The histone deacetylase inhibitor vorinostat (SAHA) increases the susceptibility of uninfected CD4+ T cells to HIV by increasing the kinetics and efficiency of post-entry viral events.

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Running Title: Vorinostat increases the susceptibility of uninfected CD4+ T cells to HIV
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 would be given in the context of antiretroviral therapy, 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 post-entry 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 histone deacetylase (HDAC)-6 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 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 a 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 during 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 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 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.
Introduction

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 (11) or possibly during intermittent viremia (10), although this 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, gastrointestinal tissues or mucosal target cell populations from this patient (15). However, two factors make replicating the success of the Berlin patient difficult. First, ablative chemo- and radiation therapy in conjunction with 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 more rare 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 non-infected 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 post-translational modifications, 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-kB 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, 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 effects 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 post-entry events including reverse transcription and integration. The enhanced kinetics of infection correlated with increased efficiency of viral events leading to higher levels 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.
Methods

Production of viruses. Combination reporter viruses were produced as previously detailed (31). Briefly, 293 T cells were transfectected with 10 μg pNL4-3-deltaE-EGFP (obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pNL4-3-delta-E-EGFP (Cat #11100) from Drs. Haili Zhang, Yan Zhou, and Robert Siliciano), 7.5 μg bla-Vpr plasmid, and 6.0 ug of HIV Envs REJO.D12.1972(32) (CCR5-tropic) or JOTO.TA1.2247(33) (CXCR4-tropic) using calcium phosphate methods. Virus was harvested 72 hours after transfection, filtered, and concentrated by ultracentrifugation through a 20% sucrose cushion according to published protocols (34). Viral concentrations were determined by p24 ELISA (Cell Biolabs) and empirical virus titers determined on purified CD4+ T cells to ensure the dose used was within the linear range of the assay VSV-G viruses were produced in a similar way. Briefly, 293 T cells were transfectected with 10μg pNL4-3-d2EGFP-Nef, 6.5μg pCMVdelta8.91, and 3.5μg pMDG. Virus was harvested 72 hours after transfection, filtered, and concentrated by ultracentrifugation at 28K for 1.5 hours. Virus was titered by qPCR (Lentivirus qPCR Titer Kit, abm) and by GFP expression in Jurkat cells.

Cells. CD4+ T cells were obtained by leukopheresis from ALLCELLS, LLC. All subjects were healthy control donors negative for HBV, 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. Cells were cryopreserved and treated with benzonase prior to infection. For experiments using polarized primary CD4+ T cells, naïve 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 cytokines IL-6, IL-23, IL-1β, IL-2, TGF-β, anti-IL-4.
antibody, and anti-IFN-γ antibody for 6 days. Beads were removed using Dynal magnet and cells were resuspended in RPMI with 10% 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 CD4+ T cells were infected in parallel with 125 ng p24 equivalent of viruses bearing R5 tropic (REJO.D12.1972) or X4-tropic HIV Envs. Unless stated otherwise, cells were incubated for 4h with 2 μM vorinostat or 2 μM tubacin prior to addition of virus. Cells were spinoculated with virus at 1200g at 24°C for two hours unless otherwise stated and incubated at 37°C for 1 hour. The first plate of the parallel infections–measuring fusion–was then washed with CO2-independent media (Gibco), resuspended in media containing CCF2-AM (Invitrogen) for 1 hour at room temperature, washed, and incubated overnight at room temperature in CO2-independent media containing probenicid. 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 hours 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 viruses at an MOI of 1. Cells were incubated for 4h with 1 μM vorinostat prior to addition of virus. Cells were spinoculated with virus at room temperature for 1.5 hours. At 72 hours after infection, cells were harvested, washed, and flow cytometry analysis was performed. 

