A Novel Histone Deacetylase Inhibitor, AR-42, Reactivates HIV-1 from Chronically and Latently Infected CD4+ T-cells

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ABSTRACT: Human immunodeficiency virus type 1 (HIV-1) latency is a major barrier to a cure of AIDS. Latently infected cells harbor an integrated HIV-1 genome but are not actively producing HIV-1. Histone deacetylase (HDAC) inhibitors, such as vorinostat (SAHA), have been shown to reactivate latent HIV-1. AR-42, a modified HDAC inhibitor, has demonstrated efficacy against malignant melanoma, meningioma, and acute myeloid leukemia and is currently used in clinical trials for non-Hodgkin's lymphoma and multiple myeloma. In this study, we evaluated the ability of AR-42 to reactivate HIV-1 in the two established CD4+ T-cell line models of HIV-1 latency. In HIV-1 chronically infected ACH-2 cells, AR-42-induced histone acetylation was more potent and robust than that of vorinostat. Although AR-42 and vorinostat were equipotent in their ability to reactivate HIV-1, AR-42-induced maximal HIV-1 reactivation was twofold greater than vorinostat in ACH-2 and J-Lat (clone 9.2) cells. These data provide rationale for assessing the efficacy of AR-42-mediated HIV-1 reactivation within primary CD4+ T-cells.

KEYWORDS: HIV-1, histone deacetylase, HIV reactivation, kick and kill, AR-42

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During primary infection, human immunodeficiency virus type 1 (HIV-1) infects permissive cells and converts its single-stranded RNA genome into a double-stranded DNA genome that integrates into the host-cell genome.[1] A subset of the cells harboring integrated HIV-1, termed the latent reservoir, does not actively produce HIV-1 progeny and is thus refractory-to-current antiviral therapy.[2,3] The posttranslational modifications of chromatin, such as histone deacetylation, cause chromatin condensation, which restricts RNA polymerase-mediated HIV-1 transcription and results in viral latency (reviewed in Siliciano and Greene).[4] Previous reports have demonstrated the ability of histone deacetylase (HDAC) inhibitors, including vorinostat (also known as SAHA) and valproic acid, to reactivate latent HIV-1 through the reversal of chromatin condensation, although there have been inconsistent reports on the effectiveness of valproic acid.[5,6] Clinical studies of vorinostat investigating the kick and kill strategy indicate consistent HIV-1 reactivation from cell lines and HIV-infected patients, but at high dosages.[7,8] Additionally, recent studies with panobinostat and romidepsin in patients on suppressive antiretroviral therapy indicate the potential utility of more potent HDAC inhibitors.[5]

The histone deacetylation activity within chromatin indicates HDAC inhibitors as potentially valuable therapeutic agents for HIV-1 reactivation.[9–11] Currently, the most potent HDAC inhibitors belong to the hydroxamic acid family.[12] This class of HDAC inhibitors includes the US Food and Drug Administration-approved vorinostat and a novel compound AR-42.[5,10] AR-42 is a novel anticancer drug candidate that inhibits deacetylation on both histone and nonhistone proteins.[13,14] AR-42, a modified hydroxamic acid, was rationally designed with an aromatic linker and two Zn^{2+}-binding motifs that bind a zinc cation in the catalytic domain of class I and II HDACs with an IC_{50} value of 30 nM.[15]

Published data indicated that AR-42 induces histone H3 acetylation in mouse and canine mast cells.[16] To determine if AR-42 induces acetylation in cells harboring a HIV-1 provirus, we treated chronically and latently infected ACH-2 cells[17] (obtained from Dr. Thomas Folks through the NIH AIDS Research and Reference Reagent Program) with a range of AR-42 (1 nM–5 μM). Following the treatment, cell lysates (15 μg) were electrophoresed on a 10% SDS-PAGE gel and transferred to nitrocellulose. Histone H3 acetylation on lysine 9 was assayed by western blot with the AcH3K9 antibody (Santa Cruz Biotechnology, Inc., 1:1500 dilution) and goat–anti-rabbit immunoglobulin/horseradish peroxidase secondary antibody (cell signaling, 1:5000 dilution). Equivalent protein loading was verified by western blot against actin (cell signaling 4967, 1:1500). Histone acetylation was quantified as a ratio to actin loading control by ImageJ densitometry analysis.

