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The Histone Deacetylase Inhibitor Vorinostat (SAHA) Increases the Susceptibility of Uninfected CD4$^+$ T Cells to HIV by Increasing the Kinetics and Efficiency of Postentry Viral Events

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ABSTRACT

Latently infected cells remain a primary barrier to eradication of HIV-1. Over the past decade, a better understanding of the molecular mechanisms by which latency is established and maintained has led to the discovery of a number of compounds that selectively reactivate latent proviruses without inducing polyclonal T cell activation. Recently, the histone deacetylase (HDAC) inhibitor vorinostat has been demonstrated to induce HIV transcription from latently infected cells when administered to patients. While vorinostat will be given in the context of antiretroviral therapy (ART), infection of new cells by induced virus remains a clinical concern. Here, we demonstrate that vorinostat significantly increases the susceptibility of CD4$^+$ T cells to infection by HIV in a dose- and time-dependent manner that is independent of receptor and coreceptor usage. Vorinostat does not enhance viral fusion with cells but rather enhances the kinetics and efficiency of postentry viral events, including reverse transcription, nuclear import, and integration, and enhances viral production in a spreading-infection assay. Selective inhibition of the cytoplasmic class IIb HDAC6 with tubacin recapitulated the effect of vorinostat. These findings reveal a previously unknown cytoplasmic effect of HDAC inhibitors promoting productive infection of CD4$^+$ T cells that is distinct from their well-characterized effects on nuclear histone acetylation and long-terminal-repeat (LTR) transcription. Our results indicate that careful monitoring of patients and ART intensification are warranted during vorinostat treatment and indicate that HDAC inhibitors that selectively target nuclear class I HDACs could reactivate latent HIV without increasing the susceptibility of uninfected cells to HIV.

IMPORTANCE

HDAC inhibitors, particularly vorinostat, are currently being investigated clinically as part of a "shock-and-kill" strategy to purge latent reservoirs of HIV. We demonstrate here that vorinostat increases the susceptibility of uninfected CD4$^+$ T cells to infection with HIV, raising clinical concerns that vorinostat may reseed the viral reservoirs it is meant to purge, particularly under conditions of suboptimal drug exposure. We demonstrate that vorinostat acts following viral fusion and enhances the kinetics and efficiency of reverse transcription, nuclear import, and integration. The effect of vorinostat was recapitulated using the cytoplasmic histone deacetylase 6 (HDAC6) inhibitor tubacin, revealing a novel and previously unknown cytoplasmic mechanism of HDAC inhibitors on HIV replication that is distinct from their well-characterized effects of long-terminal-repeat (LTR)-driven gene expression. Moreover, our results suggest that treatment of patients with class I-specific HDAC inhibitors could induce latent viruses without increasing the susceptibility of uninfected cells to HIV.

Human immunodeficiency virus type 1 (HIV-1) establishes a pool of latently infected resting memory CD4$^+$ T cells during primary infection that persist during treatment with antiretroviral therapy (ART) (1–3) and can resume active viral replication within weeks following treatment interruption (4–7). Latently infected cells are the primary barrier to eradication of HIV infection and are a principal reason that lifelong treatment with ART is typically required in the vast majority of patients to prevent disease progression. The latent reservoir is very stable, with a half-life of 44 months (8, 9), and can be replenished by homeostatic proliferation of latently infected cells (10) or possibly during intermittent viremia (11), although the latter mechanism remains controversial. The reservoir is not significantly diminished by intensified ART regimens (12, 13).

Interest in eradicating HIV infection has been rekindled due to the unprecedented success of the "Berlin patient," an HIV-infected patient treated for acute myelogenous leukemia with allogeneic stem cell transplantation consisting of cells from a *ccr5Δ32* homozygous donor that do not express functional CCR5 (14). Despite not receiving antiretroviral therapy for years, no HIV RNA or DNA has been detected in plasma, peripheral blood mononuclear cells (PBMCs), gastrointestinal tissues, or mucosal target cell populations from the patient (15). However, two factors make replicating the success of the Berlin patient difficult. First, ablative chemotherapy and radiation therapy in conjunction with

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stem cell transplantation is an expensive medical procedure with significant risks to the patient. Second, *ccr5Δ32* homozygous patients are present at a frequency of only ~1% of the Caucasian population and are considerably rarer in other racial and ethnic groups; therefore, finding appropriately matched donors will be extremely challenging.

An alternative approach being investigated is to purge latent reservoirs by specifically inducing HIV transcription without inducing polyclonal activation of uninfected $CD4^+$ T cells. Once the virus has been reactivated, the immune system, viral cytopathic effects, or cytotoxic drugs could theoretically eliminate infected cells. This strategy, referred to as "shock and kill" or "kick and kill," would be performed in the presence of ART to block infection of new target cells. Advances in our understanding of the molecular mechanisms underlying HIV latency have led to the identification of several drugs that might be efficacious at triggering HIV transcription in latently infected cells.

HIV latency is established by a combination of events that reduce initiation and prevent elongation of transcripts from the viral long-terminal-repeat (LTR) promoter and is subsequently reinforced by epigenetic modifications of the promoter region, as has been recently reviewed (16, 17). The N-terminal tails of histones are subject to multiple forms of posttranslational modification, including acetylation, methylation, and phosphorylation. Histone acetyltransferases (HATs) act to acetylate histone tails; histone deacetylases (HDACs) remove acetyl groups. One of the epigenetic mechanisms limiting HIV transcriptional initiation is the recruitment of HDACs to the LTR (17–20) via interactions with cellular proteins, including LSF and YY-1, NF-κB p50 homodimers, and CBF-1 (18–20). HDAC1 recruitment results in hypoacetylation of nuc-1, a nucleosome interacting with the HIV proviral DNA immediately downstream of the transcriptional start site, leading to transcriptional repression (19). In contrast, Tat-mediated recruitment of HATs, including CBP/p300 and PCAF, enhances expression from the viral LTR (21–23) via recruitment of the chromatin-remodeling complex BAF and remodeling of nuc-1 (24).

The relationship between histone acetylation and HIV transcription regulation raised the possibility that HDAC inhibitors could reactivate latent proviruses. Indeed, HDAC inhibitors were subsequently found to disrupt HIV latency in cell lines and primary cell models *in vitro* (25–29). More recently, *in vivo* administration of the HDAC inhibitor vorinostat (suberoylanilide hydroxamic acid; SAHA) was well tolerated and induced a 4.8-fold increase in HIV RNA expression in resting $CD4^+$ T cells in patients on combination antiretroviral therapy (cART) (30), demonstrating that targeted reactivation of latent HIV is feasible in patients. The safety and efficacy of vorinostat in patients on stable ART is being investigated in ongoing clinical trials.

