

Future approaches to clearing the latent human immunodeficiency virus reservoir: Beyond latency reversal

**Author:**Alexander M.L. Hayes¹ **Affiliation:**

¹Medical Sciences Division,
Faculty of Clinical Medicine,
University of Oxford,
Oxford, United Kingdom

Corresponding author:

Alexander Hayes,
Alexander-hayes@keble.ox.
ac.uk

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Background: While combined antiretroviral therapy (cART) allows near-normal life expectancy for people living with human immunodeficiency virus (HIV), it is unable to cure the infection and so life long treatment is required.

Objectives: The main barrier to curing HIV is the latent reservoir of cells, which is stable and resistant to cART.

Method: Current approaches under investigation for clearing this reservoir propose a 'Shock and Kill' mechanism, in which active replication is induced in latent cells by latency reversal agents, theoretically allowing killing of the newly active cells.

Results: However, previous studies have failed to achieve depletion of the T central memory cell reservoir, are unable to target other latent reservoirs and may be causing neurological damage to participants.

Conclusion: Future approaches to clearing the latent reservoir may bypass latency reversal through the use of drugs that selectively induce apoptosis in infected cells. Several classes of these pro-apoptotic drugs have shown promise in *in vitro* and *ex vivo* studies, and may represent the basis of a future functional cure for HIV.

Keywords: HIV; latency reversal; viral reservoir; pro-apoptotic drugs; cART.

Introduction

The global HIV/AIDS pandemic has caused over 25 million deaths since its origins in the 20th century and remains without a cure. Combined antiretroviral therapy (cART) is the current treatment of choice for human immunodeficiency virus (HIV) infection¹ and suppresses viral load below detectable levels with correct administration.^{1,2} However, lifelong treatment is required, at considerable expense to healthcare systems,¹ as the cessation of therapy causes a rapid rebound of viraemia within days³ and eventual progression of the disease to AIDS. Furthermore, escape mutations of the virus are common and reduce efficacy,^{4,5} necessitating expensive combination therapy with multiple drugs. Viral rebound occurs due to the presence of latent viral reservoirs, rather than continuing low-level replication during cART, as shown by clonal evolutionary studies.⁶ On therapy cessation these latently infected cells clonally expand, seeding a population of infected cells containing intact provirus.⁷ Whilst cART has been successful in extending life expectancy of people living with HIV in high-income countries, this remains impaired compared to healthy adults.⁸ As cART is limited by mutations, is an expensive lifelong treatment and may not be available to those in countries with poorly developed healthcare systems, development of a cure for HIV is desirable. Cure research would be of considerable impact in South Africa, which has the highest number of people living with HIV in the world, at 7.7 m as of 2018, a prevalence of 20.4% in adults (UNAIDS 2019)⁹. In that year 77 000 deaths were recorded due to AIDS-related illnesses (UNAIDS 2019), although this number is coming down and life expectancy is rising as the cART programme continues to grow.

Reservoirs of latently infected cells are the main barrier to cART curing HIV. The first latently infected cells to be identified were CD4⁺ central memory T cells (T_{CM}) containing replication-competent, integrated provirus.¹⁰ These were found to be present in patients on cART, with no change in reservoir size with continuous therapy¹¹ as the quiescent cells do not contain actively replicating virus, and so are unaffected by cART. The T_{CM} reservoir is present even if cART is initiated within the first week post exposure,¹² so is established early in infection. It is also extremely stable, as the latent cells have a half-life of around 44 months,¹³ so is unlikely to

be cleared in a patient's lifetime. The T_{CM} reservoir contains the majority of latent cells, but other reservoirs are also present and form barriers to cure research. Several myeloid cell lines have been shown to harbour latent HIV in chronic infection and contribute to viral rebound on cART cessation.^{14,15} There is evidence for the presence of latent reservoirs in tissue macrophages¹⁶ and microglial cells and astrocytes in the central nervous system (CNS).^{17,18} There is no *in vivo* evidence of latently infected tissue dendritic cells but infection has been shown *in vitro*,¹⁹ and it has been proposed that this may be transferred to $CD4^+$ cells via a virological synapse.²⁰ Follicular dendritic cells have been shown to maintain a stable pool of virus on their surface without being infected, providing a latent reservoir within secondary lymphoid tissue.²¹ Further reservoirs have been suggested, such as within fibrocytes,²⁰ but it is now apparent that the T_{CM} reservoir is not the only source of latently infected cells. Potential cures must, therefore, target all identified reservoirs to be successful.

