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Between a shock and a hard place: challenges and developments in HIV latency reversal

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Latently infected cells that persist in HIV-infected individuals on antiretroviral therapy (ART) are a major barrier to cure. One strategy to eliminate latency is by activating viral transcription, commonly called latency reversal. Several small non-randomised clinical trials of latency reversing agents (LRAs) in HIV-infected individuals on ART increased viral production, but disappointingly did not reduce the number of latently infected cells or delay time to viral rebound following cessation of ART. More recent approaches aimed at reversing latency include compounds that both activate virus and also modulate immunity to enhance clearance of infected cells. These immunomodulatory LRAs include toll-like receptor agonists, immune checkpoint inhibitors and some cytokines. Here, we provide a brief review of the rationale for transcription-activating and immunomodulatory LRAs, discuss recent clinical trials and some suggestions for combination approaches and research priorities for the future.

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Introduction: HIV persistence and latency

Antiretroviral therapy (ART) can suppress HIV replication but treatment is required lifelong due to the persistence of long-lived and replicating CD4+ T cells that contain integrated HIV DNA, termed a provirus. Although the majority of integrated HIV DNA that persists on ART is defective, 2–5% of proviruses are intact and potentially replication-competent [1–3]. HIV DNA and replication competent virus has been detected in essentially every CD4+ T cell subset analysed in HIV-infected individuals on ART, including naïve T cells [4–7]. Cells other than CD4+ T cells, including macrophages, astrocytes and hepatocytes may also contribute to HIV persistence [8–12]. Finally, the frequency of HIV-infected cells that persist on ART is much higher in lymphoid tissue, including lymphoid follicles in lymph node and in the gastrointestinal tissue [13–20]. The heterogeneity of where and how HIV persists on ART has significant implications for developing a robust strategy to eliminate HIV.

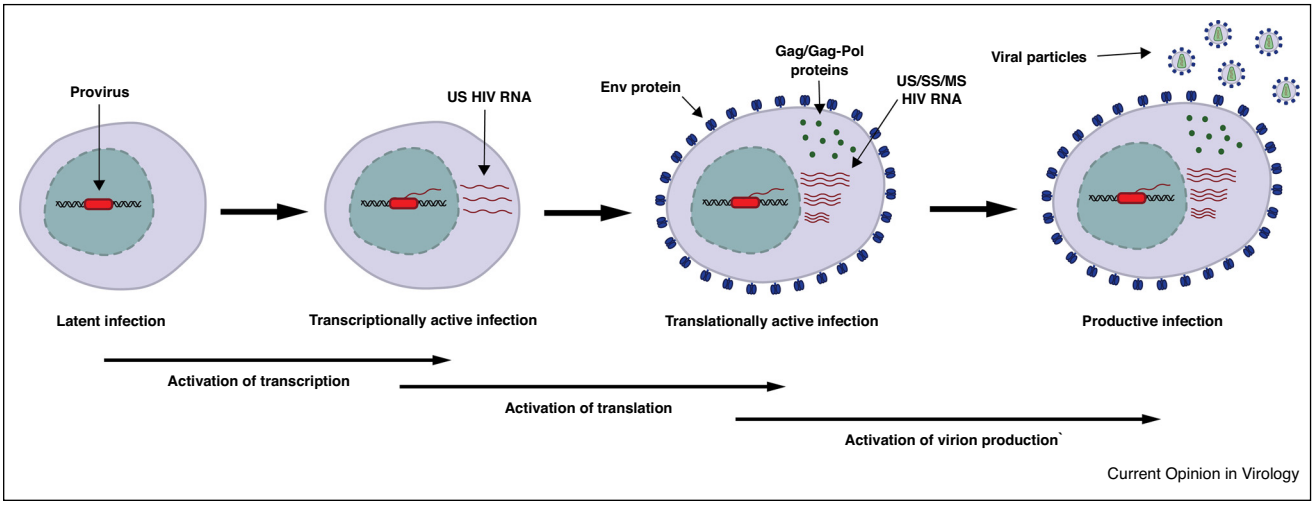
Latency reversal and intracellular blocks

Reversing HIV latency to eliminate latently infected cells has been an actively pursued strategy in HIV cure research for the last 10 years. This approach, known as 'shock and kill', involves activating latent HIV through administration of a latency reversing agent (LRA) with the aim of facilitating cell death by viral cytopathic effects or immune-mediated killing [21,22]. This is done in the presence of ART so there are no further rounds of HIV replication.