One of the key concerns with the shock-and-kill strategy is that uninfected $CD4^+$ T cells will become infected by HIV as it is purged from latent reservoirs, potentially due to noncompliance with cART, to drug resistance, or in sanctuary sites with suboptimal drug concentrations. In light of this concern, we investigated the effect of vorinostat on the susceptibility of uninfected cells to HIV. Here, we report that, in addition to its well-characterized effect of stimulating HIV transcription in latently infected cells, vorinostat dramatically increased the vulnerability of uninfected primary $CD4^+$ T cells to HIV in a dose- and time-dependent manner and promoted viral replication in a spreading-infection assay. This effect was independent of receptor and coreceptor usage. Vorinostat did not affect viral fusion with target cells but increased the kinetics of postentry events, including reverse transcription and integration. The enhanced kinetics of infection was correlated with increased efficiency of viral events, leading to higher levels of nuclear import, as reflected by 2-LTR circles. Selective inhibition of the cytoplasmic class IIb HDAC6 with tubacin recapitulated these features of vorinostat-mediated enhancement of HIV infection. These findings reveal that HDAC inhibitors have a previously unknown cytoplasmic effect promoting productive infection of $CD4^+$ T cells that is distinct from their effects promoting histone acetylation and LTR activity. From a clinical perspective, these results raise a cautionary note for the use of vorinostat to purge latent reservoirs and suggest that ART intensification or careful monitoring of new infection events may be warranted. Furthermore, they indicate that treatment with class I-specific HDAC inhibitors could be effective at reactivating latent viruses without increasing the susceptibility of uninfected cells to HIV.

MATERIALS AND METHODS

Production of viruses. Combination reporter viruses were produced as previously detailed (31). Briefly, 293 T cells were transfected with 10 μg pNL4-3-delta-E-enhanced green fluorescent protein (EGFP) (obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH [catalog number 11100] from Haili Zhang, Yan Zhou, and Robert Siliciano), 7.5 μg virion-associated β-lactamase–Vpr (bla-Vpr) plasmid, and 6.0 μg of HIV Env REJO.D12.1972 (32) (CCR5 tropic) or JOTO.TA1.2247 (33) (CXCR4 tropic), using calcium phosphate methods. Virus was harvested 72 h after transfection, filtered, and concentrated by ultracentrifugation through a 20% sucrose cushion according to published protocols (34). Viral concentrations were determined by p24 enzyme-linked immunosorbent assay (ELISA) (Cell Biolabs), and empirical virus titers were determined on purified $CD4^+$ T cells to ensure the dose used was within the linear range of the assay. Vesicular stomatitis virus (VSV) G was produced in a similar way. Briefly, 293 T cells were transfected with 10 μg pNL4-3-d2EGFP-Nef, 6.5 μg pCM-Vdelta8.91, and 3.5 μg pMDG. Virus was harvested 72 h after transfection, filtered, and concentrated by ultracentrifugation at 28 K for 1.5 h. The titer of virus was determined by quantitative PCR (qPCR) (Lentivirus qPCR Titer Kit; abm) and by green fluorescent protein (GFP) expression in Jurkat cells.

Cells. $CD4^+$ T cells were obtained by leukapheresis from Allcells, LLC. All subjects were healthy control donors negative for hepatitis B virus (HBV), hepatitis C virus (HCV), and HIV. PBMCs were isolated by Ficoll gradient separation, while $CD4^+$ T cells were purified by adding RosetteSep $CD4^+$ T cell enrichment kit antibodies (Stemcell Technologies) and autologous red blood cells. The cells were cryopreserved and treated with benzonase prior to infection. For experiments using polarized primary $CD4^+$ T cells, naive $CD4^+$ T cells were isolated from frozen leukapheresis samples using a RoboSep instrument (Stemcell Technologies). Cells were stimulated using Dynal CD3/28 beads in the presence of the cytokines interleukin 6 (IL-6), IL-23, IL-1β, IL-2, transforming growth factor β (TGF-β), anti-IL-4 antibody, and anti-gamma interferon (IFN-γ) antibody for 6 days. The beads were removed using a Dynal magnet, and the cells were resuspended in RPMI with 10% fetal bovine serum (FBS) supplemented with IL-2 and IL-23. This study was approved by the Case Western Reserve University Institutional Review Board.

Infection experiments. Unstimulated primary $CD4^+$ T cells (10^6) were infected in parallel with 125 ng p24 equivalent of viruses bearing R5-tropic (REJO.D12.1972) or X4-tropic HIV Env. Unless otherwise indicated, cells were incubated for 4 h with 2 μM vorinostat or 2 μM tubacin prior to addition of virus. The cells were spinoculated with virus at 1,200 × *g* at 24°C for 2 h unless otherwise indicated and incubated at 37°C for 1 h. The first plate of the parallel infections, measuring fusion, was then

washed with CO_2-independent medium (Gibco), resuspended in medium containing CCF2-AM (Invitrogen) for 1 h at room temperature, washed, and incubated overnight at room temperature in CO_2-independent medium containing probenecid. The cells were then prepared for flow cytometric analysis as described below. The second plate of parallel infections, to measure LTR-driven EGFP expression, was incubated for 72 h at 37°C prior to preparation for flow cytometry. Jurkat cells or polarized primary $CD4^+$ T cells were infected in parallel with VSV-G or HIV Env at a multiplicity of infection (MOI) of 1. The cells were incubated for 4 h with 1 μM vorinostat prior to addition of virus. The cells were spinoculated with virus at room temperature for 1.5 h. At 72 h after infection, cells were harvested and washed, and flow cytometry analysis was performed.

Viral-kinetics experiments. Experiments were performed on 10^6 cells spinoculated as described above. The cells were incubated 4 h prior to infection with 2 μM vorinostat and spinoculated with virus, and AMD3100 (20 μM), enfuvirtide/T20 (20 μM), raltegravir (10 μM), and efavirenz (100 nM) were added at various time points before or after spinoculation. The inhibition kinetics graphs represent averaged infection levels normalized to no-inhibitor controls. Error bars are excluded to prevent figure congestion.

