

# Eliminating the latent HIV reservoir by reactivation strategies

## Advancing to clinical trials

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Combination antiretroviral therapy (cART) has transformed HIV from a deadly to a chronic disease, but HIV patients are still burdened with excess morbidity and mortality, long-term toxicities from cART, stigmatization, and insufficient access to cART worldwide. Thus, a cure for HIV would have enormous impact on society as well as the individual. As the complexity and mechanisms of HIV persistence during therapy are being unraveled, new therapeutic targets for HIV eradication are discovered. Substances that activate HIV production in the latently infected cells have recently received much attention. By turning on expression of latent HIV proviruses, reactivation strategies could contribute to the eradication HIV infection. Compounds that are currently being or soon to be tested in clinical trials are emphasized in this review. The results from these trials will provide important clues as to whether or not reactivating strategies could become significant components of a cure for HIV.

### Introduction

The realization that prolonged combination antiretroviral treatment (cART) did not lead to eradication of HIV infection has spurred an impressive scientific effort in characterizing latent HIV reservoirs and understanding the intricate mechanisms that establish HIV latency and enable the virus to persist for decades evading host immune responses and potent cART. In terms of defining latent HIV reservoirs it is useful to distinguish between proviral latency, referring to the presence of replication competent but transcriptionally silent provirus within resting cells,<sup>1</sup> and residual viremia, referring to the continuous existence of trace levels of extracellular HIV-RNA in plasma during suppressive cART.<sup>2,3</sup>

Whereas the pool of latently infected memory CD4+ T-cells is now the most well-defined latent HIV reservoir and presumably the primary obstacle to the eradication of HIV infection,<sup>4,5</sup> the origin and significance of the residual viremia, in particular whether this is caused by on-going replication, is still debated. Yet, the lack of genetic evolution<sup>6,7</sup> and absence of resistance

development strongly suggest that effective rounds of new replication are not occurring in patients on suppressive cART. Finally, other cellular reservoirs have been suggested to persist in monocytes,<sup>8</sup> macrophages,<sup>9,10</sup> astrocytes,<sup>11</sup> hematopoietic stem cells,<sup>12</sup> naïve T cells,<sup>13</sup> and regulatory T cells,<sup>14</sup> but opposing findings are also reported.<sup>15</sup> Importantly, contrary to latently infected memory CD4+ T cells, these cellular reservoirs have not been longitudinally quantified and, therefore, it remains uncertain whether these cells carry inducible replication competent virus for prolonged periods in vivo.

Several therapeutic strategies are pursued to achieve a cure for HIV (Table 1). First, intensification studies have explored whether adding an extra antiretroviral drug to an already suppressive cART regimen can reduce the residual viremia or the latent HIV reservoir. Overall, there seems to be little or no effect from these interventions,<sup>16-21</sup> but there are conflicting results.<sup>22,23</sup> Second, the remarkable case report of an HIV-infected patient who was cured for HIV after receiving bone marrow transplantation containing a 32 base pair deletion in the HIV co-receptor CCR5 gene<sup>24</sup> has inspired studies entailing infusion of autologous CCR5-deleted CD4+ T cells<sup>25,26</sup> and studies of chemotherapy in HIV infected patients with lymphoma.<sup>27</sup> Third, elimination of latently infected T cells by reactivating HIV-1 expression using agents like histone deacetylase inhibitors (HDACi),<sup>28-40</sup> *IL-7*,<sup>41,42</sup> disulfiram<sup>43</sup> or prostratin<sup>31,44-46</sup> have been investigated in numerous studies in vitro, ex vivo and in vivo. Finally, as reactivation of HIV-1 expression in latently infected cells may be insufficient to ensure the removal of these cells,<sup>47</sup> immunotherapy to enhance HIV specific immunity are continuously being developed and tested.<sup>48</sup>

This review will be focused on reactivation strategies describing compounds that are being considered for the eradication of HIV infection by turning on expression of latent HIV proviruses with emphasis on agents that are currently being or soon to be tested in clinical trials. Immunotherapy and immunomodulatory effects will be dealt with in detail. The majority of HIV infected individuals reside in areas with deprived health care systems and inadequate infrastructure, and, therefore, the development of an HIV cure will ultimately be faced with the challenge of global accessibility and low cost. As most currently investigated reactivation compounds can be produced on a large scale and administered irrespective of HIV-subtype and HLA-profile, they do seem to possess this potential.

