Histone Deactylase Inhibitor SAHA Induces a Synergistic HIV-1 Reactivation by 12-O-Tetradecanoylphorbol-13-Acetate in Latently Infected Cells

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Key Words
HIV latency · Reactivation · Histone deacetylase inhibitor · Histone methyltransferase inhibitor · DNA methyltransferase inhibitor · 12-O-tetradecanoylphorbol-13-acetate

Abstract
Objectives: Recent studies have reported that human immunodeficiency virus type 1 (HIV-1) proviruses are strongly suppressed in the unique epigenetic environments caused by chromatin modifications such as acetylation and methylation. Therefore, optimized therapeutic strategies directed against the virus reservoir using these epigenetic modifying agents (EMAs) should cure HIV infection. Methods: Cytotoxicity and HIV-1 reactivation were determined using the PrestoBlue™ Cell Viability Reagent and p24 HIV ELISA, respectively. Results: EMAs, including histone deacetylase inhibitors (VPA and SAHA), DNA methyltransferase inhibitor (S′-Aza-CdR), histone methyltransferase inhibitor (ADOX) and 12-O-tetradecanoylphorbol-13-acetate (TPA), were used to reactivate proviruses in HIV-1 latently infected cells. The effect of monotreatment with these EMAs on HIV-1 reactivation was VPA or SAHA > S′-Aza-CdR > ADOX. Even though cotreatment with these potential HIV-1 reactivating agents did not show any significant reactivation effects in HIV-1 latently infected cells, employing SAHA under TPA treatment demonstrated a dramatic synergistic effect on purging HIV-1 proviruses in all HIV-1 latently infected cells via the ERK and AP-1 pathways. Conclusions: These results suggest that the combined approaches of EMAs, cotreatment of SAHA and TPA, could provide an effective way to lead a decline of HIV-1 reservoirs in patients.
cells which were derived from A3.01 cells. ACH2 cells were constructed for studying the effects of various physiological stimuli on HIV expression in infected T cells [3]. The effects of downregulation of CD4 receptor signaling molecules and histone modifications on HIV-1 latency were investigated using NCHA cells [4, 5]. The eukaryotic genome is organized in a highly complex nucleoprotein structure, chromatin, which can be modified by DNA methylation, covalent histone modification, and nucleosome remodeling. Histone proteins of the nucleosome core can be changed by such modifications including methylation, acetylation, ubiquitylation, sumoylation, and phosphorylation on specific residues [6]. Van Lint et al. [7] reported that the treatment of HIV-1 latently infected cells with histone deacetylase inhibitors (HDACis) induced viral transcription and the remodeling of the repressive nucleosome, nuc-1. Also, DNA methylation of the viral genome is known to play a major role in virus-associated human cancers and virus silencing [8]. It has been suggested that chromatin silencing is closely related with HIV-1 latency and that the chromatin modification can be a new target of novel drug development for HIV-1 therapy [9, 10]. Two categories of drugs that affect histone acetylation and histone or DNA methylation are currently considered as epigenetic therapies for HIV-1 infection. DNA methyltransferase inhibitors (DNMTis) and HDACis are already used in clinical practice for myelodysplastic syndrome and cutaneous T-cell lymphoma [11, 12]. In particular, DNMTis can act synergistically with prostratin or tumor necrosis factor-α to reactivate HIV-1 proviruses [13]. Based on these findings, we tried to define the combination effects of epigenetic modifying agents (EMAs) in order to find the optimal condition for reactivation of HIV-1 proviruses.