**Viral kinetics experiments.** Experiments were performed on 10^6 cells spinoculated as described above. Cells were incubated 4 hours prior to infection with 2 μM vorinostat, spinoculated with virus, and AMD3100 (20 μM), enfuvirtide/T20 (20 μM), raltegravir (10 μM), and efavirenz (100 nM) added at varying time points before or after spinoculation. Inhibition kinetics graphs
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 APC (eBioscience), CD45RO ECD (Beckman Coulter), CD27 PE-Cy7 (eBioscience), and anti-IgM PE (Invitrogen) for 4°C at 30 min. All 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 indicated time points, washed with PBS, and DNA 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’- CAAGCAGAAGACGGCATACCAGATAACTAGGGAA CCCACTGC-3’; 2-LTR circle reverse: 5’- CCTCTCTATGGGCAGTCGGTGATTC CACAGATCAAGGATATCTTGTC - 3’ (Integrated DNA Technologies). Purified DNA was amplified in a qPCR machine using 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 barcoded products were pooled, column purified, and run on a gel to select amplicons and remove residual primer. Fragments were gel purified and DNA concentration determined on a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific). 2-LTR products were
sequenced using Ion Torrent PGM NGS following manufacturers protocol. Transcript abundances were determined using Genomics Workbench 5 (CLC bio). Number of reads between experiments were normalized to sequencing chip size.

Replication competent experiments. 10x10^6 primary CD4+ T cells were treated with 100 IU/mL rIL-2 and infected with 2700 ng p24 equivalent of replication competent virus bearing X4 tropic envelope (HIV-NLG-Nef). Cells were incubated for 4h with 2 uM vorinostat prior to addition of virus and spinoculated as described above. Following incubation at 37°C for one hour, cells were supplemented with additional media to a final concentration of 1x10^6 cells/mL. At indicated time points, supernatant was preserved at -80°C and cells were washed and stained with anti-human CD3 BV650 (Biolegend), CD4 APC (eBioscience), and live/dead near-IR viability dye (Invitrogen). All 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. ELISA experiments performed in duplicate using two independent supernatant collections.

Statistics. Data in figures represent mean values and standard error of the mean unless stated otherwise. All differences with a p value of <0.05 were considered statistically significant, correcting for multiple comparisons when appropriate. Statistical analyses were performed using the paired T-test using GraphPad Prism v5.0d unless otherwise specified.
Results

Virus and cell models. The histone deacetylase (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 \textit{ex vivo} (26). Although vorinostat will be administered in the presence of cART 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 (Figure 1A). Fusion of viral and cellular membranes is identified by virion-associated \(\beta\)-lactamase–Vpr (bla-Vpr)–mediated cleavage of the \(\beta\)-lactamase substrate CCF2 (35), which alters the fluorescence characteristics of the cell (Figure 1B, top). In cells successfully completing post-entry events including uncoating, reverse transcription, integration, and LTR-driven gene expression, EGFP protein accumulates and can be detected. Spontaneous EGFP expression requires sufficient Tat and Rev levels to promote LTR transcription and export of incompletely and unspliced mRNAs, respectively. Since the NL4-3 core used to produce the combination reporter viruses contains an intact \textit{nef} gene, CD4 downregulation is also observed in EGFP+ cells (Figure 1B, bottom). By incorporating conjugated antibodies directed against CCR7 and CD45RO, naïve and memory subsets of primary CD4+ T cells undergoing fusion or LTR-driven EGFP expression can be precisely determined (Figure 1C). Using this combination reporter virus system and traditional GFP reporter viruses, we examined the effects of vorinostat on three different cell models: (1) unstimulated primary CD4+ T cells, (2) polarized, activated CD4+ T cells, and (3) Jurkat cells. Unstimulated primary CD4+ T cells are predominantly resting cells, typically with greater than 96% lacking expression of CD25 or CD69.
The histone deacetylase (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 hours prior to infection significantly increased EGFP+ cell frequencies compared to untreated controls at doses as low as 500 nM (Figure 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 (Figure 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 hour pretreatment with vorinostat significantly increased the percentage of EGFP+ activated primary cells and Jurkat cells compared to untreated controls (p=0.003 and p=0.03, respectively, Figure 2C).