At 10 nM, AR-42 treatment increased histone 3 acetylation, while vorinostat induced acetylation at ~100 nM (Fig. 1). Within the concentrations tested, AR-42-induced histone 3 acetylation was more robust than vorinostat-induced acetylation. As expected, phorbol 12-myristate 13-acetate (PMA)-mediated HIV reactivation did not increase histone 3-acetylation.

An outcome of histone acetylation in latently and chronically infected $CD4^+$ T-cells is the reactivation of HIV-1. Expanding on AR-42's ability to acetylate histone 3 (Fig. 1), we determined AR-42-induced HIV-1 reactivation within two well-established $CD4^+$ T-cell models of HIV-1 latency.[17,18] ACH-2 cells were maintained in Roswell Park Memorial Institute medium with 10% fetal bovine serum and penicillin–streptomycin at 37°C under 5% CO_2. ACH-2 cells were treated with the indicated concentrations of vorinostat or AR-42 for 48 hours, in triplicate, at a final dimethyl sulfoxide (DMSO) concentration of 0.1%. A total of 100 ng/mL PMA (Sigma-Aldrich), also in 0.1% DMSO, was used as a positive control. After incubation, 10 μL of culture supernatant was removed, frozen at −80°C, thawed at room temperature, and then assayed for reverse transcriptase (RT) activity assays as described in Ball et al.[19] HIV-1 reactivation was quantified using density (counts/mm^2) counts computed by the Typhoon Scanner (GE Healthcare Life Sciences) and the Quantity One software (Bio-Rad Life Science Research). In the ACH-2 cell model, AR-42 reactivated HIV-1 in a dose-dependent manner, while vorinostat achieved a plateau at 500 nM (Fig. 2A). Although both AR-42 and vorinostat