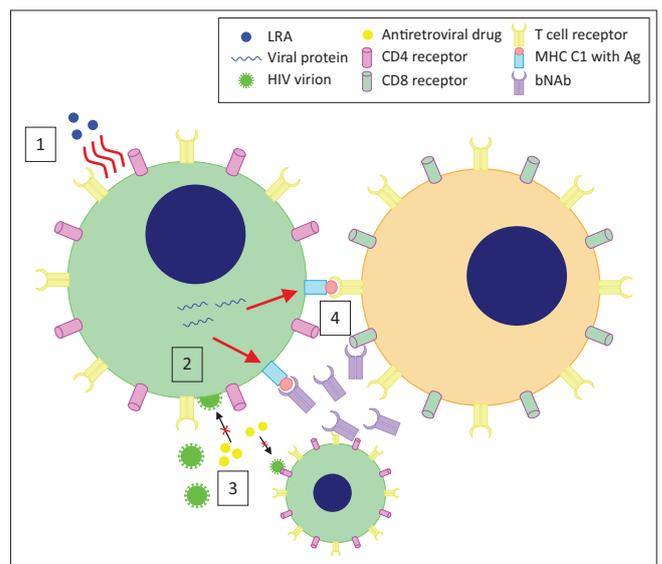
Several approaches to clearing the latent reservoirs have been investigated, with the 'Shock and Kill' combination receiving particular interest. However, recent findings seem to suggest this is unlikely to ever provide a cure for HIV^{22,23,24,25,26,27,28,29,30} and may have unresearched deleterious side effects.^{31,32,33} An emerging class of drugs targeting the apoptosis pathways in latently infected cells may allow reservoir depletion without latency reversal, and therefore merit further research as a potential cure for HIV.

Shock and Kill

A potential approach to clearing HIV latent reservoirs is 'Shock and Kill', in which the reservoir would be 'shocked' with latency-reversing agents (LRAs) to induce replication of the provirus. 'Killing' of the activated cells would then be achieved through viral cytopathic effects, $CD8^+$ T cells, cART or other agent, resulting in clearance of the latent cells and curing the infection. This mechanism is outlined in Figure 1. Several LRAs have been developed, many using Yang's *in vitro* model of latently infected $CD4^+ T_{CM}$ ³⁴ to identify compounds that selectively induce viral replication in latent cells. These include disulfiram²² and histone deacetylase inhibitors (HDACIs) such as vorinostat and panobinostat. Early *in vivo* studies were promising as latency reversal was demonstrated with an increase in viral gene expression in the resting cells observed with disulfiram,^{23,24} vorinostat²⁵ and panobinostat²⁶ administration. However, the key caveat is that none of these studies decreased reservoir size *in vivo* as the number of latent cells did not decrease following latency reversal. Whilst replication was induced in a proportion of the latent cells, this did not lead to immune or viral-mediated killing of reactivated cells.

Several reasons have been proposed for the failure of LRAs alone to reduce reservoir size. The initial principle of 'Shock

and Kill' was that viral cytopathic effects and lysis, due to HIV replication, aided by cytotoxic T lymphocyte (CTL)-mediated killing of actively infected cells, would be sufficient to kill reactivated, formerly latent, cells. Shan et al.²⁷ showed that the viral cytopathic effect alone is insufficient to kill cells, and the vigorous CTL response is lost in chronic HIV infection. However, CTL stimulation increased latent cell clearance, leading to the suggestion that some form of immune stimulation may be required alongside latency reversal to achieve a reduction in reservoir size. The CTL response to reactivated cells may be insufficient to reduce reservoir size on latency reversal, due to 98% of latent T cells carrying escape mutations to CTL killing²⁸ and T cell exhaustion in chronic infection²⁹ reducing the efficacy of the CTL response. Furthermore, LRAs may not affect non- T_{CM} reservoirs³⁰ as macrophages are resistant to the cytopathic effects of HIV, and dendritic cells do not integrate viral DNA, meaning that LRAs would be unable to cause viral replication and subsequent targeting by the immune system. It has also been proposed that continued cART may not completely inhibit infection of further cells by virions produced from reactivated, formerly latent cells.³⁰ Thus, LRA administration may lead to infection of previously uninfected cells. It is therefore clear that LRA administration alone does not reduce reservoir size, due to a lack of viral cytopathic effects and lysis, insufficient CTL response and the potential failure of cART to suppress replication. As such, stages 3 and 4 from Figure 1 are unlikely to be successful and an additional killing agent is required to achieve the second goal of 'Shock and Kill'. This may involve immune stimulation, with natural killer (NK) cell stimulation showing some promise *ex vivo*³⁵ or other agents, such as broadly neutralising antibodies (bNAbs), which appear to mediate



1. Latency reversing agents (LRAs) reactivate viral replication in latently infected cells.
2. Viral proteins are produced which are assembled into HIV virions. Antigen (Ag) processing also occurs, leading to presentation on Major Histocompatibility Complex Class 1 (MHC CI).
3. Combined antiretroviral therapy (cART) inhibits virion release. Virions which are released are unable to infect other cells due to cART.
4. Actively replicating cells are cleared by HIV-specific $CD8^+$ T cells (which may be pre-stimulated) or a killing agent such as a broadly neutralising antibody (bNAb).