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; Figure 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, pre-treatment with 2 μM vorinostat significantly enhanced LTR-driven EGFP expression compared to treatment 24 or 48 hours after infection (p=0.02 and p=0.006, respectively), conditions typically used to activate silent proviruses (Figure 2E). Raltegravir reduces EGFP+ cell frequencies by >95% when added as late as 30 hours post-infection; therefore, the observation that a 4 hour pre-incubation with vorinostat was significantly more effective at increasing EGFP+ cell frequency compared to addition 24 hours 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 infection, we measured its effect on fusion using combination reporter viruses pseudotyped with either R5- and X4-tropic Envs. 4h pre-incubation with vorinostat had no effect on fusion (Figure 3A). However, our infection protocol involved a 2h 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 effects of vorinostat on fusion and therefore repeated the experiment in the absence of spinoculation. A 10-15-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 (Figure 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 naïve 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 (Figure 3C). Interestingly, the effect of vorinostat appeared most pronounced in CCR7+CD45RO- naïve (TN) and CCR7+CD45RO+ central memory (T<sub>CM</sub>) subsets that are less activated than the CCR7-CD45RO- effector memory (T<sub>EM</sub>) and CCR7-CD45RO+ terminal effector (T<sub>TE</sub>) subsets. Together, these data suggest that vorinostat increases productive infection by modifying the efficiency of post-entry steps of the viral life cycle and may have the greatest effects on unstimulated naïve 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 post-entry events in the viral life cycle.**

To further investigate the post-entry events influenced by vorinostat, we performed experiments where the CXCR4 antagonist AMD3100, fusion inhibitor T20, reverse transcriptase inhibitor efavirenz, and integrase inhibitor raltegravir were added to unstimulated primary CD4+ cells prior to or at varying time points following infection with HIV, either in the absence or presence of vorinostat. As expected, the kinetics of HIV binding to CXCR4 or fusion of the viral and host cell membranes were unaffected by the presence of vorinostat (Figure 4A and 4B, respectively). In contrast, cells pre-treated with vorinostat became insensitive to efavirenz more rapidly than control cells (t<sub>1/2</sub> = 35.4 h vs. t<sub>1/2</sub> = 41.8 h), indicating that the kinetics of reverse transcription were accelerated in the presence of vorinostat (Figure 4C). Similarly, cells became refractory to...
raltegravir more rapidly when pretreated with vorinostat (t1/2 = 35.9 h vs. t1/2 = 47.2 h) reflecting enhanced integration kinetics (Figure 4D).

Since the kinetics of viral post-entry 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 post-entry 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 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 (Figure 5). Treatment with vorinostat resulted in a ~3-fold increase in the number of sequence reads by 72 hours. In both cell types, the accumulation of 2-LTR circles was not observed until at least 24 hours post infection, consistent with the kinetics data regarding the timing of reverse 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 post-entry 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 (Figure 6). Briefly, 10x10^6 primary CD4+ T cells were stimulated with 100 IU/mL IL-2 and infected with a replication competent HIV reporter virus (HIV-NLG-Nef), spinoculated, and incubated at 37°C
for up to 13 days. On d1, d3, d5, d7, d9, d11, and d13, cells were removed to determine EGFP
expression and supernatant harvested and stored at -80° for p24 ELISA. As expected, treatment
with vorinostat significantly increased EGFP expression of replication-competent HIV beyond a
single round of infection, evident by a steady rise in EGFP+ cells from days 7-13 when
compared to untreated controls (Figure 6a). This finding was further supported by viral p24
protein concentration in cell supernatants. Treatment with vorinostat dramatically increased the
level of p24 produced by day 13, indicating that vorinostat is acting to both augment LTR driven
EGFP expression and amplify virion release throughout multiple rounds of HIV replication.
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 (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
NAD+ -dependent sirtuin family members SIRT1-7, 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 activity of HDAC1-9, 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 of the HDAC6-selective inhibitor tubacin (43) for 4h 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 (Figure 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, Figure 7B), indicating enhanced HIV infection as was seen with vorinostat. Together, our results indicate that inhibitors targeting cytoplasmic HDACs promote HIV infection by enhancing efficiency of post-entry events in the viral life cycle, a previously unknown mechanism 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-life, 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 this 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 post-entry 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 non-compliance, 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 where 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 4h prior to infection compared to 24h after infection. This is unlikely to be a result of enhanced LTR transcription since raltegravir time-of-addition studies revealed that >95% of 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 effects 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 viral 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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Competing Financial Interests

The authors declare no competing financial interests.
Figure 1. A. Combination reporter viruses are produced by cotransfection of 293T cells with plasmids encoding an egfp-containing HIV core lacking an envelope gene (pNL4-3-ΔE-EGFP), β-lactamase–vpr, and an HIV env with known coreceptor tropism. Virions package 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. C. In combination with the memory markers CCR7 and CD45RO, naïve (TN), central memory (TCM), effector memory (TEM), and terminal effector (TTE) subsets can be identified. Cells undergoing fusion or LTR-driven EGFP expression are shown in blue.