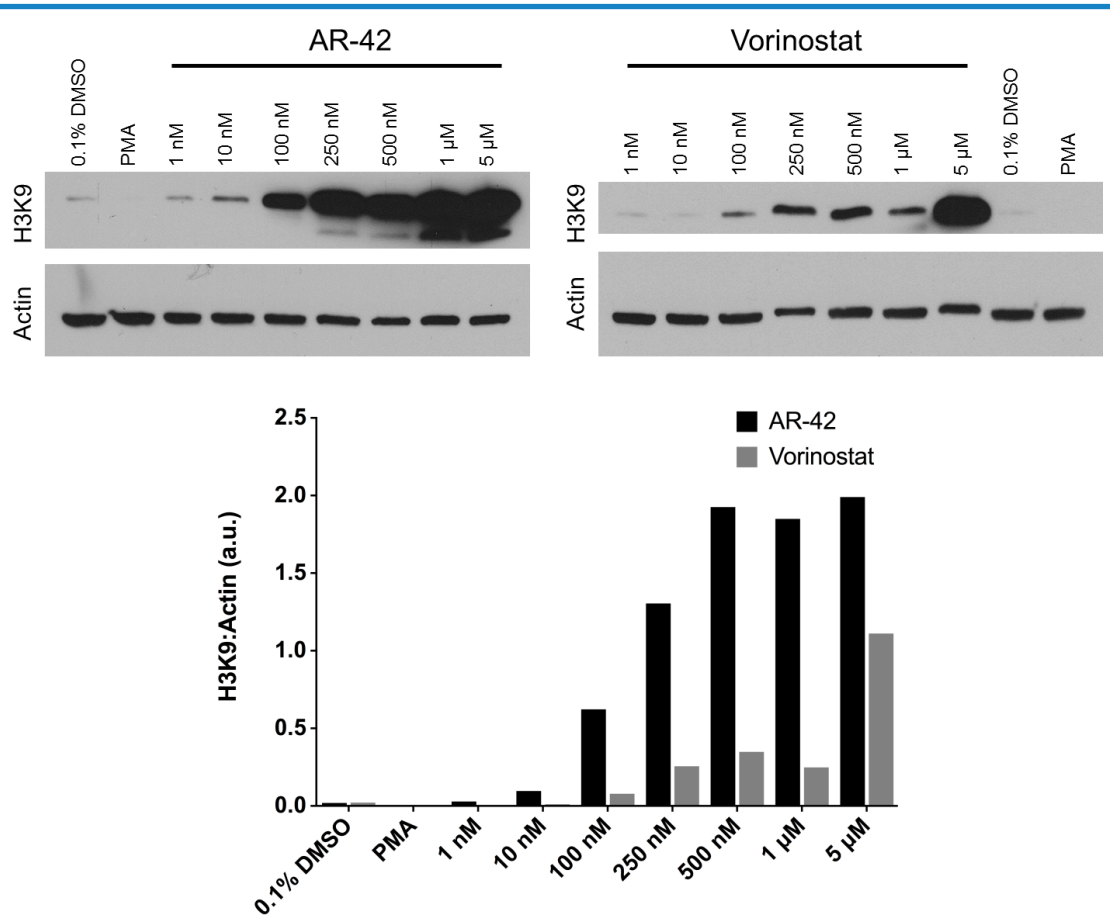

Figure 1. Vorinostat and AR-42 increase histone acetylation. Cellular lysates (15 μg) from ACH-2 cells were loaded per lane and probed with antibodies against acetylated histone H3 and actin. PMA treatments (0.1% DMSO and 100 ng/mL) were negative controls. AR-42 and vorinostat concentrations range from 1 nM to 5000 nM. Densitometry quantification of the actin-loading control and histone 3 acetylation (ImageJ) is displayed as the ratio of histone acetylation intensity to actin-loading control intensity.

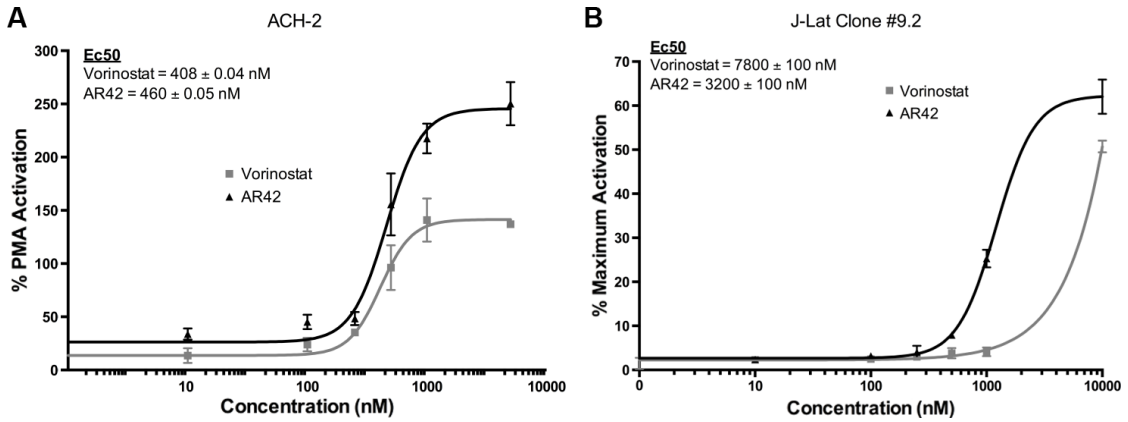

Figure 2. AR-42 more effectively induces HIV-1 reactivation and expression from latently infected CD4+ T-cells than vorinostat. (**A**) RT activity of treatment over% PMA activation after 48 hours (average ± SD, $n = 3$). Calculated EC_{50} values for both AR-42 and vorinostat are depicted. (**B**) HIV-1 latently infected J-Lat cells (clone 9.2) were treated with AR-42 or vorinostat at the indicated concentrations for 24 hours, and GFP-positive cells were scored by flow cytometry. The maximum% of GFP-positive cells was determined with the positive control TNF-α (10 ng/mL), which was set to 100%, and the percentage of activation induced by each drug relative to TNF-α is presented.

have similar potency (460 ± 0.05 nM and 408 ± 0.04 nM, respectively), at higher concentrations, AR-42 is twofold more efficacious than vorinostat in ACH-2 cells.