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**Table 1.** Recent or ongoing clinical trials for HIV eradication

Therapeutic strategy	Mechanism	Trials performed/intervention	Principal investigators	Outcome measure	Main results	
Eliminating residual viremia and/or residual viral replication by treatment intensification	Suppression of residual viral activity by adding an extra anti-retroviral drug to an already suppressive regimen	Enfuvirtide, 2 NRTI and boosted PI	Joseph J. Eron, Jr.	IUPM	No decay of the latent reservoir during 48 weeks	
		Raltegravir	Joseph J. Eron, Jr.	Plasma HIV-RNA	No decrease in plasma HIV-RNA	
		Raltegravir	Deborah McMahon	Plasma HIV-RNA	Plasma HIV-RNA	No decrease in plasma HIV-RNA
		Efavirenz, atazanavir/r or lopinavir/r	Frank Maldarelli	Plasma HIV-RNA	Plasma HIV-RNA	No decrease in plasma HIV-RNA
		Raltegravir, raltegravir/efavirenz or raltegravir/darunavir	Diane Havlir, Joseph K Wong, Steven Yukl	Plasma HIV-RNA, cell-associated HIV-RNA and HIV-DNA from PBMCs and 4 gut sites	Plasma HIV-RNA, HIV-DNA	No consistent decrease in plasma HIV-RNA, cell-associated HIV-RNA or HIV-DNA
		Abacavir	Scott Hammer	Plasma HIV-RNA and HIV-DNA in PBMCs	Plasma HIV-RNA	No decrease in HIV-DNA or plasma HIV-RNA
		Raltegravir	Javier Martinez-Picado	HIV-DNA and episomal HIV-1 cDNA	HIV-DNA and episomal HIV-1 cDNA	No decrease in HIV-DNA; increase in episomal HIV-1 cDNA in 13 of 45 subjects
		Raltegravir	Santiago Moreno	IUPM, plasma HIV-RNA, episomal HIV-1 cDNA	IUPM, plasma HIV-RNA, episomal HIV-1 cDNA	Decrease in IUPM in all 9 subjects, no decrease in plasma HIV-RNA
		Maraviroc	Martin Markowitz	Mucosal cell-associated HIV- RNA	Mucosal cell-associated HIV- RNA	Ongoing
		SB-728-T	Winson Tang	Plasma HIV-RNA during cART interruption	Plasma HIV-RNA during cART interruption	Ongoing
Host modification to confer resistance to HIV-infection	Infusion of autologous CD4+ T cells with zinc finger nuclease-mediated disruption of CCR5 expression	SB-728-T	Winson Tang	Persistence and activity of CCR5 ZFN-modified autologous T-Cells	Ongoing	
		Chemotherapy for AIDS-related lymphoma	John Mellors	Plasma HIV-RNA, HIV-DNA	No significant effect on plasma HIV-RNA or HIV-DNA	
Chemotherapy for lymphoma	Elimination of latently infected cells through chemotherapy for AIDS-related lymphoma	Allogeneic hematopoietic cell transplantation for hematological malignancies	Joseph Alvarnas, Richard Ambinder	Plasma HIV-RNA, HIV-DNA	Ongoing	
		Autologous hematopoietic stem cell transplantation for lymphoma	Amrita Krishnan	Plasma HIV-RNA	Ongoing	
Eliminating latently infected cells by reactivating HIV-1 expression	Induction of HIV-1 expression in latently infected cells to eradicate the latent reservoir, as these cells could be eliminated due to viral cytopathic effects or immune mediated killing	Vorinostat	David Margolis	Cell-associated HIV-RNA, IUPM	Significant increases in cell-associated HIV-RNA in 8 of 8 subjects receiving a single 400 mg dose	
		Vorinostat	Sharon Lewin	Cell-associated HIV-RNA	Ongoing	
		Panobinostat	Thomas Rasmussen	Cell-associated HIV-RNA, HIV-DNA, IUPM	Ongoing	
		Disulfiram	Steven Deeks, Adriana Andrade	IUPM, plasma HIV-RNA	No consistent significant decrease in plasma HIV-RNA or HIV-DNA	
Enhance innate immunity	Suppression of viral replication by administering cytokines that are part of the host's innate antiviral response	Interferon α2A	Luis Montaner	Viral rebound during cART interruption	Lower proportions of viral rebound than a historical cohort	
		Interferon α2B	Frank Maldarelli	Plasma HIV-RNA	Ongoing	
Enhance HIV-specific immunity and combination approaches	Combining therapeutic HIV vaccination with other therapeutic strategies to enhance the host's adaptive HIV-specific immunity	IL-7 + HIV vaccine + intensification	Christine Katlama (Eramune 1)	HIV-DNA	Ongoing	
		HIV vaccine + intensification	Robert Murphy (Eramune 2)	HIV-DNA	Ongoing	

cART: combination antiretroviral therapy; NRTI: nucleoside reverse transcriptase inhibitor; IUPM: infectious units per million;