FIGURE 1: Proposed mechanism of 'Shock and Kill'.

antibody-dependent cellular cytotoxicity of activated latently infected cells in mice³⁶ but human studies are required to assess their efficacy. Killing agents that selectively induce apoptosis of HIV-infected cells have also shown promise *in vitro* (see Pro-apoptotic drugs [PADs]).

Limitations of latency-reversing agents

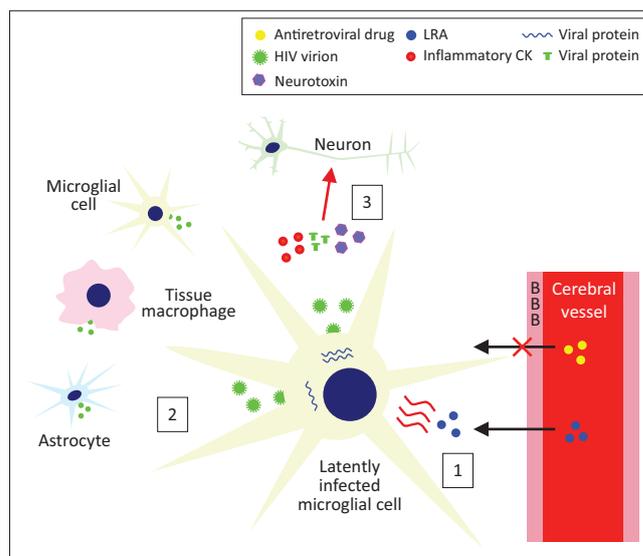
Whilst it has been repeatedly shown that LRAs alone are unable to reduce the size of the latent HIV reservoir, administration of a killing agent alongside an LRA has been proposed as a method of clearing the reactivated cells.³⁷ However, it seems likely that even with killing agents LRAs cannot clear enough latent cells to achieve a functional cure and may cause complications due to non-T cell HIV reservoirs. Future studies in humans to test the efficacy of killing agents administered alongside LRAs could be carried out by administering a previously tested LRA, such as disulfiram, alongside a killing agent such as a bNAb to participants living with HIV and measuring the change in reservoir size using a technique such as TILDA.³⁸ However, the combination of an LRA with, for example, a bNAb, is unlikely to clear the myeloid reservoir as dendritic cells are likely to be unaffected by LRAs.³⁰ Therefore, even if functional elimination of the T_{CM} reservoir is achieved, viral rebound on cART cessation may still occur due to the presence of the myeloid reservoir, which would likely not be cleared by current 'Shock and Kill' approaches under investigation.

Human immunodeficiency virus-associated neurological disorders (HANDs) are well-observed in patients on cART and may lead to widespread neurological impairment,³⁹ suggesting significant damage to the CNS even when viraemia elsewhere is suppressed below detectable levels. The cause of this appears to be relatively poor blood-brain barrier (BBB) penetrance by antiretrovirals,³¹ allowing continued HIV replication alongside the presence of latently infected microglial cells, perivascular macrophages and astrocytes.²⁰ Recent research suggests that impaired neurogenesis may underpin HAND persistence even with cART.³² A study on the CNS penetrance of LRAs³³ showed that disulfiram and vorinostat are able to cross the BBB relatively easily. Whilst this could enable targeting of the CNS latent reservoir, it may mean that current *in vivo* studies of LRAs are damaging to the patients involved, as there is no reduction in latent reservoir size, whilst LRAs are able to penetrate the CNS and increase viral replication with no inhibition from antiretroviral drugs. This may potentially lead to an increase in viral protein, inflammatory cytokine (CK) and neurotoxin release from infected macrophages and microglia, which could increase the probability of HAND development (personal communication, Grant Campbell) as shown in Figure 2. Therefore, in addition to their inability to affect all HIV reservoirs, even with the accompanying administration of killing agents, LRAs may cause increased neurological impairment of patients.

Furthermore, if the killing agent used in a hypothetical cure is unable to penetrate the BBB, there would be no reduction in the size of the CNS reservoir. This makes the development of a cure strategy distinct from 'Shock and Kill', involving the direct killing of latent cells without the need for latency reversal, desirable. The PAD class mentioned earlier has shown promise in achieving this.