HIV latency is defined as cells that contain integrated HIV DNA but are transcriptionally silent and, therefore, there is no protein expression or virus production. It is now apparent that there is a spectrum of transcriptional activity in latently infected cells in HIV-infected individuals on fully suppressive ART with some cells truly transcriptionally silent while others produce low levels of cell-associated HIV RNA or HIV proteins [23–25]. In order for an HIV-infected cell to produce progeny virions, more than 40 different mRNA species are produced as a result of RNA splicing [26]. Unspliced (US) HIV RNAs encode the structural proteins, gag and pol, and serve as the genome for new virions. Singly spliced (SS) or incompletely spliced HIV RNAs encode the accessory proteins, Vif, Vpr, Vpu, as well as Env. Multiply spliced (MS) HIV RNAs encode the regulatory proteins Tat, Rev, and Nef [26]. For virion production to occur efficiently, all HIV RNA species need to be produced (Figure 1).

HIV latency is maintained largely by transcriptional repression through a variety of mechanisms [27,28]. The HIV LTR promotor can be suppressed through increased acetylation or reduced methylation leading to epigenetic silencing [29,30] or due to the limited availability of key transcription factors in resting CD4+ T cells

Figure 1



Reversing HIV latency requires multiple steps to produce a productively infected cell. In vivo, HIV-infected cells that persist on ART are at various stages on this spectrum ranging from truly latent (DNA+, RNA-) to transcriptionally active (DNA+, US RNA+) to translationally active (DNA+, US RNA+, MS RNA+, protein+). Latency reversing agents can have activity at different stages along this transition. An LRA that initiates transcription only will unlikely be effective. Combination latency reversal strategies should ideally target multiple steps to generate viral proteins or productive infection but without activating the T-cell directly. Env = envelope protein, US = unspliced, SS = singly spliced, MS = multiply spliced.

[31]. HIV RNA trafficking from the nucleus, a key step for the production of multiply spliced HIV RNA, can also be impaired through cellular inhibition of Rev [32,33]. Unspliced HIV RNAs are readily detectable in total CD4+ T cells in most individuals on ART, but the majority of these are incomplete transcripts, highlighting that HIV transcription in latently infected cells is inefficient [24,34,35]. In blood CD4+ T cells there is a higher frequency of short transcripts followed by a successively lower frequency of complete transcripts, polyadenylated transcripts and multiply spliced transcripts [24]. These transcriptional blocks differ in CD4+ T cells isolated from blood and rectal tissue [36]. Collectively, these data demonstrate that latency is not all or nothing as originally thought, but rather there is a spectrum with some cells actively transcribing low levels of the viral genome and others maintaining a deeper state of latency. The implications of this are that there are multiple blocks to viral RNA and protein expression that must be overcome to efficiently reverse latency and these differ in different tissue sites.

Transcription-activating LRAs

Multiple classes of drugs have been shown to activate HIV transcription *in vitro*, including epigenetic modifiers (such as histone deacetylase inhibitors (HDACi), methyl transferase inhibitors, methylation inhibitors and bromodomain inhibitors), protein kinase C agonists, activators of the PI3K pathway (including disulfiram and mTOR inhibitors), NFkB agonists (including SMAC mimetics and maraviroc) [reviewed in Ref. [37]]. Multiple early non-randomised clinical trials of HDACi and disulfiram in HIV-infected individuals on ART all demonstrated a modest increase in cell-associated HIV RNA and in some cases plasma HIV RNA, however, there was no decline in infected cells or time to virus rebound during ART interruption (reviewed in Ref. [38]). It thus appears unlikely that these agents will significantly impact the latent reservoir on their own, but they may still have a role in combination with other interventions. Ongoing studies are testing this concept (Table 1).