Flow cytometry. Cells were stained with anti-human CCR7 IgM (Becton, Dickinson) and LIVE/DEAD fixable yellow viability dye (Invitrogen) at 37°C for 30 min, washed, and incubated with anti-human CD3 BV650 (Biolegend), CD4 allophycocyanin (APC) (eBioscience), CD45RO electron-coupled dye (ECD) (Beckman Coulter), CD27 phycoerythrin (PE)-Cy7 (eBioscience), and anti-IgM PE (Invitrogen) at 4°C for 30 min. All the cells were washed with PBS-BSA and resuspended in 1% paraformaldehyde prior to analysis. At least 50,000 events were collected per sample. All infection conditions were performed in triplicate. FlowJo version 9.6 (Tree Star, Inc.) was used for analysis of flow cytometry experiments.

Determination of 2-LTR-circle products. Primary $CD4^+$ T cells and polarized activated T cells were spinoculated in parallel in the presence or absence of 2 μM vorinostat with CXCR4-tropic HIV-1. Following spinoculation, cells were harvested at the indicated time points and washed with PBS, and DNA was extracted. All extracts were incubated at 55°C with proteinase K prior to purification (Thermo Fisher Scientific GeneJet PCR purification kit). 2-LTR-circle products were amplified using the following primer set: 2-LTR circle forward, 5′-CAAGCAGAAGACGGCATACCAGATAACTAGGGAACCCACTGC-3′; 2-LTR circle reverse, 5′-CCTCTCTATGGGCAGTCGGTGATTCCACAGATCAAGGATATCTTGTC-3′ (Integrated DNA Technologies). Purified DNA was amplified in a qPCR machine using the 12.5 μM primers specified above, in addition to barcoded sequencing adapters to allow multiplexing of samples. PCR was stopped during the log phase of amplification, and the barcoded products were pooled, column purified, and run on a gel to select amplicons and remove residual primer. Fragments were gel purified, and DNA concentrations were determined on a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific). 2-LTR products were sequenced using an Ion Torrent Personal Genome Machine (PGM) NGS (Life Technologies) following the manufacturer's protocol. Transcript abundances were determined using Genomics Workbench 5 (CLC Bio). The numbers of reads between experiments were normalized to the sequencing-chip size.

Replication-competent experiments. Primary $CD4^+$ T cells (1×10^7) were treated with 100 IU/ml recombinant IL-2 (rIL-2) and infected with 2,700 ng p24 equivalent of replication-competent virus bearing X4-tropic envelope (HIV-NLG-Nef). Cells were incubated for 4 h with 2 μM vorinostat prior to addition of virus and spinoculated as described above. Following incubation at 37°C for 1 h, the cells were supplemented with additional medium to a final concentration of 1×10^6 cells/ml. At the indicated time points, the supernatant was preserved at −80°C, and the cells were washed and stained with anti-human CD3 BV650 (Biolegend), CD4 APC (eBioscience), and LIVE/DEAD near-infrared (IR) viability dye (Invitrogen). All the cells were washed with PBS-BSA and resuspended in 1% paraformaldehyde prior to analysis on a BD Fortessa flow cytometer. Infection conditions were performed in triplicate. Viral concentrations in supernatants were quantified by p24 ELISA (Cell Biolabs) and normalized to cell viability as determined by flow cytometry. ELISAs were performed in duplicate using two independent supernatant collections.

Statistics. The data in the figures represent mean values and standard errors of the mean unless otherwise indicated. All differences with a P value of <0.05 were considered statistically significant, with correction for multiple comparisons when appropriate. Statistical analyses were performed using a paired t test with GraphPad Prism v5.0d unless otherwise specified.

RESULTS

Virus and cell models. The HDAC inhibitor vorinostat is currently being investigated as a treatment strategy to purge latent reservoirs in HIV-infected patients (30) due to its efficacy in activating latent proviruses *ex vivo* (26). Although vorinostat will be administered in the presence of ART to block infection of uninfected cells, seeding of new reservoirs remains a clinical concern. To determine if vorinostat affects the susceptibility of uninfected $CD4^+$ T cells to HIV, we employed a novel flow cytometric combination reporter virus system (31) that can measure viral fusion and LTR-driven EGFP expression using a single reporter virus construct (Fig. 1A). Fusion of viral and cellular membranes is identified by bla-Vpr-mediated cleavage of the β-lactamase substrate CCF2 (35), which alters the fluorescence characteristics of the cell (Fig. 1B, top). In cells successfully completing postentry events, including uncoating, reverse transcription, integration, and LTR-driven gene expression, EGFP accumulates and can be detected. Spontaneous EGFP expression requires sufficient Tat and Rev levels to promote LTR transcription and export of incompletely spliced and unspliced mRNAs, respectively. Since the NL4-3 core used to produce the combination reporter viruses contains an intact *nef* gene, CD4 downregulation is also observed in $EGFP^+$ cells (Fig. 1B, bottom). By incorporating conjugated antibodies directed against CCR7 and CD45RO, naive and memory subsets of primary $CD4^+$ T cells undergoing fusion or LTR-driven EGFP expression can be precisely determined (Fig. 1C). Using this combination reporter virus system and traditional GFP reporter viruses, we examined the effects of vorinostat on three different cell models: (i) unstimulated primary $CD4^+$ T cells; (ii) polarized, activated $CD4^+$ T cells; and (iii) Jurkat cells. Unstimulated primary $CD4^+$ T cells are predominantly resting cells, typically with greater than 96% lacking expression of CD25 or CD69.

The HDAC inhibitor vorinostat increases the vulnerability of uninfected $CD4^+$ T cells to HIV. To investigate the effects of vorinostat on $CD4^+$ T cell susceptibility to HIV, we infected purified, unstimulated primary $CD4^+$ T cells with combination reporter viruses bearing a patient-derived CXCR4-tropic Env, JOTO.TA1.2247 (33). Addition of vorinostat to $CD4^+$ T cells 4 h prior to infection significantly increased $EGFP^+$ cell frequencies compared to untreated controls at doses as low as 500 nM (Fig. 2A). Similar results were seen with the pan-HDAC inhibitors panobinostat and romidepsin, indicating that the enhancement of HIV infection was related to the HDAC-inhibitory activity of vorinostat (Fig. 2B). Unstimulated $CD4^+$ T cells are primarily in a resting state; to determine whether vorinostat had similar effects on activated cells, we infected polarized, activated primary $CD4^+$ T cells and Jurkat cells. Similar to its effects on unstimulated cells, 4-h pretreatment with vorinostat significantly increased the percentages of $EGFP^+$ activated primary cells and Jurkat cells compared to untreated controls ($P = 0.003$ and $P = 0.03$, respectively) (Fig. 2C).