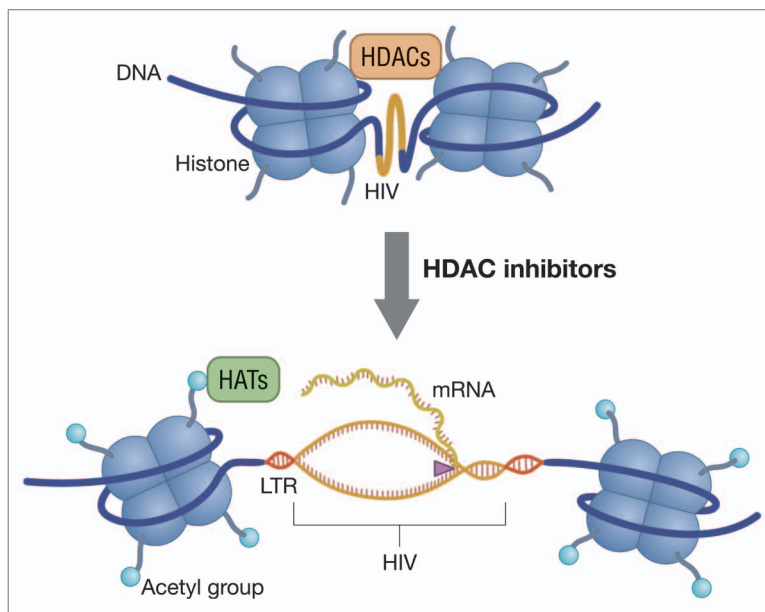
## HDAC Inhibitors

### Role of histone deacetylases and HDACi in HIV Latency.

There are 11 known histone deacetylase (HDAC) metalloenzymes, which are classified into class I (HDAC 1, 2, 3, and 8), class IIa (HDAC 4, 5, 7 and 9), class IIb (HDAC 6 and 10), and class IV (HDAC 11).<sup>49</sup> The counteracting mechanisms of HDACs and histone acetyl transferases (HAT) exert a key function in regulating gene expression by controlling the degree of acetylation/deacetylation of histone tails, which in turn influences chromatin condensation. The HIV 5' long-terminal repeat (LTR) that contains promoter and enhancer elements and has binding sites for several transcription factors is arranged in two nucleosomes, nuc-0 and nuc-1.<sup>50</sup> In the transcriptionally silent state of HIV latency various transcription factors recruit HDACs to the HIV-1 5' LTR where they induce chromatin condensation by promoting deacetylation of lysine residues on histones<sup>51-57</sup> keeping nuc-1 in the hypoacetylated state and preventing HIV transcription. HDACi offsets these mechanisms by inhibiting HDACs (Fig. 1). Chromatin immunoprecipitation assays have shown that the class I HDACs, HDAC1, 2 and 3, may be particularly important to maintaining latency.<sup>53,58</sup> Notably, a recent study correlating HDACi isoform specificity with the ability to reactivate latent HIV-1 expression, showed that potent inhibition or knockdown of HDAC1 was not sufficient to disrupt HIV latency. Instead, HDAC3 inhibition was found to be essential for reactivating viral expression.<sup>59</sup> Class I HDACs are ubiquitously expressed<sup>60</sup> and deacetylation of lysine residues on histones is a key function of class I HDACs. However, recent data suggest that they may deacetylate more than 1750 non-histone proteins.<sup>61</sup> To which degree, if any, the non-histone effects of HDACi contribute to the desired circumvention of HIV latency is largely unknown.

The HDACi acting on HDAC metalloenzymes may be categorized according to their chemical structure into short chain fatty acids, hydroxamic acids and cyclic tetrapeptides,<sup>62</sup> and are further characterized as selective or pan-inhibitors according to their spectrum of action. Consistent with the role histone deacetylases play in repressing transcription, HDAC inhibitors have been shown to disrupt HIV-latency and induce virus HIV-1 expression in latently infected cell lines, latently infected primary T-cells, resting CD4+ T-cells isolated from HIV-infected donors and, recently, in vivo.<sup>28-36,40</sup>