In this study, we investigated the effects of EMAs on HIV-1 reactivation in HIV-1 latently infected cells such as ACH2, J1.1, and NCHA cells. Reference cell lines, A3.01, ACH2 [14, and J1.1 cells [3, were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. NCHA cells were established in our laboratory [2]. The HIV-1 reactivating capacities and cytotoxicities of HDACis, DNMTis, and HMTis were examined in HIV-1 latently infected cells. The cytotoxicity and proviral HIV reactivation assays were performed by PrestoBlue Cell Viability Reagent and HIV-1 p24 ELISA kit, respectively. The optimal concentration of these agents was determined (table 1) as the dose with no cytotoxic effect (cell viability >80%) (fig. 1a) and a high reactivation effect (high HIV-1 p24 levels). As previous studies have shown that the long terminal repeats (LTRs) of HIV-1 proviruses in the latent state accumulate HDACs at a high level [15], we first validated the effect of HDACi on HIV-1 reactivation in HIV-1 latently infected cells. In the monotherapy with EMAs, SAHA showed remarkable HIV-1 reactivating ability in all HIV-1 latently infected cells. Even though VPA induced a high level of HIV-1 p24 antigen, it required a higher concentration than SAHA for effective HIV-1 reactivation. Other studies have reported that VPA has unacceptable adverse effects when given as a long-term oral treatment [16]. It means that the clinical utility of high doses of VPA must be considered with caution. Thus, VPA was excluded from the combined assays of EMAs. Next, we determined the effect of histone methylation on HIV-1 reactivation in HIV-1 latently infected cells. Cells were treated with 5′-Aza-CdR or histone methyltransferase inhibitor (ADOX). As a result, 5′-Aza-CdR could reactivate slightly HIV-1 proviruses in all HIV-1 latently infected cells, but ADOX only in ACH2 cells (fig. 1b). To investigate how the combinations of SAHA, 5′-Aza-CdR, and ADOX affect HIV-1 reactivation in HIV latently infected cells, the combinations of SAHA, 5′-Aza-CdR, and ADOX were performed with optimal concentrations without cytotoxicity. Cotreatment with these EMAs did not appear to have significant impacts on reactivation of HIV-1 proviruses (fig. 1b). In addition, cotreatment of SAHA and 5′-Aza-CdR or SAHA and ADOX showed no synergistic reactivating effects on HIV-1 latently infected cells. In some combinations, EMAs exhibited the different patterns as if cotreatment of SAHA, 5′-Aza-CdR and ADOX affect HIV-1 reactivation in both ACH2 and J1.1 cells but not NCHA cells. ACH2 and J1.1 cells have a single copy of HIV-1 proviruses, whereas NCHA cells harbor two copies of HIV-1 proviruses. Some studies have reported that methylation inhibitors have complicated effects since the activation of some

### Table 1. Optimal concentrations of EMAs

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>ACH2, mM</th>
<th>NCHA, mM</th>
<th>J1.1, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDACs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VPA</td>
<td>2.5</td>
<td>2.5</td>
<td>1</td>
</tr>
<tr>
<td>SAHA</td>
<td>0.1</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>DNMT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5′-Aza-CdR</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>HMT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADOX</td>
<td>5</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

n.s. = No significance.
genes may inhibit others [17, 18]. Therefore, the different effects of these EMAs on HIV-1 reactivation may be explained by the various methylation effects and the unique chromatin structure of the LTR on HIV-1 proviruses in latently infected cell lines with different HIV-1 integration sites [10]. Reuse et al. [19] have also reported that no synergistic reactivation of latent virus was observed when 5′-Aza-CdR was combined with VPA. As 12-O-tetradecanoylphorbol-13-acetate (TPA), a global T-cell activator, can powerfully express HIV-1 proviruses [2], we assessed the combined effect of TPA and EMAs. The combined use of SAHA and TPA showed a more significant synergistic effect in HIV-1 reactivation than TPA alone in all HIV-1 latently infected cells (fig. 1c). When HIV-1 latently infected cells were cotreated with SAHA and TPA, HIV-1 reactivating effects were 1.5-fold higher in ACH2 cells, 2.7-fold higher in NCHA cells, and 2.1-fold higher in J1.1 cells compared to those treated with TPA alone. Generally, HIV-1 proviral DNA is synthesized and incorporated into nucleosomes after HIV-1 infection, and the transcriptional activity of the provirus is under the control of the regional nucleosomal structure [10, 20]. HIV-1 transcription is driven by promoter activity in the 5′-LTR of the integrated provirus and regulated by cellular factors such as NF-κB, CREB, Sp1 and AP-1 [10]. But, DNA methylation can regulate HIV-1 transcription by impairing the binding of transcription factors [21]. A previous study reported that the presence of nuc-1 is the primary cause for the suppression of transcription at the HIV promoter in basal conditions and TPA can disrupt nuc-1 rapidly to reactivate HIV-1 proviruses [22]. One possible explanation for the synergistic effect in cotreatment of SAHA and TPA may be that a TPA-induced release of condensed chromatin structure allows SAHA to access LTR site of the integrated HIV-1 proviruses easily, which results in increasing reactivation of HIV-1 proviruses. To examine whether MAPK or PI3 kinase pathway is involved in HIV-1 reactivation pathway, we pretreated HIV-1 latently infected cells with ERK inhibitor (PD98059), p38 MAPK inhibitor (SB203580), and P13 kinase inhibitor (LY294002) for 2 h and then treated with SAHA and TPA (fig. 2a). Though ERK and p38 MAPK signaling molecules can modulate T-cell