PKC agonists non-specifically alter gene expression levels and can also induce non-specific T cell activation. They include prostratin [39], bryostatin-1 (BRY-1) [40], *Euphorbia kansui* [41], ingenol-3-angelate (PEP005) [42], Ingenol-B [43], and ingenol 3,20-dibenzoate [44] and have been evaluated in both *in vitro* and animal models. Only BRY-1 has been evaluated in a clinical trial [45]. While BRY-1 was well-tolerated, it was only administered at a low concentration and there was no activation of PKC or reversal of viral latency [45]. More recently, the HIV entry inhibitor maraviroc (MVC) was shown to increase cell-associated unspliced HIV RNA, potentially mediated through activation of NF-κB via the CCR5 receptor [46]. An intensification study of MVC demonstrated an increase in NF-kB activation, and a modest increase in cell-associated unspliced HIV RNA [47], but the effect on plasma viremia or frequency of latent infection has not been assessed.

Recently, it has been shown that certain biological processes including circadian rhythm and sex hormones can affect HIV transcription. These insights may be exploited

Table 1

Ongoing combination clinical trials including a latency reversing agents in HIV

LRA	Additional intervention	*n*	Institution	Status	Identifier
HDACi					
Nicotinamide	Dendritic cell vaccine + auranofin + ART intensification (DTG + MVC)	30	Federal University of Sao Paolo	Recruiting	NCT02961829
Romidepsin	3BNC117	30	Rockefeller University	Recruiting	NCT02850016
Romidepsin	MVA.HIVconsv vaccine	15	IrsiCaixa	Completed	NCT02616874
Panobinostat	Peg-IFNα-2a	34	Massachusetts General Hospital	Recruiting	NCT02471430
Vorinostat	ChAdV63.HIVconsv (ChAd) prime and MVA.HIVconsv boost vaccines	60	Imperial College London	Active, not recruiting	NCT02336074
Vorinostat	Hydroxychloroquine + maraviroc	15	Thai Red Cross AIDS Research Centre	Ongoing, not recruiting	NCT02475915
Romidepsin	MVA vector HIV vaccine + HIVACAR01 (personalised HIV RNA vaccine) + 10-1074	56	David Garcia Cinca, Hospital Clinic of Barcelona	Not yet recruiting	NCT03619278
Vorinostat	Disulfiram	15	Peter Doherty Institute for Infection and Immunity	Suspended during review of AE	NCT03198559
Valproic acid	Pyrimethamine	28	Erasmus Medical Center	Recruiting	NCT03525730
Vorinostat	HXTC (HIV antigen expanded specific T cell therapy)	12	University of North Carolina, Chapel Hill	Recruiting	NCT03212989
Vorinostat	Tamoxifen	30	NIAID	Active, not recruiting	NCT03382834
Romidepsin	3BNC117	60	Aarhus University Hospital	Recruiting	NCT03041012
Interleukin					
IL-2	Ex vivo activated NK cells	5	University of Minnesota	Recruiting	NCT03346499
TLR9 agonist					
Lefitolimod (MGN1703)	10-1074 + 3BNC117	48	Aarhus University Hospital	Not yet recruiting	XXXX

HDACi, histone deacetylase inhibitor; TLR, toll-like receptor; IL, interleukin; NK, natural killer; DTG, dolutegravir; MVC, maraviroc.

further. We demonstrated significant variation in cell associated unspliced HIV RNA in HIV-infected individuals on ART which varied with the time of blood collection [48] and changes in the expression of the circadian-locomotor-output-cycles-kaput (CLOCK)-associated gene and brain-and-muscle-ARNT-like-1 (BMAL1), a circadian regulator of gene expression [49]. Using an *in vitro* system we then demonstrated that CLOCK and BMAL1 can activate HIV transcription through direct activation of the HIV LTR [49]. Further studies will reveal whether these processes can be targeted to optimise latency reversal.