**Valproic acid and vorinostat.** Valproic acid (VPA), a known anticonvulsant that also exerts weak HDAC inhibition, was the first HDACi to be tested in a clinical study with the objective of depleting the latent reservoir of HIV-1 infection. Whereas a substantial decline was seen in the frequency of replication competent HIV in circulating resting CD4+ T cells in the initial study,<sup>37</sup> additional studies failed to demonstrate any effect of VPA, even in the setting of intensified cART.<sup>38,39,63</sup> HDACi with much higher potency are now being investigated. Vorinostat is a hydroxamic acid containing pan-HDACi with activity against



**Figure 1.** Disruption of HIV latency by HDAC Inhibitors. In the latent state HDACs suppresses HIV-1 expression by catalyzing deacetylation of histone tails and keeping the chromatin in a compacted state. Inhibition of HDACs by HDACi promotes histone acetylation by HATs leading to relaxation of the chromatin and initiation of transcription. HDACs: histone deacetylases; HDACi: histone deacetylase inhibitors; HATs: histone acetyl transferases; LTR: long-terminal repeat.

class I and II HDACs.<sup>64</sup> Having received FDA-approval in 2006 for the treatment of cutaneous T cell lymphoma there is now considerable clinical experience with the use of this drug. It is the most extensively investigated HDACi in HIV context having consistently shown the ability to reactivate HIV-1 expression at therapeutic concentrations in latently infected cell lines, latently infected primary cells, and resting CD4+ T-cells from HIV-infected patients on suppressive HAART.<sup>28,29,35,65</sup> In contrast, a recent study, investigating the HDACi vorinostat, VPA and oxamflatin, found that the levels of HIV production by HDAC inhibitor stimulated resting CD4+ T-cells from aviremic donors were not significantly different from those of cells treated with media alone.<sup>66</sup> Of note, in this study virion-associated (extracellular) HIV-RNA rather than cell-associated HIV-RNA was quantified. Two clinical trials are currently undertaken to evaluate whether vorinostat can reactivate latent HIV in vivo. The first data from one these trials was recently published showing that a single dose of 400 mg vorinostat significantly increased expression of HIV-RNA in isolated resting CD4+ T cells in 8 of 8 evaluated subjects without any safety issues.<sup>40</sup> This is a very important result establishing proof-of-concept for the use vorinostat to reactivate latent HIV. However, as the 8 evaluated subjects were selected from a total of 16 based upon demonstrable virus production following 335 nM vorinostat ex vivo stimulation, the effect on a non-selected study group may be of less magnitude. The results from a clinical study (NCT01365065) conducted in Melbourne, Australia in which HIV infected patients on suppressive cART receive 400 mg vorinostat daily for 14 consecutive days are awaited with much anticipation.

**Givinostat, panobinostat and belinostat.** Givinostat, panobinostat and belinostat are all hydroxamic acid containing pan-HDACi. Givinostat was initially compared with VPA in an in vitro study employing the latently infected cell lines, ACH2 and U1. Robust induction of HIV-1 expression was shown, approximately 10 times more efficient than VPA at clinically relevant concentrations.<sup>30</sup> These results were confirmed recently in the same cell lines showing higher potency for HIV reactivation than vorinostat.<sup>67</sup> In addition, givinostat was shown to decrease CXCR4 and CCR5 expression,<sup>30</sup> which is probably owing to its anti-inflammatory properties. At nanomolar concentrations, this compound inhibits production of pro-inflammatory cytokines and reduces systemic inflammation.<sup>68,69</sup> Furthermore, givinostat was used in a clinical study to treat systemic-onset juvenile arthritis with an acceptable safety profile at a therapeutically effective dose of 1.5 mg/kg.<sup>70</sup> Chronic immune activation as evidenced by higher levels of pro-inflammatory biomarkers and T-cell activation is a hallmark of HIV infection and contributes to HIV disease progression,<sup>71-74</sup> but may also promote HIV persistence by inducing homeostatic proliferation of latently infected cells<sup>5</sup> and inhibiting the function of HIV-specific effector T-cells. Whether givinostat has any effect on these HIV-related pathological processes is unknown, but would be important to explore in future HIV-related trials. In the latently infected cell lines, ACH2 and U1, belinostat has activity against class I and II HDACs with similar potency to givinostat<sup>36,67</sup> and also displayed ability to induce HIV production at therapeutic concentrations in a primary CD4+ T cell model of latency (Rasmussen et al., unpublished). However, as belinostat has primarily been used intravenously, published pharmacokinetic information on the oral formulation of belinostat is limited.

