24 production than vorinostatin, panobinostat, and romidepsin at reversing HIV-1 latency in vitro.

Characterization of full-length proviruses has illustrated that only 10 to 12% of proviruses were inducible, while only a fraction of them were induced following VOA (29). Interestingly, the reactivated provirus induced by givinostat represented 57 to 74% of that induced by anti-CD3/CD28 MAb treatment and was significantly higher than the fraction of HIV-1 proviruses induced by vorinostat or romidepsin. In addition, the combined treatment of resting CD4 T cells with givinostat and bryostatin (a PKC agonist) did not significantly increase HIV-1 replication compared to bryostatin treatment alone, suggesting that givinostat may synergize with mechanistically different LRAs such as bryostatin. However, further investigations may be required.

Of note, givinostat has not been evaluated in an HIV clinical
FIG 7 Assessment of potential synergistic effect between givinostat treatment and TCR stimulation or PKC agonist on the reactivation of HIV-1 replication. (A) Proportion of HIV-1 RNA-positive wells following treatment with givinostat and/or anti-CD3/anti-CD28 MAbs (n = 4). Wells with detectable HIV-1 RNA (≥200 HIV-1 RNA copies/ml) are indicated as HIV-1 RNA-positive wells for the condition tested. (B) Proportion of P24-positive wells following treatment with givinostat and/or anti-CD3/anti-CD28 MAbs (n = 4). Wells with detectable P24 (≥1 ECL unit/ml) are indicated as P24-positive wells for the condition tested. (C) HIV-1 RNA (copies per milliliter) induced following treatment with givinostat and/or anti-CD3/anti-CD28 MAbs (n = 4). (D) Levels of P24 (ECL units per milliliter) induced following treatment with givinostat and/or anti-CD3/CD28 MAbs (n = 4). (E) Proportion of HIV-1 RNA-positive wells following treatment with anti-CD3/CD28 MAbs or givinostat (Givi) and/or bryostatin (Bryo) (n = 3). Wells with detectable HIV-1 RNA (≥200 HIV-1 RNA copies/ml) are indicated as HIV-1 RNA-positive wells for the condition tested. US, unstimulated. (F) Proportion of P24-positive wells following treatment with anti-CD3/CD28 MAbs or givinostat and/or bryostatin (n = 3). (G) Levels of HIV-1 RNA copies per milliliter induced following treatment with anti-CD3/CD28 MAbs or givinostat and/or bryostatin (n = 3). Wells with detectable P24 (≥1 ECL unit/ml) are indicated as P24-positive wells for the condition tested. (H) Levels of HIV-1 P24 (ECL units per milliliter) induced following treatment with anti-CD3/CD28 MAbs or givinostat and/or bryostatin (n = 3). Subjects were color coded, and each color corresponds to a subject (C and D and G and H). Red error bars correspond to means ± SEM. Red asterisks indicate statistical significance (P < 0.05). Green asterisks indicate statistical significance compared to the givinostat (18-h) condition (P < 0.05). Statistical significance (P values) was obtained using two-tailed chi-square analysis for comparison of positive proportions (A, B, E, and F) or by one-way ANOVA (Kruskal-Wallis test) followed by Wilcoxon matched-pair two-tailed signed rank test (C, D, G, and H).
trial but is currently being evaluated in a phase II clinical trial for leukemia and has been approved by the European Union for treatment of juvenile arthritis since 2010 (30). Givinostat has been shown to be safe upon single or repeated oral dose administration, is eliminated slowly (half-life of 6 to 7 h), and shows robust dose-dependent pharmacokinetics up to a dose of 600 mg daily, achieving a maximum concentration of 542 ± 93 ng/ml in the blood of healthy individuals. Similar to its short-term use, there have been no adverse effects associated with long-term use of givinostat (30). Its administration has been associated with mild but reversible effects on platelet and neutrophil levels in blood and with a transient decrease in the levels of proinflammatory cytokine production (30).

We have also demonstrated that HIV-1 reactivated in the VOA cell culture supernatants was not only replication competent but also infectious using an in vitro HIV-1 infection assay. In addition, the P24 levels detected in the cell culture supernatants from the new in vitro infection correlated with the P24 levels measured in the modified VOA supernatants regardless of the original VOA treatment, thus suggesting that the presence of infectious particles was directly associated with the efficiency of the reactivation.

Finally, we have evaluated the contribution of the parameters modified in the present VOA: i.e., (i) prolonged/repeated HDACi treatment and (ii) use of allogeneic CD8-depleted blood mononuclear cells as a provider of an activation signal and/or as a source of de novo CD4 T-cell targets for HIV-1. We provide evidence that the prolonged treatment with HDACis is the primary mechanism responsible for the reactivation of HIV-1 replication in resting memory CD4 T cells of aviremic long-term-ART-treated patients. The use of allogeneic nonirradiated blood mononuclear cells appears to have a secondary effect since it was associated with only a moderate increase in the percentage of activated CD4 T cells and a minor effect on HIV-1 replication.

In summary, the present modified VOA represents a powerful tool in order to evaluate consistently the potency of novel LRAs and/or combination therapies to reactivate HIV-1 replication in resting memory CD4 T cells of long-term-treated aviremic HIV-1 infected subjects. Using this assay, we demonstrated that prolonged/repeated treatment of resting memory CD4 T cells with HDACis induced HIV-1 replication and production from primary resting memory CD4 T cells isolated from aviremic long-term-treated HIV-1-infected subjects. Interestingly, the viral particles produced were not only replication competent but also infectious. Notably, givinostat, an HDACi that has not been investigated in clinical trials, was more efficient than vorinostat, panobinostat, and romidepsin in reversing HIV-1 latency in vitro. The reason why givinostat harbors enhanced efficacy of latency reversal in vitro remains to be established. However, the fact that givinostat was used at a higher concentration (i.e., 400 nM) than panobinostat and romidepsin might have contributed to its enhanced in vitro efficacy of latency reversal. Indeed, the concentrations of panobinostat and romidepsin used in our experimental settings were about 26- and 80-fold lower than that of givinostat. Of note, all HDACis were used at clinically relevant concentrations.

Taken together, these results support further evaluation of givinostat as a latency-reversing agent in aviremic long-term-treated HIV-1-infected subjects.

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