Sex hormones may also significantly affect HIV transcriptional activity, as illustrated by the higher levels of cell-associated HIV RNA, residual plasma viremia, immune activation and immune exhaustion in men compared to women on ART [50]. Moreover, estrogen and other agonists of estrogen receptor-1 (ESR-1), including β-estradiol, inhibit HIV latency reversal in contrast to antagonists of ESR-1 such as tamoxifen, which activate HIV transcription [51]. These findings suggest that control of latent infection differ in men and women and, therefore, LRAs may act differently. Further work is underway to determine if targeting ESR-1 could lead to a novel LRA.

LRAs studied to date in clinical trials (specifically HDACi, disulfiram and maraviroc) may not have effectively cleared latently infected cells for several reasons. These include limited potency when used alone, some dose-limiting toxicities, potential insufficient delivery to lymphoid tissue, post-transcriptional blocks limiting viral protein expression [52], competing mechanisms of HIV persistence such as clonal expansion [53,54] and immune exhaustion limiting immune-mediated clearance of virus-expressing cells [55–57]. According to these findings, there is an ongoing search for new drug targets and also active efforts to test LRAs in combination with other LRAs or immune enhancement strategies (Table 1).

Immunomodulatory latency reversing agents

Newer approaches to target latent HIV that appear to be more promising, at least in animal models, include compounds that activate immune function in combination with induction of viral expression. These include toll-like receptor (TLR) agonists, immune checkpoint (IC) inhibitors and cytokine-based therapy such as interleukin (IL)-15.

Toll-like receptor agonists

Therapeutic stimulation of TLRs may lead to dendritic cell (DC) maturation, natural killer (NK) cell activation, enhanced antigen presentation, and enhanced adaptive immune responses [58,59]. In HIV, TLR agonists may play a role both as LRAs and as immunotherapy.

The TLR7 agonists GS-986 and GS-9620 activated multiple immune cell populations in simian immunodeficiency virus (SIV)-infected rhesus macaques on ART initiated during chronic infection and also induced cyclical increases in CD4+ T cell activation and plasma viremia [60]. Other studies in non-human primate (NHP) models have investigated the combination of GS-9620 with a therapeutic vaccine. When a TLR7 agonist was administered with an Ad26/MVA vector to SIV-infected NHPs who initiated ART during acute infection, there was a marked increase in both the magnitude and breadth of SIV-specific immune responses and also delayed viral rebound following ART discontinuation [61]. Even more exciting results were reported when GS-9620 was administered in combination with the broadly neutralising antibody (bNAb) PGT121 to SHIV-infected NHPs on ART initiated within seven days of infection [62]. Whereas almost all NHP receiving placebo, GS-9620-only or PGT121-only had viral rebound after ART was stopped, 5/11 NHP receiving both GS-9620 and PGT121 did not rebound during ART interruption, even after CD8-depletion. Whether the TLR7 agonist was acting as an LRA in both combination studies is unclear as blips in plasma virus were not observed. It is possible that the favourable effects of the TLR7 agonist were a result of immune enhancement, potentially through activation of NK cells. GS-9620 is currently being tested in phase 1 studies in HIV-infected individuals on ART (NCT02858401 and NCT03060447).

The TLR9 agonist lefitolimod (MGN1703) has also been explored in a non-randomised single arm study of HIV-infected individuals on ART. There was a significant induction of antiviral innate immune responses — including activation of plasmacytoid DCs, NK cells and T cells and an increase in plasma levels of interferon-α [63]. Interestingly, in 6 of the 15 study participants, there was a quantifiable increase in plasma HIV RNA during lefitolimod administration ranging from 21 to 1571 copies/mL, but no change in cell-associated HIV RNA and actually a slight decrease in cell-associated HIV-RNA post-treatment compared to baseline.

In summary, TLR agonists can activate multiple immune cell populations and may also reverse HIV latency, although it is unclear why this effect was primarily seen as increases in plasma HIV RNA and not cell-associated HIV RNA. This is in contrast to clinical trials of HDACi where an increase in cell-associated HIV RNA was observed more frequently than plasma HIV RNA. These findings may relate to timing for collection of specimens or the rapid elimination of cells that express viral RNA or protein. New tools that can detect the number of cells that express HIV RNA could potentially assist in future studies [64]. Whether the combination of TLR7 agonists with vaccines or antibodies has a similar effect on the reservoir in NHPs treated during chronic infection or in human studies remains eagerly awaited. There are multiple ongoing studies that include TLR7 or TLR9 agonists alone or in combination with other interventions (Table 1).