Panobinostat has recently displayed considerable potency in reactivating HIV-1 expression in latently infected cell lines and primary resting CD4+ T cells as compared with other HDACi in clinical development.<sup>67</sup> In this study, panobinostat reactivated HIV-1 expression at concentrations as low as 8–32nM – well below the levels obtained with oral clinical dosing. Panobinostat is likely one of the most potent pan-HDAC inhibitors in clinical development and as the elimination time of panobinostat is relatively long, prolonged histone hyper acetylation can be observed 7 d after a second dose with this compound.<sup>75</sup> This allows for dose reductions or intermittent dosing schedules to diminish the problematic thrombocytopenia seen with all HDAC inhibitors. A clinical trial to investigate the in vivo effect of panobinostat on HIV-1 expression and HIV reservoir size has been initiated by our group at Aarhus University Hospital, Denmark (NCT01680094). This study entails 8 week of cyclic panobinostat therapy with a primary endpoint of change from baseline in cell-associated unspliced HIV-RNA and will also provide a unique opportunity for studying the effect on host immune responses.

**Other HDAC inhibitors.** An increasing number of other HDACi have been tested in different models for the ability to reactivate HIV-1 expression in latently infected cells, but most of these compounds have never been administered to humans. These investigations include sodium butyrate (cell lines),<sup>31</sup>

entinostat (cell lines and primary T cells),<sup>31,65</sup> trichostatin A (cell lines and primary T cells),<sup>31,59,76,77</sup> oxamflatin (cell lines and patient cells),<sup>34,66</sup> apicidin (cell lines),<sup>78,79</sup> NCF-51 (cell lines)<sup>80</sup> and scriptaid (cell lines).<sup>76</sup> Also, explorations of HDACi generated by Merck (MRK1, MRK4, MRK10–14) with varying degree of HDAC selectivity showed that inhibitors of class I HDACs were more efficient inducers of HIV-1 expression than inhibitors of class II HDACs in cell lines and resting CD4+ T cells from patients on cART.<sup>78</sup> Similarly, givinostat analogs ITFa, ITFb and ITFc increased HIV-1 expression in latently infected cell lines and higher levels of virus production was seen with compounds that exhibited the highest inhibitory potential for class I HDACs.<sup>30</sup> Romidepsin (Istodax®), like vorinostat, is an FDA-approved HDACi for the treatment of cutaneous T cell lymphoma. Romidepsin has high potency specificity for HDAC1 and HDAC2,<sup>81</sup> but there is no published data on the effect on latent HIV. The Aids Clinical Trial Group (ACTG) is reportedly making preparations toward a romidepsin ascending single dose study to investigate the in vivo effect on virus production.

### Immune Modulatory Effects of HDAC Inhibitors

While HDACi initially attracted attention in the oncology field due to their proapoptotic and cell cycle arrest actions on malignant cells, their potential as immunotherapy is now also being intensively tested focusing on anti-inflammatory effects. Clinical and experimental studies have identified a range of immune modulatory effects of HDACi involving both specific inflammation signaling pathways (e.g., regulation of NF- $\kappa$ B via I $\kappa$ B $\alpha$  or p65) as well as epigenetic mechanisms.<sup>82,83</sup> Most of these effects are anti-inflammatory but the biologic roles of individual HDAC isoforms and their corresponding selective inhibitors are complex and show great diversity.

In the context of HIV, HDACi's action on T cells and regulatory T cells (Tregs) in particular is highly relevant. Therapy with a pan-HDACi (e.g., SAHA or panobinostat) can stimulate thymic production of Foxp3+ Tregs, promote conversion of T cells into Tregs, and enhance the immune suppressive function of human Tregs. In addition, HDACi increase Foxp3 acetylation thereby protecting it from proteasomal degradation.<sup>84</sup> Thus, HDACi induced immune suppression via Tregs may impact the course of HIV infection given the fact that the virus induces excess inflammation that drives disease progression in untreated HIV infection and causes premature immunosenescence and morbidity in persons on HAART.<sup>71</sup> In HIV eradication, the consequences of HDACi induced Treg expansion and/or function, could be either beneficial, by suppressing generalized T-cell activation, or detrimental, by weakening HIV-specific responses and thereby hindering immune-mediated clearance of latently infected reactivated CD4+ T cells. However, predicting different HDACi's in vivo anti- or pro-inflammatory effects in HIV may prove challenging since even structurally related compounds have been shown to have opposing actions. For example, in a rodent model of graft-vs.-host disease (GVHD) vorinostat reduced inflammation and GVHD-related mortality<sup>85</sup> while Wang et al. found that panobinostat induced a Th1-directed