The second T-cell model, Jurkat CD4+ T-cell-derived J-Lat cells (full length clone 9.2),[18] was obtained from Dr. Eric Verdin through the NIH AIDS Research and Reference Reagent Program. J-Lat cells (clone 9.2) were cultured for 24 hours in the presence of 0.1% DMSO with or without AR-42 or vorinostat. Treatment with tumor necrosis factor alpha (TNF-α) (10 ng/mL) served as a positive control.[18] Following the treatment, the cells were washed, fixed in 4% paraformaldehyde, and quantified by flow cytometry using Guava EasyCyte Mini (EMD Millipore). HIV-1 reactivation [green fluorescent protein (GFP) expression] was determined using the FlowJo software (Tree Star) with the gate equivalent to 0.1% DMSO-treated control cells. Additionally, the PRISM software was used to determine the half maximal effective concentration (EC_{50}) for AR-42 and vorinostat. Flow cytometry analysis determined that in the J-Lat (clone 9.2) cell model, AR-42 is 2.4-fold more potent at HIV-1 reaction than vorinostat (EC_{50} values of 3200 ± 100 nM and 7800 ± 100 nM, respectively; Fig. 2B). Together, the ACH-2 and J-Lat (clone 9.2) data demonstrate that AR-42 can be more potent and efficacious than vorinostat in these HIV-1 reactivation cell line models.

To determine the effect of treatments on cell viability, AR-42-treated cells were assayed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)/3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. The effects of AR-42 and vorinostat were tested for 48 hours and 24 hours, respectively, in ACH-2 and J-Lat (clone 9.2) cells. In ACH-2 cells, both vorinostat and AR-42 caused approximately similar reduction in MTT/MTS activity at 5 μM; although at lower treatment concentrations, vorinostat did not lower MTT/MTS activity >0.1% DMSO after 48 hours (Fig. 3A). In the J-Lat cells (clone 9.2), after 24 hours of treatment, the half cytotoxicity concentration (CC_{50}) of AR-42 was 300 ± 100 nM, while that of vorinostat was 1300 ± 100 nM (Fig. 3B).

In addition to MTT/MTS cell viability analysis, early apoptosis and necrosis studies were performed on AR-42-treated ACH-2 cells using annexin V and propidium iodide staining. Flow cytometry parameters for annexin V and propidium iodide were set based on heat-killed cells (incubated at 50°C for one hour) and performed using Beckman Coulter Cytomics FC500. Similar to the MTT/MTS results, AR-42 reduced the cell viability of ACH-2 cells at the CC_{50} of 217 ± 1 nM (Fig. 3C). These data suggest that AR-42 is more toxic than vorinostat in these two HIV-infected cell lines.

This study was designed to assess the ability of a novel HDAC inhibitor (AR-42) to reactivate HIV-1. We observed the following: AR-42 more potently induces histone 3 acetylation than vorinostat, AR-42 is more efficacious and equipotent than vorinostat in its ability to induce HIV-1 gene expression, and AR-42 is more toxic than vorinostat in two CD4+ T-cell line models of HIV-1 latency.

In the cellular models of schwannoma and meningioma, AR-42 inhibited cellular growth (IC_{50} values between 250 nM and 1 μM, depending on the cell line).[20] In several models of non-Hodgkin's lymphoma, AR-42 significantly enhanced the antitumor activity of HB22.7, an anti-CD22 monoclonal biologic.[21] AR-42 is currently in two clinical trials: one for the treatment of non-Hodgkin's lymphoma (NCT01798901) and the other for multiple myeloma (NCT01129193, www.clinicaltrials.gov). In the multiple myeloma phase I trial, a 40-mg dose of AR-42 achieved a maximum concentration (C_{max}) of 1 μM, a concentration that is sufficient to reactivate HIV in the ACH-2 model.[22,23] In the MT-2 and C8166 cellular models of cancers associated with the deltaretrovirus human T-lymphotropic virus type 1 (HTLV-1), AR-42 induces both histone acetylation and