Immune checkpoint blockade as a means to reverse HIV latency

Signalling through ICs provides inhibitory signals to T cells and blockade of these pathways has shown great promise in the treatment of some malignancies [65]. In untreated HIV infection, increased expression of ICs is associated with immune exhaustion and disease progression [55,56,66–70]. This may be partially reversed through blocking ICs, which reinvigorates virus-specific T cell function *ex vivo* and in SIV-infected rhesus NHPs [71–75]. In addition, CD4+ T cells expressing IC, especially PD-1, lymphocyte activation gene 3 (LAG-3) and T cell immunoreceptor with Ig and ITIM domains (TIGIT), are enriched for HIV and, therefore, these proteins may play a role in the establishment and maintenance of HIV latency [4,56,76]. Using an *in vitro* model, we recently demonstrated that blocking PD-1 and TIM-3 before infection, reduced the establishment of HIV latency [77]. We and others have also shown that antibodies to PD-1 or PD-L1 can enhance virus production from CD4+ T cells collected from HIV-infected individuals on ART, but only in the presence of an additional stimuli such as bryostatin or Staphylococcal enterotoxin A/B (SEA/SEB) [78]. In contrast, the administration of anti-PD1 alone seems to have limited effects on virus production *ex vivo* [79].

A limited number of case reports or small case studies have examined the effects of IC blockers on HIV persistence in HIV-infected individuals on ART receiving IC blockade for cancer. These studies have showed an increase in cell-associated unspliced HIV RNA following anti-CTLA4 (ipilimumab) and anti-PD-1 (nivolumab) [77,80] and a decline in the frequency of infected cells after repeated administration of anti-PD-1 [78,81]. In contrast, another study found no consistent change in cell associated or plasma HIV RNA following anti-PD-1 [82].

Collectively, these studies suggest that IC inhibitors may enhance immune effector functions and perturb HIV latency, but larger clinical trials in HIV-infected individuals are still needed to fully determine whether IC blockers can be used to eliminate latently infected cells. A very significant limitation with these antibodies is the risk of immune-related adverse events which may occur with the currently available antibodies.

Cytokine therapy

Several clinical studies have investigated the effect of IL-2 or IL-7 on HIV latency and have overall not been

successful. Three clinical trials of recombinant human IL-7, one in the setting of intensified ART, showed that the homeostatic effects of this cytokine induced proliferation of CD4+ T cells and actually expanded the pool of latently infected cells [83–85]. More recently, IL-15 and IL-15 superagonists have been explored in cure-related research. In rhesus NHPs infected with SHIV or SIV, both native heterodimeric IL-15 and the IL-15 superagonist ALT-803 increased levels of virus-specific CD8+ T cells in lymph node tissue, including in B cell follicles [86,87], an important anatomical site for SIV persistence [88]. These studies were done in infected NHP not on ART and thus were not designed to investigate the latency-reversing effects of IL-15. However, therapeutically relevant concentrations of ALT-803 were previously shown to reverse latency and even sensitise latently infected CD4+ T cells for CD8+ T cell recognition *ex vivo* [89]. ALT-803 is currently being investigated in a clinical trial for its effect on HIV persistence in HIV infected individuals on ART (NCT02191098).

Combination strategies to optimise shock and kill

Combination LRAs

Several *in vitro* and *ex vivo* studies have shown that combinations of LRAs can act synergistically to enhance latency reversal [40,90–96], particularly when combining a PKC agonist with either a bromodomain inhibitor or an HDACi [91,93,96]. It is therefore possible that combining LRAs with different mechanisms of action will significantly enhance latency reversal, although safety remains a limiting factor for advancing this approach. Other combination approaches include combining LRAs with therapeutic vaccines, IC inhibitors, TLR agonists, interferon, bNAbs or pro-apoptotic compounds.