Interactions of human immunodeficiency virus with apoptosis

The human immunodeficiency virus has complex interactions with apoptosis pathways in infected cells.⁴⁰ In latently infected cells the increase in longevity observed is partially the result of avoidance of apoptosis.³⁷ A number of specific effects on apoptosis pathways have been suggested as the cause of this. The intrinsic apoptosis pathway is a response to cellular damage and involves pro-apoptotic factors causing mitochondrial outer membrane permeabilisation (MOMP), triggering the release of soluble proteins from the mitochondrial intermembrane space. These include the cytochrome c and second mitochondria-derived activator of caspase (SMAC, also known as DIABLO⁴¹). Cytochrome c binds cytosolic proteins to form the apoptosome, which activates the 'initiator' enzyme caspase-9. This, in turn, cleaves the 'executioner' enzymes caspase-3 and caspase-7 which trigger the cleavage cascade of other caspase enzymes resulting in cell death. The second mitochondria-derived activator of caspase activates this process through disinhibition of caspases 3, 7 and 9 via binding and inactivating the X-linked inhibitor of apoptosis (XIAP). The X-linked inhibitor of apoptosis binds these caspases in the absence of SMAC, inhibiting their activity



1. LRAs cross the blood brain barrier (BBB) but antiretrovirals do not. This induces viral replication in latently infected cells (for example microglial cells), leading to production of HIV virions, viral proteins, inflammatory cytokines (CKs) and neurotoxins.
2. HIV virions infect other central nervous system (CNS) cells.
3. Viral proteins, inflammatory CKs and neurotoxins damage neurons, leading to HAND development.

FIGURE 2: Proposed mechanism for human immunodeficiency virus associated neurological disorder development on latency reversing agent administration.

and directing their degradation by the proteasome. The second mitochondria-derived activator of caspase antagonises this activity, allowing the progression of the apoptotic process.⁴¹ Several other IAPs are also inhibited by SMAC, including the baculoviral IAP repeat-containing protein 2 (BIRC, also known as cellular IAP1).⁴¹

Viral proteins, in particular Tat and Nef, cause inhibition of this intrinsic apoptosis pathway in latently infected cells. Tat upregulates host production of anti-apoptotic proteins including XIAP and Bcl2.⁴² The X-linked inhibitor of apoptosis inhibits caspase activity, as detailed earlier, whilst Bcl2 inhibits MOMP,⁴³ thus preventing the release of cytochrome c and SMAC. Tat also inhibits the pro-apoptotic proteins p53 and the Bcl2-associated antagonist of cell death (BAD).⁴⁰ Nef causes p21-activated kinase activation, leading to phosphorylation of BAD, thus inhibiting its pro-apoptotic activity (binding to and inhibition of Bcl2),⁴⁴ and also inhibits caspase-8 and caspase-10.⁴⁰ Conversely, in the CNS, it appears that Tat and Nef may induce autophagy and apoptosis in HAND pathogenesis.^{45,46} Expression and activation of anti-apoptotic proteins are therefore increased in latently infected cells, making this an attractive therapeutic target.

Pro-apoptotic drugs

Several classes of pro-apoptotic compounds have been developed recently for cancer treatment,^{47,48} as tumour cells often over-express or activate IAPs allowing the evasion of apoptosis, a key hallmark of cancer. Some of these have been shown to induce apoptosis of latent HIV infected cells *in vitro*, leading to speculation that PADs may contribute to a future cure.

Second mitochondria-derived activator of caspase mimetics (SMs) are a class of drugs which bind IAPs in a similar fashion to the endogenous molecule.⁴⁸ They cause caspase activation via IAP binding⁴⁹ and appear to reduce IAP activity through both degradation and inhibition of the proteins,⁵⁰ and therefore induce apoptosis. Second mitochondrias have recently been developed for cancer treatment, inducing apoptosis⁴⁸ of tumour cells which over-express or activate IAPs. The SMs birinapant, embelin and GDC-0152 were investigated for their efficacy to induce apoptosis in latently infected T_{CM} by Campbell et al.⁵¹ All were found to cause rapid degradation of XIAP and BIRC2, which were upregulated in the infected cells, and importantly showed selective killing of HIV-infected T_{CM} compared to uninfected T_{CM} with the doses required for 90% clearance of HIV-infected cells causing a small increase of T_{CM} cell death, of 3.5% – 4.6% above basal levels. This occurred in the absence of increased virus production, despite findings in models of latency by Pache et al.⁵² suggesting that SMs may act as LRAs due to the reduction in BIRC2 leading to increased NF κ B signalling, resulting in an increased viral transcription. However, in latent HIV- T_{CM} from patients undergoing cART, the Pache study showed that SMs only caused latency reversal when used in combination with an LRA such as vorinostat.