Fig. 1. Effects of EMAs on HIV-1 reactivation. a The 5×10^4 cells/wells were treated with the inhibitors for 48 h. Cell viability was assessed in all HIV-1 latently infected cells with various EMAs, VPA, SAHA, 5′-Aza-CdR, and ADOX.
Fig. 1. Effects of EMAs on HIV-1 reactivation. **b** HIV-1 latently infected cells were treated for 48 h with SAHA, 5′-Aza-CdR, and ADOX. **c** HIV-1 latently infected cells were treated for 48 h with TPA, SAHA, 5′-Aza-CdR, and ADOX. The concentration of SAHA was used differently depending on cell lines (ACH2 cells, 0.1 μM; NCHA and J1.1 cells, 0.5 μM). A statistical analysis was performed with Student’s t test (# p < 0.05 and ## p < 0.005 vs. control; * p < 0.05 vs. TPA treatment). All experiments were performed in triplicate and error bars indicate the standard deviation.
Fig. 2. Induction of HIV-1 reactivation by ERK and AP-1 pathways. 

a HIV-1 latently infected cells were pretreated for 2 h with the indicated inhibitors, LY294002 (PI3 kinase inhibitor), PD98059 (ERK inhibitor), and SB203580 (p38 MAPK inhibitor), followed by treatment with TPA and SAHA for 48 h. 

b HIV-1 latently infected cells were treated with TPA. The expression levels of ERK were determined by Western blot assay. 

c HIV-1 latently infected cells were pretreated with ERK inhibitor for 2 h, followed by treatment with TPA. The expression levels of c-Jun were also determined by Western blot assay. A statistical analysis was performed with Student’s t test (* p < 0.05 and ** p < 0.005 vs. control; * p < 0.05 and ** p < 0.005 vs. TPA and SAHA treatments, respectively).
function [14], these molecules have different effects on T-cell development, cell cycle progression, and apoptosis [23–25]. Also, it is significant that the MAPK signaling pathway is known to interact with HIV-1 viral proteins [26]. As shown in figure 2a, cotreatment of SAHA and TPA could induce HIV-1 reactivation via not the p38 MAPK pathway but the ERK pathway in HIV-1 latently infected cells (fig. 2a). However, LY294002 caused the decrease of HIV-1 p24 antigens induced by cotreatment of SAHA and TPA in NCHA and J1.1 cells except ACH2 cells. Finally, we investigated the relationship between the ERK signaling pathway and HIV-1 reactivation. In treatment of PD98059, HIV-1 p24 antigens were decreased to 20–50% in all HIV-1 latently infected cells compared to control. We confirmed that c-Jun, one of components of AP-1, and ERK were phosphorylated after TPA treatment (fig. 2b, c) and PD98059 reduced the phosphorylation levels of c-Jun in HIV-1 latently infected cells (fig. 2c), while cotreatment of SAHA and TPA did not show a synergistic ERK and c-Jun phosphorylation and SAHA failed to show an effect on the ERK pathway in HIV-1 latently infected cells (data not shown). Contreras et al. [27] reported that SAHA reactivates HIV-1 via the PI3 kinase/AKT pathway, not the ERK1/2 pathway in the primary memory T-cell subpopulation. These results suggest that TPA can induce HIV-1 reactivation by activating AP-1 via the ERK signaling pathway.

Our findings suggested that the combined approaches of EMAs, cotreatment of SAHA and TPA, may be a more effective way to reactivate HIV-1 proviruses in HIV-1 latently infected cells. In these strategies, a ‘shock’ phase is used to reactivate latent proviruses, while a ‘kill’ phase is used to eliminate the induced cells through immune responses. For example, valproic acid was tested in clinical trial in combination with the fusion inhibitor enfuvirtide [28]. Although multiple agents are reported to activate HIV-1 latently infected cells in vitro, novel therapeutic approaches are required for targeting HIV-1 reservoirs and development of relevant in vivo animal models for evaluating the safety and effectiveness of these compounds before clinical application.

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References