Combining LRA with therapeutic vaccination or interferon

The therapeutic peptide-based HIV vaccine, Vacc-4x, given with rhGM-CSF as local adjuvant, was tested in a study where a prime-boost regimen of six vaccine administrations were followed by three infusions of the HDACi romidepsin [97]. This combined intervention was associated with a moderate decrease in the frequency of latently infected CD4+ T cells but did not delay time to virus rebound during ATI [97]. Therapeutic HIV vaccination combined with vorinostat or romidepsin is also being investigated in several ongoing studies in HIV-infected individuals who started ART <6 months or <4 weeks after primary HIV infection. Finally, based on post hoc observations of the effects of panobinostat [98], panobinostat is tested in combination with pegylated interferon-α2a in an ongoing study (NCT02471430). Additional studies are investigating other combinations that include an LRA and are summarised in Table 1.

Using pro-apoptotic drugs to enhance killing of virus-expressing cells

As latency reversal may effectively shift virus-expressing cells from a pro-survival to a pro-death state, it is conceivable that combining an LRA with a pro-apoptotic drug may promote selective killing of virus-expressing cells, as recently reviewed by Kim *et al.* [37]. Several pro-apoptotic drugs have been developed for use in cancer treatment, including Bcl-2 antagonists, Pi3K/Akt inhibitors, Smac mimetics, and RIG-I inducer [37,99]. Preliminary analyses using the pro-apoptotic BCL-2 antagonists either alone or in combination with an LRA, have demonstrated a decrease in HIV-infected cells, both *in vitro* and *ex vivo* [100–105]. However, further evaluation is needed to better understand the efficacy and safety profile of this approach.

Areas for future research

Work to date has showed evidence of latency reversal *in vivo* in both blood and tissue-derived CD4+ T cells, however it is unclear if LRAs have different effects in clonally expanded infected cells; different T cell subsets; in transcriptionally silent or actively transcribing cells; or on intact or defective proviruses [3,106]. These are all important sources of latent virus to understand. Given that a fraction of latently infected cells contain full-length intact proviruses that are not easily induced even by maximal T cell stimulation [1], it is unclear if these cells can be ignored or need to be specifically targets. It is also unknown whether latently infected CD4+ T cells in anatomic compartments such as lymphoid follicles respond to LRAs, as few studies have examined tissue sites before and following LRA treatment. Advances in imaging the reservoir with radiolabelled antibodies that bind to HIV envelope [107] could potentially answer this question should these tools work in human clinical trials. Finally, virus rebound after cessation of ART can occur even after dramatic reductions in reservoir size, for example following very early ART or stem cell transplantation [108,109]. These sobering case reports of prolonged absence of viremia and sudden rebound many months after cessation of ART suggest that unless every virus is eliminated, potent immune surveillance will be required to keep whatever virus remains in check.

Other important considerations for future studies include timing of LRA administration and selection of participants who have a high likelihood of response to an LRA. HIV-infected individuals who initiated ART in acute infection have a lower frequency of latently infected cells, better preserved T cell function and little or no accumulation of immune escape mutations [110]. They are, therefore, more likely to eliminate virus-expressing cells and have a higher likelihood of spontaneous post-treatment control after cessation of ART [111]. These observations underscore the need for a placebo control group when studying this population. Interestingly, modelling and NHP studies have

suggested that latently infected cells turn over at a higher rate during productive infection, that is, before ART is initiated, which has led to the speculation that administration of an LRA intervention during this labile phase might have greater potency [112,113]. An ongoing study of romidepsin and 3BNC117 is investigating this hypothesis (Table 1).

Conclusion

Overall, we believe that LRAs play a key role in HIV cure strategies as a component of a combination approach and to provide a mechanism to 'expose' virus. The potency of LRAs can be potentially enhanced through development of compounds with increased specificity for infected cells, ideally through an HIV-specific mode of action; by improving delivery to key tissue sites, potentially through nanoparticle technology; or by using LRAs in conjunction with other interventions to enhance killing of virus-expressing cells. Immunomodulatory LRAs have several advantages and results of human clinical trials with these agents are eagerly awaited.

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Conflict of interest

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