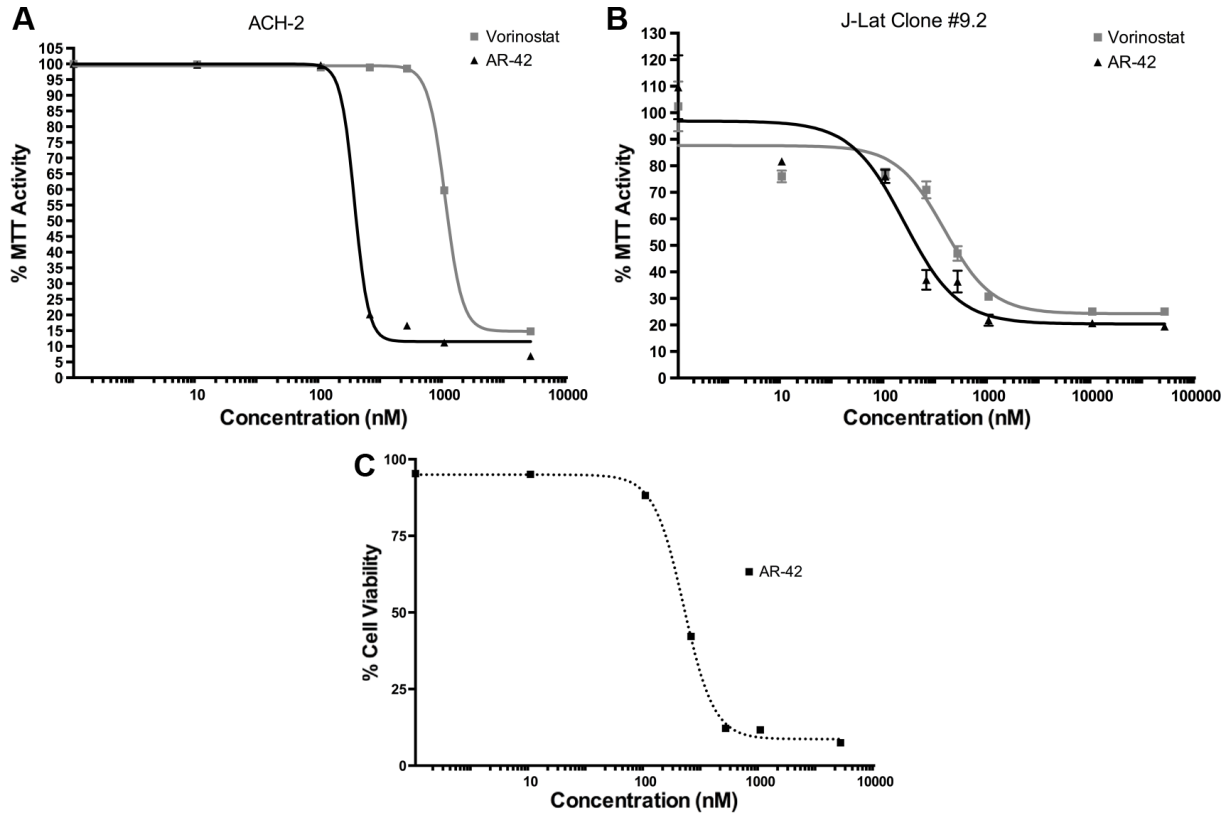

Figure 3. AR-42 reduces the viability of latently infected CD4⁺ T-cells. (**A**) ACH-2 latently infected cells (48 hours). (**B**) J-Lat (clone 9.2) latently infected cells (24 hours). MTT or MTS cell viability assays were tested using vorinostat (SAHA) as a positive control. DMSO (0.1%) was used as a vehicle control. (**C**) Early apoptosis and necrosis (annexin V and propidium iodide staining) were tested in ACH-2 latently infected cells (black dotted) treated with 0.1% DMSO ± AR-42 for 48 hours.

apoptosis; this study did not assess the ability of AR-42 to reactivate HTLV-1 gene expression.[11] Furthermore, in a mouse model of HTVL-1-associated adult T-cell leukemia/lymphoma, AR-42 significantly increased animal survival compared to vehicle-treated control animals.[11] Thus, AR-42 has promising activity against the cancers of various etiologies.