pro-inflammatory response and augmented GVHD progression.<sup>86</sup> This divergent effect of two hydroxamic acid containing pan-HDACi may be explained by differences in their isotype-specific HDAC inhibitory potential. Knockout of HDAC3 have been shown to induce upregulation of NF- $\kappa$ B, one of the main pro-inflammatory pathways. Panobinostat inhibits HDAC3 at 10-fold lower EC<sub>50</sub> concentrations than vorinostat suggesting that panobinostat may have more pronounced pro-inflammatory effects than vorinostat.<sup>64</sup> On the other hand, panobinostat's EC<sub>50</sub> for inhibition of HDAC9 is approximately 30–40 fold lower than the EC<sub>50</sub> for vorinostat.<sup>64</sup> Inhibition of HDAC9 leads to enhanced suppressive Tregs' function and proliferation pointing toward a more potent induction of anti-inflammatory T-regs by panobinostat than vorinostat.<sup>83</sup>

Nevertheless, in oncologic studies various HDACi have repeatedly been shown to enhance anti-tumor effects by stimulating antigen-presenting cell and T cell activity.<sup>87</sup> Collectively, the current literature suggest that the anti-inflammatory effects of HDACi in vivo generally tend to target pathologic inflammatory responses while preserving normal immune cell function.

## Immunotherapy

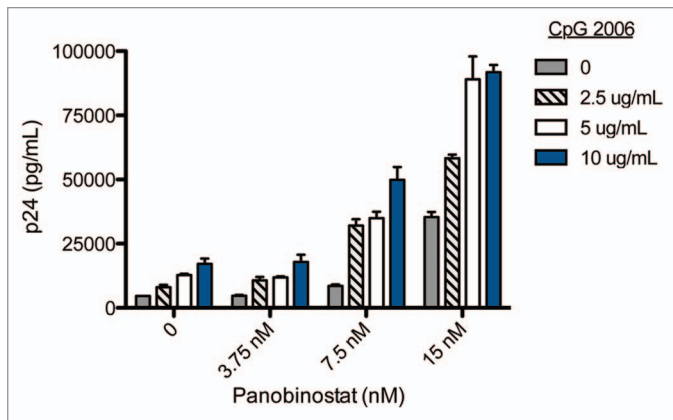
**Cytokines.** Early studies suggested that interleukin (IL)-2 therapy might impact on the frequency of resting cells harboring replication competent virus,<sup>88</sup> but rebound viremia occurred rapidly in these subjects upon interruption of cART.<sup>89</sup> Moreover, additional studies could not establish an effect of IL-2 on the pool of latently infected CD4+ T cells or HIV production,<sup>90,91</sup> and when IL-2 was used in combination with anti-CD3 antibody OKT3 this led to detrimental T cell activation and irreversible CD4+ T cell depletion.<sup>92</sup> Currently, there is more focus on the prospects of the homeostatic cytokine, IL-7. Several studies have shown that IL-7 induce virus outgrowth ex vivo in the resting CD4+ T cells of HIV infected patients on cART.<sup>41,42</sup> Two small clinical trials conducted in HIV infected patients reported that IL-7 administration increased CD4+ and CD8+ T cells with a memory phenotype. Furthermore, transient increases in plasma HIV-RNA was seen in 4 of 13 and 6 of 11 study subjects, respectively.<sup>93,94</sup> To identify the sources of HIV detected, HIV-RNA and HIV-DNA sequences present before, during and after transient viremic episodes were analyzed and these results indicated that the release of virus originated from a preexisting pool of HIV-RNA rather than activation of silent proviruses.<sup>95</sup> Also, a recent study showed that, whereas partial reactivation of latent HIV-1 can be achieved with IL-2 and IL-7 in combination, this does not reduce the pool of latently infected cells.<sup>96</sup> Rather, homeostatic proliferation induced by these cytokines may favor the maintenance of the latent HIV-1 reservoir.<sup>5,96</sup> Collectively, these findings indicate that the homeostatic proliferation induced by IL-7 therapy could be counterproductive in HIV eradication therapy. A recent clinical study among 32 HIV-infected subjects confirmed that administration of recombinant human IL-7 increases CD4+ T cells of predominantly naïve and central memory phenotype.<sup>97</sup> Interestingly, transient low-level viremia was seen in a minority of study subjects and, moreover, levels of total HIV-DNA per

milliliter blood, but not per 10<sup>6</sup> CD4+ T cells, increased suggesting that IL-7 treatment could have induced homeostatic proliferation of latently infected cells. An ongoing clinical trial (ERAMUNE) is currently investigating IL-7 for its effect on the latent HIV reservoir.