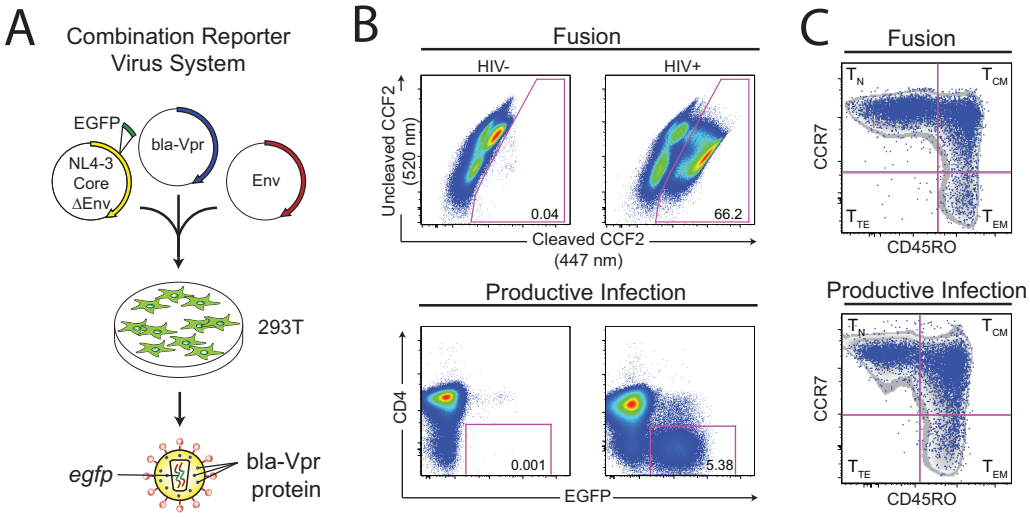

FIG 1 (A) Combination reporter viruses are produced by cotransfection of 293T cells with plasmids bearing an *egfp*-containing HIV core lacking an envelope gene (pNL4-3-ΔE-EGFP), bla-Vpr, and an HIV *env* with known coreceptor tropism. Virions package the bla-Vpr protein and the *egfp*-HIV core. (B) Fusion between virions and $CD4^+$ T cells is detectable by flow cytometry, as bla-Vpr-mediated CCF2 cleavage alters cellular fluorescence. Cells undergoing reverse transcription, uncoating, nuclear import, integration, and LTR-dependent gene expression are identified by Nef-mediated CD4 downregulation and EGFP accumulation. Numbers represent percentages of positive fusion and productive infection events (top and bottom, respectively) within the total number of events recorded. (C) In combination with the memory markers CCR7 and CD45RO, T_N, T_{CM}, T_{EM}, and T_{TE} subsets can be identified. Cells undergoing fusion or LTR-driven EGFP expression are shown in blue.

An X4-tropic HIV Env was initially selected for examination because CXCR4 is expressed on a far higher percentage of primary $CD4^+$ T cells than HIV. However, R5-tropic viruses are more common *in vivo*, particularly among patients in early stages of infection. To determine whether vorinostat-mediated enhancement of T cell vulnerability was dependent on receptor or coreceptor expression, we infected Jurkat cells with viruses pseudotyped with the CD4-independent VSV-G envelope or with a patient-derived R5-tropic Env, REJO.D12.1972 (32). As was observed for X4-tropic virus, vorinostat significantly increased the percentage of $EGFP^+$ cells following infection with VSV-G and R5-tropic pseudotyped viruses ($P = 0.01$ and $P = 0.04$, respectively) (Fig. 2D), suggesting that the enhancement of $CD4^+$ T cell vulnerability to infection is receptor and coreceptor independent.

Since vorinostat would be expected to promote transcription from viral LTRs that might otherwise become silenced soon after infection, these findings were not entirely unexpected. However, pretreatment with 2 μM vorinostat significantly enhanced LTR-driven EGFP expression compared to treatment 24 or 48 h after infection ($P = 0.02$ and $P = 0.006$, respectively), conditions typically used to activate silent proviruses (Fig. 2E). Raltegravir reduces $EGFP^+$ cell frequencies by >95% when added as late as 30 h postinfection; therefore, the observation that a 4-h preincubation with vorinostat was significantly more effective at increasing $EGFP^+$ cell frequency than addition 24 h after infection was surprising and suggested that vorinostat might have additional, unknown activities at a stage of the viral life cycle proximal to integration into the host chromosome.

Vorinostat does not enhance viral fusion with $CD4^+$ T cells. To investigate how vorinostat promotes susceptibility to infec-

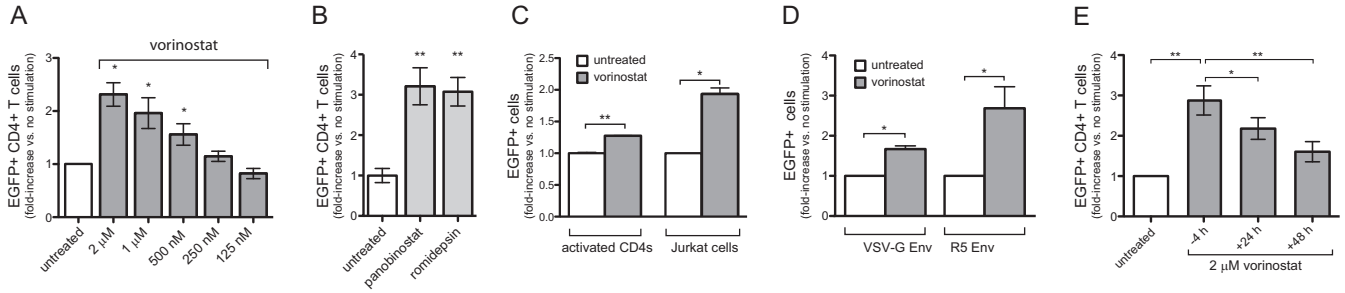

FIG 2 Vorinostat enhances HIV infection of $CD4^+$ T cells in a dose- and time-dependent manner. (A) Four-hour pretreatment of unstimulated primary $CD4^+$ T cells with vorinostat enhanced LTR-driven EGFP expression by combination reporter viruses pseudotyped with a patient-derived CXCR4-tropic Env in a dose-dependent manner. (B) Four-hour pretreatment of unstimulated $CD4^+$ T cells with 200 nM panobinostat or romidepsin also enhanced EGFP expression. (C) Infection of activated, polarized $CD4^+$ T cells and Jurkat cells with reporter viruses bearing CXCR4-tropic Envs is also enhanced by 4-h pretreatment with vorinostat. (D) Pretreatment of cells with vorinostat increases their susceptibility to reporter viruses pseudotyped with VSV-G or CCR5-tropic HIV Env, suggesting enhanced infection is independent of receptor or coreceptor usage. (E) HIV infection of unstimulated primary $CD4^+$ T cells was enhanced by 4-h pretreatment with vorinostat compared to addition 24 or 48 h after infection. The data represent means and standard errors of the mean. *, $P < 0.05$; **, $P < 0.01$. Exact *P* values are provided in the text.