AR-42 treatment decreased MTT activity and cell viability at the treatment concentrations of 250 nM–1000 nM, although the cellular damage would not be attributed solely to drug treatment, because AR-42-induced HIV-1 release can also result in cell death. Previous studies have indicated that activated latently infected cells are presumed to die due to viral pathogenic effects, apoptosis, or pyroptosis.[4,24] A strength of this study is that rather than assessing the supernatant-associated HIV RNA concentration following the reactivation, we assessed either intracellular GFP production (J-Lat cells clone 9.2) or RT activity deposited into the supernatant (ACH-2); both of these assays would not be confounded by HIV RNA or DNA, which could be liberated by cell death.

HIV-1 latently infected cell line models, as used in this study, have proven to be useful in investigating the induced reactivation of HIV from latently infected cells.[25] Recognizing that individual HIV-1 latently infected cell models have limitations, we tested the ability of AR-42 to reactivate the HIV-1 gene expression in both the J-Lat cells (clone 9.2) and the ACH-2 models. Although there are slight differences between the results from the two cell lines, compared to vorinostat, AR-42 had at least one favorable pharmacological attribute in each model [ie, better efficacy in ACH-2 and better potency in J-Lat cells (clone 9.2)].

In summary, AR-42 potently induces histone acetylation in the ACH-2 cells and HIV-1 gene expression in the two models of latently infected CD4⁺ T-cells. These results (ie, favorable efficacy and toxicity profiles), combined with the ongoing AR-42 clinical studies, suggest that AR-42 should be tested in the primary cell models of HIV-1 latency.[26]

Author Contributions

Conceived and designed the experiments: JMM, SdeS, LW, JJK. Analyzed the data: JMM, SdeS, LW, JJK. Wrote the first draft of the manuscript: JMM, SdeS, LW, JJK. Contributed to the writing of the manuscript: ML, KVD, RAB. Agree with manuscript results and conclusions: JMM, SdeS, ML, KVD, RAB, LW, JJK. Jointly developed the structure and arguments for the paper: LW, JJK. Made critical revisions and approved final version: JMM, SdeS, ML, KVD, RAB, LW, JJK. All authors reviewed and approved of the final manuscript.