Therefore, it appears that the major effect of SMs on latently infected T_{CM} is to induce apoptosis rather than reactivate transcription.

Furthermore, Campbell et al. demonstrated the mechanism of apoptosis on SM administration is dependent on the induction (but not completion) of autophagy. Wortmannin (an inhibitor of early stages of autophagy) led to a reduction in apoptosis despite the degradation of IAPs by SMs. Further findings suggest that, following IAP degradation, autophagy proteins form a scaffold for the assembly of a ripoptosome-like death-inducing signaling complex (DISC), which can then initiate apoptosis via caspase 8 induction. This is an additional mechanism to the simple removal of IAP inhibition due to their degradation and may explain the selectivity of SMs for inducing death of HIV- T_{CM} . Inhibitors of later stages of autophagy did not reduce apoptosis, suggesting that the process of autophagy itself is not required for this mechanism of cell death. Induction of autophagy is sufficient for apoptosis, as autophagy proteins allow DISC formation. IAPs are degraded by SMs in uninfected T_{CM} but this does not lead to the same degree of cell death due to reduced autophagy induction and therefore decreased probability of DISC formation. HIV- T_{CM} are more prone to autophagy induction than uninfected cells as a result of the effects of viral proteins. Gag and Nef interact with autophagy factors to increase autophagy induction, which in turn augments HIV yields in cells containing the actively replicating virus.⁵³ Nef and Vif inhibit later stages of autophagy to prevent HIV degradation.^{53,54} Therefore, HIV- T_{CM} are more prone to autophagy induction than uninfected cells, providing a potential explanation for the selective apoptosis observed in latently infected cells on SM administration. Over-expression or activation of IAPs in infected cells may also contribute to the selectivity of SMs.

These findings present *ex vivo* evidence of the selective killing of latently infected T_{CM} by SMs, along with a convincing mechanism of how uninfected cells are spared. Clinical trials in humans may now be justified, but should be performed with caution as the side effects of these drugs in people living with HIV are unknown. A number of SMs have been used in phase I trials for various cancers, with a generally tolerable safety profile, although the occasional occurrence of cytokine release syndrome in patients is of some concern.⁵⁵ *In vitro* and *ex vivo* findings suggest SMs may be able to deplete the T_{CM} reservoir *in vivo*, and, whilst if 90% of latently infected cells are killed (as in the Campbell study) the patient will not be cured of HIV, this eclipses reservoir clearance demonstrated by current 'Shock and Kill' approaches. However, it is unclear if these drugs would induce apoptosis in non-T cell reservoirs to the same degree, which may prevent the development of a functional cure. Future *in vitro* and *ex vivo* studies of PADs using latently infected myeloid cells from people living with HIV would be advisable to further test their suitability.

In vitro and *ex vivo* studies of other PADs have also shown promise. Bcl-2 antagonists such as venetoclax caused disinhibition of MOMP and led to the depletion of latent HIV- T_{CM} from patients on cART in an *ex vivo* study.⁵⁶ P13K/Akt inhibitors increase the expression of pro-apoptotic genes downregulated by HIV and have shown promise in predisposing latently infected macrophages and microglia to cell death.^{57,58} RIG-1 inducers activate an innate immune response to viral RNA, triggering apoptosis. An *ex vivo* study demonstrated selective depletion of latent HIV- T_{CM} using the RIG-1 inducer acitretin.⁵⁹ These classes of PADs also merit further investigation and could be used in combination with SMs in a future approach to clearing the latent reservoir to induce apoptosis in a greater portion of latently infected cells.

Conclusions

Recent 'Shock and Kill' research has failed to demonstrate significant depletion of the viral reservoir, and it remains unclear if the use of LRAs may cause HANDs. PADs may represent a promising future approach to curing HIV given their *in vitro* and *ex vivo* success in selectively causing apoptosis in latently infected cells, with SMs appearing effective against the T_{CM} reservoir and P13K/Akt inhibitors against myeloid reservoirs. Several of these drugs have been used in clinical trials for other conditions, and further investigation into their potential for people living with HIV is merited.

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Competing interests

I declare that no competing interests exist.

Author's contributions

I am the sole author for this work.

Ethical consideration

This article followed all ethical standards for carrying out research without direct contact with human or animal subjects.

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Data availability statement

Data sharing is not applicable to this article as no new data were created or analysed in this study.

Disclaimer

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