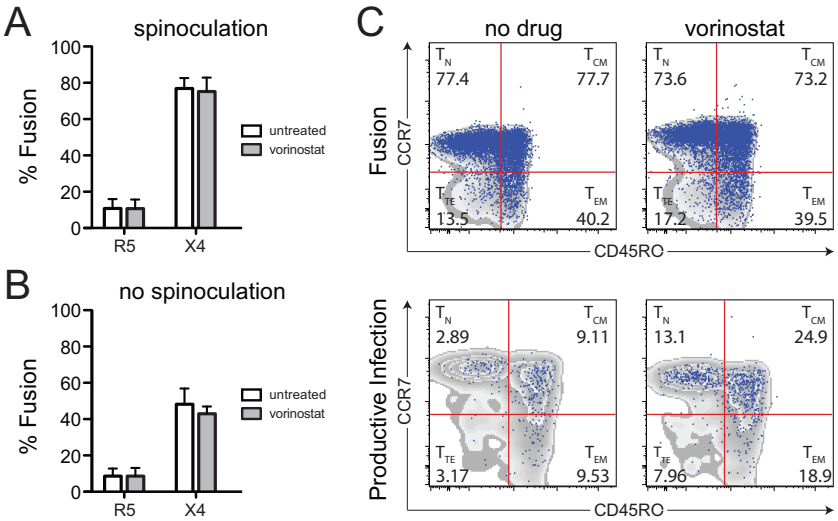

FIG 3 Vorinostat does not increase viral fusion with CD4$^+$ T cells. (A) Unstimulated primary CD4$^+$ T cells were infected with X4- or R5-tropic combination reporter viruses and spinoculated at 1,200 × *g* for 2 h, and fusion levels were determined by bla-Vpr-mediated CCF2 cleavage. Four-hour vorinostat pretreatment did not affect fusion levels of either X4- or R5-tropic HIV. (B) Vorinostat pretreatment did not increase fusion levels in the absence of spinoculation for either R5- or X4-tropic HIV. Ten- to 15-fold-higher concentrations of HIV were used to compensate for the reduction in fusion in the absence of spinoculation. The data represent means and standard errors of the mean. (C) Pretreatment with vorinostat did not affect the percentages of CD4$^+$ T cells in T_N, T_{CM}, T_{EM}, or T_{TE} subsets fusing with HIV but enhanced the likelihood of infection following fusion. Representative data from one of five patients are shown. The numbers represent the percentages of cells undergoing fusion or LTR-driven EGFP expression in each CD4$^+$ T cell subset.

tion, we measured its effect on fusion using combination reporter viruses pseudotyped with either R5- or X4-tropic Envs. Four hours of preincubation with vorinostat had no effect on fusion (Fig. 3A). However, our infection protocol involved a 2-h spinoculation step, which has recently been reported to induce remodeling of the cortical actin barrier and promote viral infection (36). We reasoned that spinoculation might mask the effects of vorinostat on fusion and therefore repeated the experiment in the absence of spinoculation. A 10- to 15-fold-higher viral inoculum was used in these studies, as fusion is significantly diminished in the absence of spinoculation in the combination reporter virus assay (31). Again, no enhancement of viral fusion was observed for reporter viruses pseudotyped with either R5- or X4-tropic Envs (Fig. 3B); in fact, there was a slight decrease in fusion of X4-tropic HIV in the presence of vorinostat, consistent with an observation that CXCR4 receptor levels can be decreased by HDAC inhibitors (37, 38).

To gain further insight into how vorinostat influences CD4$^+$ T cell vulnerability to HIV, we infected unstimulated primary CD4$^+$ T cells with an X4-tropic combination reporter virus and examined both fusion and LTR-driven EGFP expression in naive and memory subsets. Consistent with the total CD4$^+$ T cell analysis, vorinostat did not increase HIV fusion within any of the CD4$^+$ T cell subsets but did enhance reporter gene expression (Fig. 3C). Interestingly, the effect of vorinostat appeared most pronounced in the CCR7$^+$ CD45RO$^-$ naive (T_N) and CCR7$^+$ CD45RO$^+$ central memory (T_{CM}) subsets, which are less activated than the CCR7$^-$ CD45RO$^-$ effector memory (T_{EM}) and CCR7$^-$ CD45RO$^+$ terminal effector (T_{TE}) subsets. Together, these data suggest that vorinostat increases productive infection by modifying the efficiency of postentry steps of the viral life cycle and may have the greatest effects on unstimulated naive and central memory CD4$^+$ T cells, which appear to be resting, since they do not express the classical phenotypic surface markers CD25 and CD69.

Vorinostat increases the kinetics and efficiency of postentry events in the viral life cycle. To further investigate the postentry events influenced by vorinostat, we performed experiments in which the CXCR4 antagonist AMD3100, the fusion inhibitor T20, the reverse transcriptase inhibitor efavirenz, and the integrase inhibitor raltegravir were added to unstimulated primary CD4$^+$ cells prior to or at various time points following infection with HIV, in either the absence or presence of vorinostat. As expected, the kinetics of HIV binding to CXCR4 or fusion of the viral and host cell membranes was unaffected by the presence of vorinostat (Fig. 4A and B, respectively). In contrast, cells pretreated with vorinostat became insensitive to efavirenz more rapidly than control cells (half-life [$t_{1/2}$] = 35.4 h versus 41.8 h), indicating that the kinetics of reverse transcription were accelerated in the presence of vorinostat (Fig. 4C). Similarly, cells became refractory to raltegravir more rapidly when pretreated with vorinostat ($t_{1/2}$ = 35.9 h versus 47.2 h), reflecting enhanced integration kinetics (Fig. 4D).