REFERENCES

1. Chun TW, Engel D, Berrey MM, Shea T, Corey L, Fauci AS. Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1 infection. *Proc Nat Acad Sci U S A*. 1998;95:8869–8873.
2. Choudhary SK, Margolis DM. Curing HIV: pharmacologic approaches to target HIV-1 latency. *Annu Rev Pharmacol Toxicol*. 2011;51:397–418.
3. Richman DD, Margolis DM, Delaney M, Greene WC, Hazuda D, Pomerantz RJ. The challenge of finding a cure for HIV infection. *Science*. 2009;323:1304–1307.
4. Siliciano RF, Greene WC. HIV latency. *Cold Spring Harbor Perspect Med*. 2011;1: a007096.
5. Rasmussen TA, Schmeltz Søgaard O, Brinkmann C, et al. Comparison of HDAC inhibitors in clinical development: effect on HIV production in latently infected cells and T-cell activation. *Hum Vaccin Immunother*. 2013;9:993–1001.
6. Routy JP, Tremblay CL, Angel JB, et al. Valproic acid in association with highly active antiretroviral therapy for reducing systemic HIV-1 reservoirs: results from a multicentre randomized clinical study. *HIV Med*. 2012;13:291–296.
7. Archin NM, Liberty AL, Kashuba AD, et al. Administration of vorinostat disrupts HIV-1 latency in patients on antiretroviral therapy. *Nature*. 2012;487: 482–485.
8. Blazkova J, Chun TW, Belay BW, et al. Effect of histone deacetylase inhibitors on HIV production in latently infected, resting CD4(+) T cells from infected individuals receiving effective antiretroviral therapy. *J Infect Dis*. 2012;206:765–769.
9. Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. *Nat Rev Drug Discovery*. 2006;5:769–784.
10. Marks PA, Dokmanovic M, et al. Histone deacetylase inhibitors: discovery and development as anticancer agents. *Expert Opin Investig Drugs*. 2005;14: 1497–1511.
11. Zimmerman B, Sargeant A, Landes K, Fernandez SA, Chen CS, Lairmore MD. Efficacy of novel histone deacetylase inhibitor, AR42, in a mouse model of, human T-lymphotropic virus type 1 adult T cell lymphoma. *Leuk Res*. 2011;35: 1491–1497.
12. de Ruijter AJ, van Gennip AH, Caron HN, Kemp S, van Kuilenburg AB. Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem J*. 2003;370:737–749.
13. Drummond DC, Noble CO, Kirpotin DB, Guo Z, Scott GK, Benz CC. Clinical development of histone deacetylase inhibitors as anticancer agents. *Annu Rev Pharmacol Toxicol*. 2005;45:495–528.
14. Yang XJ, Seto E. HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention. *Oncogene*. 2007;26:5310–5318.
15. Lu Q, Wang DS, Chen CS, Hu YD, Chen CS. Structure-based optimization of phenylbutyrate-derived histone deacetylase inhibitors. *J Med Chem*. 2005;48: 5530–5535.
16. Lin TY, Fenger J, Murahari S, et al. AR-42, a novel HDAC inhibitor, exhibits biologic activity against malignant mast cell lines via down-regulation of constitutively activated Kit. *Blood*. 2010;115:4217–4225.
17. Folks TM, Clouse KA, Justement J, et al. Tumor necrosis factor alpha induces expression of human immunodeficiency virus in a chronically infected T-cell clone. *Proc Natl Acad Sci U S A*. 1989;86:2365–2368.
18. Jordan A, Bisgrove D, Verdin E. HIV reproducibly establishes a latent infection after acute infection of T cells in vitro. *EMBO Journal*. 2003;22:1868–1877.
19. Ball SC, Abraha A, Collins KR, et al. Comparing the ex vivo fitness of CCR5-tropic human immunodeficiency virus type 1 isolates of subtypes B and C. *J Virol*. 2003;77:1021–1038.
20. Bush ML, Oblinger J, Brendel V, et al. AR42, a novel histone deacetylase inhibitor, as a potential therapy for vestibular schwannomas and meningiomas. *Neuro Oncol*. 2011;13:983–999.
21. Kong Y, Barisone GA, Abuhay M, et al. Histone deacetylase inhibition enhances the lymphomacidal activity of the anti-CD22 monoclonal antibody HB22.7. *Leuk Res*. 2014:1320–1326
22. Cordero-Nieves H, Sborov DW, Canella A, et al. HDAC inhibitor AR-42 decreases CD44 expression and sensitizes myeloma cells to lenalidomide. *Blood*. 2014;124:3377–3377.
23. Hofmeister CC, Liu Z, Bowers MA, et al. Phase I study of AR-42 in relapsed multiple myeloma and lymphoma. *Blood*. 2012;120(21):2955.
24. Doitsh G, Galloway NL, Geng X, et al. Cell death by pyroptosis drives CD4 T-cell depletion in HIV-1 infection. *Nature*. 2014;505:509–514.
25. Spina CA, Anderson J, Archin NM, et al. An in-depth comparison of latent HIV-1 reactivation in multiple cell model systems and resting CD4+ T cells from aviremic patients. *PLoS Pathog*. 2013;9:e1003834.
26. Tyagi M, Romerio F. Models of HIV-1 persistence in the CD4+ T cell compartment: past, present and future. *Curr HIV Res*. 2011;9:579–587.