**Toll-like receptor (TLR) agonists.** Non-pathogen specific stimulation of the innate immune system via TLRs is used to treat certain viral diseases (e.g., Imiquimod against genital warts) and as adjuvant in immunization. In addition, some TLR ligands appear to modulate latent HIV infection. First, the TLR-5 agonist flagellin results in NF- $\kappa$ B activation and induces expression in latently infected cell lines and resting central memory T-cells transfected with HIV-1, but could not be shown to reactivate HIV-1 in purified resting CD4+ T cells from aviremic HIV-patients.<sup>98</sup> Second, the TLR7/8 agonist, R-848, activated HIV from cells of myeloid-monocytic origin through TLR8-mediated NF- $\kappa$ B activation.<sup>99,100</sup> Finally, synthetic CpG oligodeoxynucleotides (CpG ODNs) that stimulate immune cells via TLR9 induced HIV reactivation in vitro.<sup>101,102</sup> We recently conducted a double-blind randomized controlled vaccine trial in which 95 HIV-infected adults were randomized to receive pneumococcal vaccines with or without the synthetic CpG ODN, CPG 7909, as adjuvant.<sup>103</sup> This trial provided a unique opportunity to explore whether CpG ODNs might have impacted upon the proviral reservoir in vivo despite the limitations in dosage and sampling inherent to the vaccine trial design. Inclusion into this *post hoc* analysis was restricted to 54 participants who were on cART, had available sample material and had quantifiable HIV-DNA at the time of immunization. Indeed, we observed a moderate but statistically significant reduction in proviral HIV-DNA among CPG 7909 recipients compared with those receiving placebo adjuvant ( $p = 0.02$ ) advising that further investigation into the effect of TLR9 agonists on HIV latency is warranted (personal communication). Interestingly, in vitro studies conducted at our laboratory revealed a synergistic effect of CpG ODNs and HDACi in combination. Treating the latently infected cell line U1 with increasing concentrations of CpG ODNs produced marked increases in HIV production following stimulation with low concentrations of the HDACi panobinostat (**Fig. 2**). Briefly, cells were incubated with indicated drug concentrations for 48 h followed by cell lysis and p24 ELISA enumeration as previously described.<sup>104</sup> Combining CpG and Panobinostat induces significantly HIV production than both treatments individually ( $p < 0.001$ , ANOVA).

## Protein Kinase C (PKC) Activators

Bryostatin-1 is a natural occurring PKC activator belonging to the marine macrolide class of molecules. It is isolated from the marine bryozoan *Bugula neritina*<sup>105</sup> and has been administered in numerous clinical trials for its anti-cancer effect. In cell lines bryostatin-1 reactivated HIV-1 expression more potently than vorinostat and prostratin via activation of PKC.<sup>106</sup> In addition, lipid nanoparticles with bryostatin-2 incorporated have been developed and were shown to stimulate HIV production in T cell lines in vitro and in latently infected cells ex vivo in a humanized



**Figure 2.** Stimulation of HIV-1 expression by CpG 2006 and panobinostat. HIV-1 expression in the latently infected cell line U1 following treatment for 48 h with combinations of CpG 2006 (0–10  $\mu$ g/mL) and panobinostat (LBH589; 0–15 nM). Virus production was estimated by p24 levels in supernatant; mean  $\pm$  SEM shown in figure.

mouse model.<sup>107</sup> The limited supply of natural occurring bryostatin has impeded the clinical use of this compound, but production of bryostatin analogs that reactivate latent HIV with similar or higher potencies was described recently.<sup>108</sup> Another naturally occurring PKC activator, prostratin, is isolated from the Samoan medicinal plant, *Homalanthus nutans*. Prostratin induces HIV-1 expression in latently infected cell lines and cells isolated from aviremic HIV-infected patients on cART through PKC-mediated activation of NF- $\kappa$ B.<sup>45,46,109</sup> However, the in vivo toxicity and safety of prostratin is unknown and advancing this compound to the level of clinical testing, if feasible, will take some time. Notably, synergistic effects of activating virus expression have been described for both bryostatin-1<sup>110</sup> and prostratin<sup>31</sup> indicating that targeting mechanistically different pathways implicated in silencing HIV transcription is desirable for breaking latency.