Figure 2. Vorinostat enhances HIV infection of CD4+ T cells to HIV in a dose- and time-dependent manner. A. 4h 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. 4h pretreatment of unstimulated CD4+ T cells with 200 μM 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 4h 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 4h
pretreatment with vorinostat compared to addition 24 or 48h after infection. *p<0.05, **p<0.01.

Exact p-values are provided in the text.

**Figure 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, spinoculated at 1200g for 2 hours, and fusion levels determined by bla-Vpr–mediated CCF2 cleavage. 4h 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. 10-15–fold higher concentrations of HIV were used to compensate for the reduction in fusion in the absence of spinoculation. **C.** Pretreatment with vorinostat did not affect the percentages of CD4+ T cell 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 is shown. Numbers reflect the percentage of cells undergoing fusion or LTR-driven EGFP expression in each CD4+ T cell subset.

**Figure 4.** Vorinostat enhances the kinetics of post-entry viral events including reverse transcription and integration. Uninfected CD4+ T cells were infected with X4-tropic reporter viruses in the presence or absence of 4h pretreatment with vorinostat. Prior to infection or at varying time points after infections, the (A) coreceptor antagonist AMD3100, (B) fusion inhibitor T20, (C) reverse transcriptase inhibitor efavirenz, or (D) integrase inhibitor raltegravir were added. Infection values were normalized to uninfected controls. Graphs represent averaged levels among three replicates. Error bars are excluded to prevent figure congestion.
Figure 5. The efficiency of post-entry viral events including reverse transcription and nuclear import is improved by vorinostat. (A) Primary unstimulated and (B) polarized, activated CD4+ T cells were infected with X4-tropic reporter viruses and spinoculated. At various time points following infection, the cells were harvested and DNA extracted for qPCR of 2-LTR circles followed by deep sequencing to confirm specificity. Product abundances were determined using Genomics Workbench 5. Times are as indicated in hours. Data normalized by input DNA and sequencing chip size.

Figure 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 post-infection. (A) Pretreatment with vorinostat increased the percentage of CD4+ T cells infected by replication-competent HIV, 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, evident by a steady increase by 13 days post-infection. Data normalized to ng p24 per 1x10^6 viable cells.

Figure 7. Specific inhibition of HDAC6 by tubacin recapitulates the effect of vorinostat on uninfected CD4+ T cells. (A) 4h 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. 10-15–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.
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HIV-FusionCombination Reporter Virus System

Uncleaved CCF2 (520 nm)
Cleaved CCF2 (447 nm)

HIV+
Productive Infection
EGFP
CD4

A
Combination Reporter Virus System

B

CD4
0.007
形态
Productive Infection

Uncleaved CCF2 (520 nm)

C

CCR7
0.04
形态
Productive Infection

CD45RO

CD45RO

5.38
0.001

66.2
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**Graph:**

- **A**: Comparison of % Fusion with and without vorinostat, showing an increase in fusion with vorinostat.
- **B**: Comparison of % Fusion with and without spinoculation, showing a decrease in fusion with spinoculation.
- **C**: Productive Infection and Fusion plots showing the effect of vorinostat and spinoculation on the expression of CD45RO and CCR7.
Figure 1: Time of drug addition (minutes post infection) vs. % inhibition for AMD3100, Enfuvirtide, Efavirenz, and Raltegravir.

A. AMD3100
B. Enfuvirtide
C. Efavirenz
D. Raltegravir

Each graph shows the % inhibition over time for untreated and vorinostat-treated samples.
A
Unstimulated CD4s

B
Activated CD4s

# Reads

0 20 40 60 80

0
20000
40000
60000
80000

Time (hours)

0 20 40 60 80

0
20000
40000
60000
80000

Time (hours)

Untreated
Vorinostat

on June 29, 2017 by guest http://jvi.asm.org/ Downloaded from
A. % Fusion

B. EGFP Expression (fold-increase)

Spinoculation vs. no spinoculation:
- Untreated samples
- Tubacin-treated samples

p = 0.011