Since the kinetics of viral postentry events have been correlated with the efficiency of viral infection (39), we reasoned that the enhanced kinetics of entry in the presence of vorinostat would translate into more efficient progression through postentry stages of the viral life cycle. To directly test this hypothesis, we measured the production of 2-LTR circles, a marker of nuclear import of viral DNA, in unstimulated or activated primary CD4$^+$ T cells infected with HIV using qPCR followed by deep sequencing on an Ion Torrent Personal Genome Machine to confirm the specificity of the products. Both unstimulated primary CD4$^+$ T cells and polarized, activated CD4$^+$ T cells demonstrated increased levels of 2-LTR circles in the presence of vorinostat (Fig. 5). Treatment with vorinostat resulted in an ~3-fold increase in the number of sequence reads by 72 h. In both cell types, the accumulation of 2-LTR circles was not observed until at least 24 h postinfection, consistent with the kinetics data regarding the timing of reverse

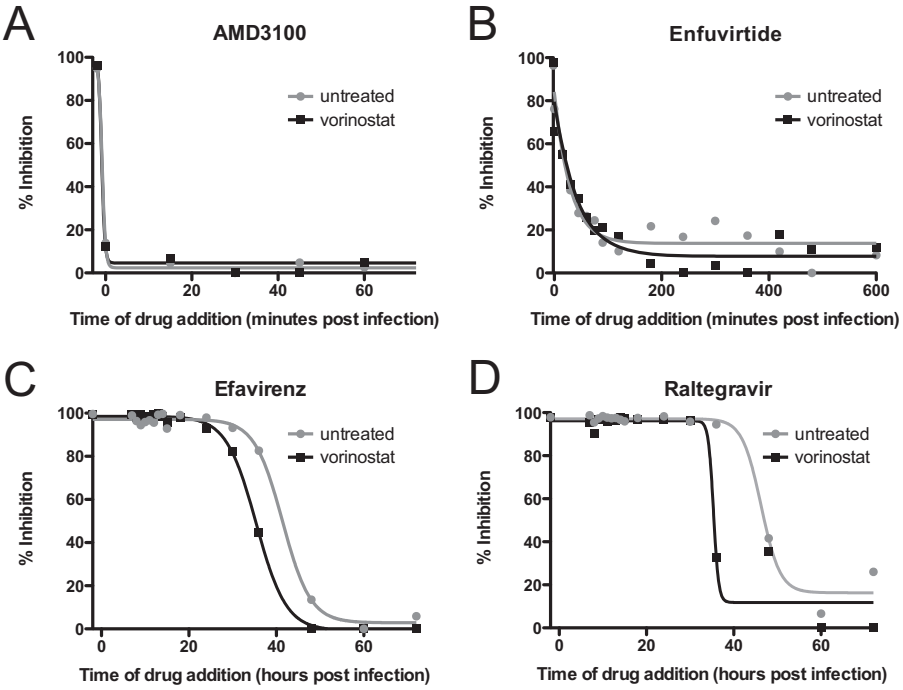

FIG 4 Vorinostat enhances the kinetics of postentry viral events, including reverse transcription and integration. Uninfected CD4$^+$ T cells were infected with X4-tropic reporter viruses with or without 4-h pretreatment with vorinostat. Prior to infection or at various time points after infection, the coreceptor antagonist AMD3100 (A), fusion inhibitor T20 (B), reverse transcriptase inhibitor efavirenz (C), or integrase inhibitor raltegravir (D) was added. Infection values were normalized to uninfected controls. The graphs represent average levels among three replicates. Error bars are omitted to prevent figure congestion.

transcription. Furthermore, polarized, activated CD4$^+$ T cells yielded greater overall sequence reads, in agreement with previous findings that activated cells are increasingly susceptible to HIV infection. Together with our kinetics data, these data suggest that vorinostat increases the efficiency of viral postentry events, including reverse transcription, nuclear import, and integration.

Vorinostat enhances productive infection of CD4$^+$ T cells and replication of HIV. As the combination reporter virus is a single-cycle assay due to the replacement of the viral *env* gene with *egfp*, we also examined the effects of vorinostat in a spreading-infection assay (Fig. 6). Briefly, 1×10^7 primary CD4$^+$ T cells were stimulated with 100 IU/ml IL-2, infected with a replication-competent HIV reporter (HIV-NLG-Nef), spinoculated, and incubated at 37°C for up to 13 days. On days 1, 3, 5, 7, 9, 11, and 13, cells were removed to determine EGFP expression, and the supernatant was harvested and stored at −80° for p24 ELISA. As expected, treatment with vorinostat significantly increased the EGFP expression of replication-competent HIV beyond a single round of infection, as evidenced by a steady rise in EGFP$^+$ cells from days 7 to 13 compared to untreated controls (Fig. 6A). This finding was further supported by the viral p24 protein concentration in the cell supernatants. Treatment with vorinostat dramatically increased the level of p24 produced by day 13, indicating that vorinostat acts to both augment LTR-driven EGFP expression and amplify virion release throughout multiple rounds of HIV repli-

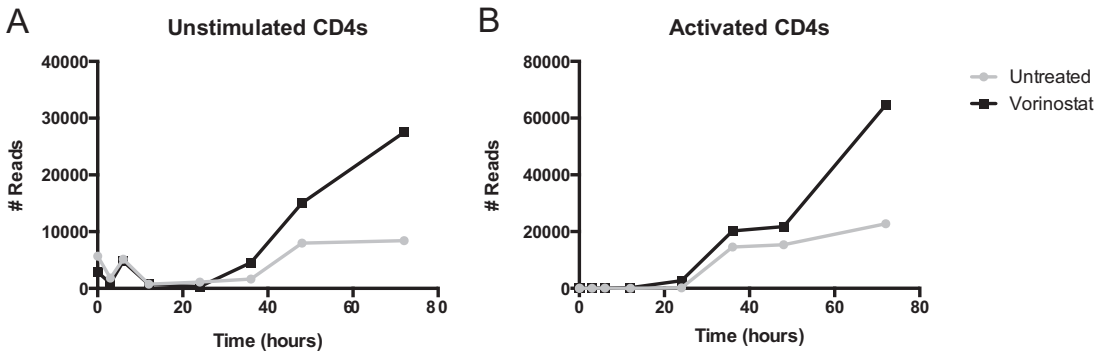

FIG 5 Efficiency of postentry viral events, including reverse transcription and nuclear import, is improved by vorinostat. Primary unstimulated (A) and polarized, activated (B) CD4$^+$ T cells were infected with X4-tropic reporter viruses and spinoculated. At various time points following infection, the cells were harvested and DNA was extracted for qPCR of 2-LTR circles, followed by deep sequencing to confirm specificity. Product abundances were determined using Genomics Workbench 5. The data were normalized by input DNA and sequencing-chip size.