### Other Activators of HIV

Recently, using a primary CD4+ T cell model, in which HIV-1 latency was established by transducing primary human CD4+ T cells with the prosurvival gene bcl-2 and infecting them with HIV-1 before allowing the cells to return to a resting state, drug libraries were screened for compounds that reverse HIV-1 latency in vitro without cellular activation.<sup>111</sup> Disulfiram, an inhibitor of acetaldehyde dehydrogenase used to treat alcoholism, was identified as a potential re-activator of latent HIV-1,<sup>43</sup> presumably owing to depletion of the phosphatase and tensin homolog (PTEN) resulting in activation of the Akt signaling pathway.<sup>112</sup> A single arm pilot study has been undertaken to evaluate whether adding 500 mg disulfiram daily for 2 weeks to stable cART will increase HIV production and decrease the HIV reservoir in vivo. Preliminary results showed no significant effect of disulfiram on these endpoints. However, increases in plasma HIV-RNA were observed among study subjects with available blood samples 1–2

h after the first dose.<sup>113</sup> Full results from this study are expected in the near future.

In addition to the actions of HDACs that repress HIV-1 transcription by keeping nuc-1 in the hypoacetylated state, histone methyltransferases (HMTs) have been shown to inhibit viral expression by promoting histone H3 methylation in nuc-1.<sup>114</sup> Two inhibitors of HMT, chaetocin and BIX-01294, have been described so far. These compounds induced virus outgrowth in resting CD4+ T cells from aviremic HIV infected donors on cART, but cannot be administered safely to humans.<sup>115</sup> Hexamethylbisacetamide (HBMA) is a kinase agonist that was tested in a few clinical studies more than 20 y ago for its effect on hematologic malignancies.<sup>116</sup> It has been shown to promote HIV-1 expression in latently infected cell lines in a Tat independent manner<sup>117</sup> and induce outgrowth of HIV-1 from resting CD4+ T cells recovered from aviremic patients on cART.<sup>118</sup> While thrombocytopenia appears to limit the clinical use of HBMA,<sup>116</sup> findings that HBMA mediates its effect on HIV latency through signaling via both protein kinase C (PKC)  $\mu$  and phosphatidylinositol 3-kinase, reveal cellular kinases that may be therapeutically exploited.<sup>118</sup>

### Discussion

Thus, while an increasing number of substances that could reactivate HIV-1 from latency are being described, there are limitations to this approach and significant gaps in knowledge. Ongoing viral replicative activity or cell-to-cell spread<sup>119</sup> is not targeted by reactivation approaches and, to the extent that this is at all occurring during suppressive therapy, must be addressed by improvements in drug delivery to tissues of residual HIV exchange. Moreover, for reactivating strategies to be successful, the induced HIV-1 expression in latently infected cells must be followed by the removal of these cells by viral cytopathic effects or immune mediated mechanisms. It is currently unknown to which extent this occurs in vivo as chronic HIV-infection is characterized by an impaired cytolytic capacity of CD8+ T cells, which is not restored by cART.<sup>120</sup> Notably, a recent in vitro study showed that reactivation of virus production in latently infected resting cells was insufficient to eliminate these cells; only after stimulation of HIV-1 specific cytolytic T cells was efficient killing of latently infected cells achieved.<sup>47</sup> This suggests that combining pharmacological reactivation of HIV-1 from latency with therapies designed to improve the killing capacity of cytolytic T cells could be needed and would be a logical next step once HDACi induced HIV reactivation in vivo is described in more detail. In addition, there are several unique challenges related to testing strategies in clinical trials that require careful consideration. While eradication therapies that could significantly impact the latent reservoir may also have associated toxicities or unknown long-term effects, there is little chance of a health benefit for study participants in the initial trials. Thus, careful consideration of acceptable risks weighed against possible long-term benefits is necessary and poses challenges for investigators and regulatory authorities. Also, difficulties in measuring the

effects on HIV transcription or the latent reservoir are significant barriers to expanding clinical trial strategies. Large cell numbers and complex assays are currently applied and these methods, and the inherent high costs, will be difficult to operate in larger clinical trials. In the end viral rebound parameters during cART interruption will be the most relevant clinical endpoint, but will require careful consideration of when this is justified and which efficacy criteria should be met.

The development and implementation of cART has been a major medical achievement that has transformed HIV from a deadly to a chronic disease, but HIV infected patients are still burdened with excess morbidity and mortality, long-term toxicities from cART, stigmatization and, finally, insufficient access to cART worldwide. Thus, a cure for HIV would have a substantial

impact on society as well as the individual and continues to be a high research priority. As the complexity and mechanisms of HIV persistence during therapy are unraveled, new therapeutic targets are discovered. A growing number of substances that could promote the eradication of HIV through activating HIV production in latently infected cells are now being described. Whether or not reactivating strategies will prove to be a significant component of a cure for HIV is a key question within this field of research. The first indications of what the answer will be will come from clinical trials currently conducted or underway.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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