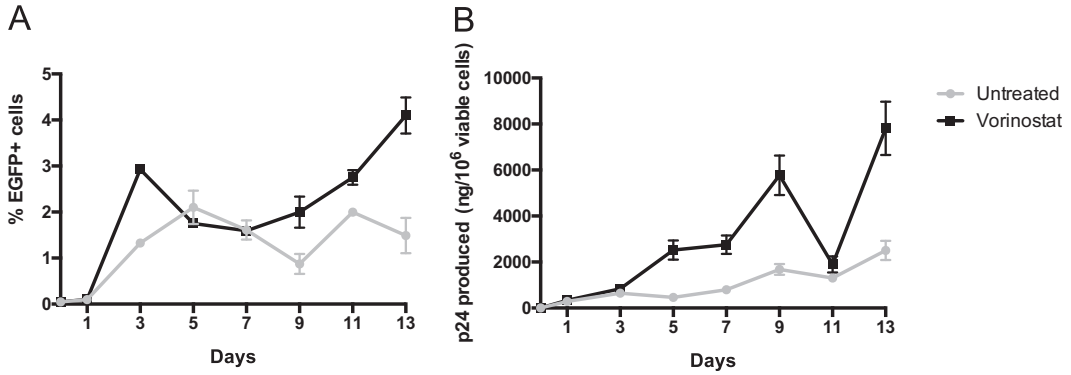

FIG 6 Vorinostat enhances productive infection and replication of HIV in a spreading infection. Primary CD4$^+$ T cells were stimulated with 100 IU/ml IL-2, infected with X4-tropic replication-competent virus, and spinoculated. Cells and supernatants were harvested at days 1, 3, 5, 7, 9, 11, and 13 postinfection. (A) Pretreatment with vorinostat increased the percentage of CD4$^+$ T cells infected by replication-competent HIV, as measured by EGFP accumulation using flow cytometry. (B) p24 produced from primary CD4$^+$ T cells, as measured by ELISA. Pretreatment with vorinostat dramatically increased the levels of p24 throughout multiple rounds of infection, as evidenced by a steady increase by 13 days postinfection. The data were normalized to ng p24 per 1×10^6 viable cells. The data represent means and standard errors of the mean.

cation. Together, these data demonstrate that, in addition to its effects promoting reverse transcription, nuclear import, and integration in a single-cycle assay, vorinostat also enhances productive infection and spread of replication-competent HIV.

Specific inhibition of HDAC6 recapitulates vorinostat-mediated enhancement of CD4$^+$ T cell susceptibility to HIV. In humans, HDACs are divided into four classes based upon their homology to yeast proteins (reviewed in reference 40). Class I HDACs, including HDAC1, -2, -3, and -8, are predominantly located in the nucleus and are the primary mediators of histone deacetylation. Class IIa HDACs shuttle between the nucleus and the cytoplasm of cells and include HDAC4, -5, -7, and -9. Class IIb HDACs are predominantly cytoplasmic and include HDAC6 and -10. Class III HDACs bear homology to the yeast Sir2 protein and include the NAD$^+$-dependent sirtuin family members SIRT1 to -7, while class IV HDACs consist of HDAC11, differentiated from other classes based upon conserved catalytic elements shared by both class I and class II HDACs. Vorinostat inhibits the activities of HDAC1 to -9, albeit with reduced potency against HDAC8. Our observation that vorinostat increased the kinetics of reverse transcription in CD4$^+$ T cells suggested that inhibition of cytoplasmic HDACs might contribute to increased susceptibility to HIV. Of these, the class IIb HDAC6 was of particular interest, because specific inhibitors have previously been reported to enhance HIV infection; however, these studies reported enhancement of viral fusion (41, 42), which we did not observe with vorinostat treatment.

HDAC6 has multiple cytoplasmic targets, including α-tubulin, the cortical actin binding protein cortactin, and heat shock protein 90 (40). To test whether inhibition of HDAC6 might contribute to the vorinostat-mediated enhancement of HIV infection, we pretreated unstimulated primary CD4$^+$ T cells with 2 μM the HDAC6-selective inhibitor tubacin (43) for 4 h prior to infection with HIV. In contrast to previously published results (41, 42), we did not observe any effect of HDAC6 inhibition on HIV fusion with target cells (Fig. 7A). The lack of an effect on fusion was observed with both R5- and X4-tropic HIV in the presence or absence of spinoculation and was consistent with our previous observations with vorinostat. Tubacin pretreatment increased EGFP$^+$ CD4$^+$ T cell frequency compared to untreated controls (R5-tropic HIV, 1.64- ± 0.35-fold increase, $P = 0.057$; X4-tropic HIV, 1.89- ± 0.41-fold increase, $P = 0.011$) (Fig. 7B), indicating enhanced HIV infection, as was seen with vorinostat. Together, our results indicate that inhibitors targeting cytoplasmic HDACs promote HIV infection by enhancing the efficiency of postentry events in the viral life cycle, a previously unknown mecha-

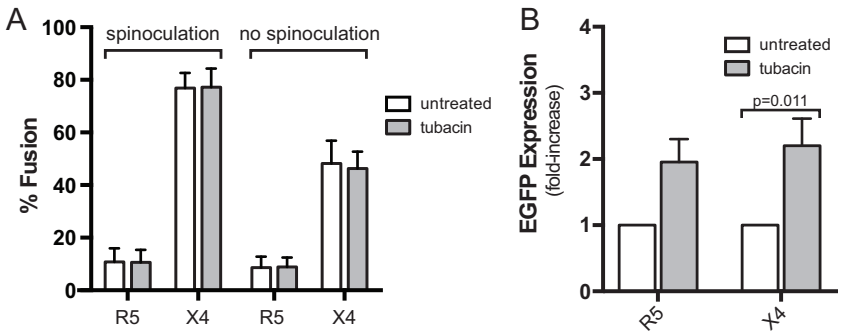

FIG 7 Specific inhibition of HDAC6 by tubacin recapitulates the effect of vorinostat on uninfected CD4$^+$ T cells. (A) Four-hour pretreatment of CD4$^+$ T cells with 2 μM tubacin did not affect fusion of R5- or X4-tropic combination reporter viruses in the presence or absence of spinoculation. Ten- to 15-fold-higher viral concentrations were used in the absence of spinoculation to compensate for lower fusion levels. (B) Pretreatment of CD4$^+$ T cells with 2 μM tubacin increased the percentage of CD4$^+$ T cells infected by HIV for R5- and X4-tropic viruses. The data represent means and standard errors of the mean.

nism distinct from reactivation of latent proviruses by histone modifications in the nucleus.

DISCUSSION

Latently infected resting CD4$^+$ T cells are a primary barrier to the eradication of HIV infection due to their long half-lives, persistence in the presence of antiretroviral therapy, and ability to renew the reservoir during periods of intermittent viremia or by homeostatic proliferation. Over the past decade, significant progress has been made in identifying compounds that are capable of reactivating latent HIV without inducing polyclonal T cell activation (26, 29, 44–47). The HDAC inhibitor vorinostat has shown efficacy both *in vitro* and *in vivo* in inducing HIV transcription in latently infected CD4$^+$ T cells (26, 30), which in combination with antiviral immune responses, antiretroviral drugs, or cytopathic effects may eradicate latent reservoirs. This strategy, known as shock and kill, is currently being investigated in HIV-infected patients. A major concern with the strategy is that uninfected CD4$^+$ T cells will become infected during periods of viral activation from latency.

In this study, we investigated the effect of vorinostat on uninfected cells using unstimulated and activated polarized primary CD4$^+$ T cells and Jurkat T cell lines. Vorinostat was found to promote HIV infection in a dose- and time-dependent manner that was independent of receptor and coreceptor usage. Vorinostat did not affect HIV fusion, but rather increased the kinetics of postentry events of the HIV life cycle, including reverse transcription and integration. This was coupled with enhanced efficiency of 2-LTR-circle formation, a measure of nuclear import. Furthermore, tubacin-mediated inhibition of HDAC6, a class IIb HDAC believed to be predominantly cytoplasmic, recapitulated the effects of vorinostat by increasing the vulnerability of CD4$^+$ T cells to infection by HIV.

Our findings raise concerns for the clinical use of vorinostat, as it could reseed the viral reservoirs intended to be purged. Although shock-and-kill strategies to eliminate latent HIV will be performed in the presence of cART, suboptimal viral inhibition could occur in the context of patient noncompliance, viral resistance, or sanctuary sites with poor drug penetration. While translating *in vitro* findings to *in vivo* effects is not straightforward, it is worth mentioning that the doses expected following a single 400-mg dose of vorinostat in patients (335 nM [26, 30]) are close to the level at which a significant enhancement of viral infection was observed in this study (500 nM). Importantly, *in vivo* administration of vorinostat did not increase residual viremia in patients on cART, raising doubts as to whether HDAC inhibitors alone will be sufficient to purge latent reservoirs. However, if vorinostat is eventually employed in therapeutic strategies to eliminate viral reservoirs, either alone or in combination with other latency-reversing agents, intensification of ART may help reduce the likelihood of reseeding viral reservoirs.

In addition to these important clinical implications, these results reveal a previously unknown mechanism of HDACs on HIV infection that is distinct from their ability to activate viral transcription in latently infected cells. Several lines of evidence support the conclusion that these effects of HDACs are separate. First, vorinostat significantly increased LTR-driven EGFP expression when administered 4 h prior to infection compared to 24 h after infection. This is unlikely to be a result of enhanced LTR transcription, since raltegravir time-of-addition studies revealed that >95% of the virus had not integrated at either time point. Second, the kinetics of reverse transcription—thought to be primarily a cytoplasmic process—were enhanced by vorinostat. Third, nuclear import of viral DNA, as estimated by 2-LTR-circle formation, and the kinetics of integration itself were also enhanced. Both of these processes lie upstream of LTR transcription in the viral life cycle. Fourth, tubacin, a specific inhibitor of the cytoplasmic class IIb HDAC6, promoted HIV infection of CD4$^+$ T cells at 2 μM, whereas it has no effect on histone acetylation at doses up to 20 μM (43, 48). Finally, previous studies with the HDAC inhibitor valproic acid (VPA) did not demonstrate increased HIV infection of uninfected cells despite pronounced effects on reactivation of latent HIV (29). Intriguingly, VPA is a potent inhibitor of nuclear class I HDACs but has minimal effects upon class IIb HDACs, including HDAC6 (49, 50). Together, these data strongly suggest that vorinostat promotes *de novo* infection of uninfected CD4$^+$ T cells through a novel mechanism distinct from nuclear histone acetylation. From a drug development standpoint, these findings also imply that an HDAC inhibitor that selectively targets nuclear class I HDACs could reactivate latent HIV without increasing the vulnerability of cells to infection.

The observation that tubacin can recapitulate the vorinostat-mediated enhancement of HIV infection strongly implicates HDAC6 as a cellular factor inhibiting viral infection. Previous studies have found that inhibition of HDAC6 enhances HIV infection by increasing fusion in cell-cell and virus-cell fusion models (41, 42), results that we were unable to replicate here. Differences in the viral or cell models used may account for these differences. Multiple cellular proteins are deacetylated by HDAC6, including α-tubulin, cortactin, and heat shock protein 90. α-Tubulin acetylated at the ε-amino group of Lys40 is preferentially found in stable microtubules, which are both essential components of the cytoskeletal architecture and conduits for trafficking of large macromolecules through the cell via the microtubule motors dynein and kinesin. HIV has been reported to hijack the microtubule network through interactions between dynein and the viral capsid protein p24, facilitating virus-trafficking movement to the perinuclear region or microtubule-organizing center (MTOC) (51). Decreased microtubule stability, such as that induced by overexpression of the ezrin-radixin-moeisin (ERM) family members ezrin and moeisin, is associated with reduced infection of cells by murine leukemia viruses and HIV at a step prior to reverse transcription (52, 53). Conversely, inhibition of HDAC6-mediated α-tubulin deacetylation by vorinostat or tubacin may favor stable microtubule tracts, facilitating HIV reverse transcription and trafficking of HIV to the nucleus (51) and promoting HIV infection. Finally, a recent paper by Sabo and colleagues has demonstrated that HIV promotes the formation of acetylated and detyrosinated stable microtubule tracts during early infection (54). Blocking the formation of these tracts using an end binding protein (EB1) dominant-negative carboxy-terminal fragment also reduced HIV infectivity. It is tempting to speculate that inhibition of cytoplasmic HDAC6 further stabilizes these stable microtubule networks and facilitates HIV reverse transcription and transport to the nucleus, enhancing viral infection. A better understanding of the role of HDACs in regulating cytoplasmic proteins and cytoskeletal architecture will provide insight into critical early events in the viral life cycle and provide strategies for the development of agents that specifically activate

HIV transcription without increasing susceptibility of cells to *de novo* HIV infection.

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We declare no competing financial interests.

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