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HIV infection cannot be cured due to the persistence of a reservoir of latently infected cells. Furthermore, virally suppressed individuals experience chronic immune activation from ongoing low-level viral expression. Drugs that inhibit HIV transcription and/or reactivation of latent HIV have been proposed as a strategy to reduce HIV-associated immune activation and/or to achieve a functional cure. We evaluated 26 small molecules, both previously reported drugs and new drug candidates, for their ability to act as “latency promoting/silencing agents (LPAs)” that can reduce or prevent HIV expression after T cell activation. Using a panel of RT-ddPCR assays, we measured the progression through HIV transcription and pinpointed the step at which each of those drugs inhibited HIV transcription, with and without prior activation. While some drugs primarily inhibited one or two steps in HIV reactivation, other drugs (CDK inhibitors, splicing inhibitors, tanespimycin, and triptolide) inhibited multiple stages of HIV transcription and blocked the production of supernatant viral RNA. Dinaciclib, AZD4573, and pladienolide B also appeared to inhibit HIV splicing in unstimulated PBMC. By selecting drugs with known mechanisms of action, we specifically identified cellular factors and pathways that may be involved in regulation of HIV expression. These drugs/targets deserve further study in strategies aimed at reducing HIV-associated immune activation or achieving a functional cure.

Mechanisms and efficacy of small molecule "latency promoting agents" to inhibit HIV reactivation ex vivo

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Abstract

Drugs that inhibit HIV transcription and/or reactivation of latent HIV have been proposed as a strategy to reduce HIV-associated immune activation and/or to achieve a functional cure, yet comparative studies are still lacking. We evaluated 26 drugs with different mechanisms, including drugs previously reported to inhibit HIV transcription (inhibitors of Tat-dependent HIV transcription, Rev, HSF-1/PTEF-b, HSP90, Jak/Stat, or SIRT1/Tat deacetylation) and other agents that were not tested before but predicted to inhibit HIV transcription initiation (inhibitors of PKC, NF-κB, SP-1, or Histone acetyltransferase; NR2F1 agonists), elongation (inhibitors of CDK9/PTEF-b), completion (inhibitors of PolyA-polymerase), or splicing (inhibitors of human splice factors). To investigate if those drugs would vary in their ability to affect different blocks to HIV transcription, we measured levels of initiated, elongated, mid-transcribed, completed, and multiply spliced HIV RNA in PBMC from ART-suppressed individuals following ex vivo treatment with each drug and subsequent T cell activation. We identified new drugs that prevent HIV reactivation, including CDK and

splicing inhibitors. While some drugs primarily inhibited one or two steps, other drugs (CDK inhibitors, splicing inhibitors, tanespimycin, and triptolide) inhibited multiple stages of HIV transcription and blocked the production of supernatant viral RNA. These drugs/targets deserve further study in strategies aimed at reducing HIV-associated immune activation or achieving a functional cure.

Summary

HIV infection cannot be cured due to the persistence of a reservoir of latently infected cells. Furthermore, virally suppressed individuals experience chronic immune activation from ongoing low-level viral expression. Drugs that inhibit HIV transcription and/or reactivation of latent HIV have been proposed as a strategy to reduce HIV-associated immune activation and/or to achieve a functional cure. We evaluated 26 small molecules, both previously reported drugs and new drug candidates, for their ability to act as "latency promoting/silencing agents (LPAs)" that can reduce or prevent HIV expression after T cell activation. Using a panel of RT-ddPCR assays, we measured the progression through HIV transcription and pinpointed the step at which each of those drugs inhibited HIV transcription, with and without prior activation. While some drugs primarily inhibited one or two steps in HIV reactivation, other drugs (CDK inhibitors, splicing inhibitors, tanespimycin, and triptolide) inhibited multiple stages of HIV transcription and blocked the production of supernatant viral RNA. Dinaciclib, AZD4573, and pladienolide B also appeared to inhibit HIV splicing in unstimulated PBMC. By selecting drugs with known mechanisms of action, we specifically identified cellular factors and pathways that may be involved in regulation of HIV expression. These drugs/targets deserve further study in strategies aimed at reducing HIV-associated immune activation or achieving a functional cure.

Introduction

The global number of Human Immunodeficiency Virus (HIV)-related deaths has been strikingly reduced by antiretroviral therapy (ART). Nonetheless, ART fails to cure HIV, since HIV persists as a reservoir of infectious proviruses, including latently infected CD4+ T cells that do not produce virions constitutively but can be induced to produce infectious virus after physiologic T cell activation (1–3). The reversible silencing of viral gene expression allows survival of latently infected cells, while reactivation from a small subset of these cells is thought to be responsible for the plasma virus that rebounds after ART interruption (4, 5). Preventing HIV rebound from intact, replication-competent reservoirs will be an absolute prerequisite and poses the main barrier to cure HIV infection.

Cell-associated HIV transcripts can be detected in almost all people with HIV (PWH) on ART, despite suppression of plasma virus (6–9). Antiretroviral drugs in current clinical use do not block HIV transcription, providing a mechanism for the persistence of HIV-transcribing cells on prolonged ART. Using a panel of assays that can simultaneously quantify multiple different regions of HIV RNA, we recently showed that most infected CD4+ T cells from the blood of ART-suppressed PWH have initiated HIV transcription, while successively smaller fractions express 5’ elongated, polyadenylated (completed), and multiply spliced HIV transcripts, likely due to reversible blocks at sequential stages of HIV transcription/splicing (8, 10). While many of the proviruses in this “active reservoir” may be defective (9), some of the defective HIV RNA may trigger intracellular pattern recognition receptors and/or be translated into viral proteins that contribute to immune activation. The mechanisms of chronic immune activation in PWH are not completely defined and may be multifactorial, but the persistence of cell-associated HIV RNA (reviewed in (11)) has been correlated with T cell activation (12). It is believed that residual immune activation contributes to “non-AIDS” morbidity and mortality observed in ART-treated PWH (13).

These findings advocate for the development of therapies that can block reactivation from latency and/or inhibit HIV transcription in order to curb immune activation in ART-treated PWH. Additionally, inhibitors that durably silence HIV transcription could be beneficial in a "block-and-lock" strategy aimed at functional HIV cure (14). The objective of this strategy is to reinforce latency and prevent HIV reactivation in order to achieve a state of HIV remission without the need for antiretroviral therapy. An example of a promising block-and-lock strategy is the inhibition of the HIV transcription factor Tat using didehydro-cortistatin A (15) since Tat is needed to recruit P-TEFb and ensure efficient elongation of HIV transcription. In addition to Tat, HIV relies heavily on cellular factors to ensure robust transcription from the LTR promoter.

As such, cellular pathways imposing restrictions at the HIV promoter and elongation phase present interesting targets to silence HIV expression. For example, the mTOR inhibitor rapamycin reduced basal transcription (16) but not activation-induced HIV transcription (17), while INK128 reduced both via inhibition of PKC and downstream NF-κB signaling (18). In addition, inhibition of HSP90 has been reported to durably block HIV reactivation *in vitro* (19) and in vivo (20), even after the removal of the drug, by impacting NF-κB, NFAT, and STAT5 signaling pathways. Alternatively, the JAK-STAT inhibitor ruxolitinib reduced viral reactivation by blocking cytokine-induced STAT signaling (21). Recently, filgotinib was shown to reduce HIV splicing through a mechanism known as intron retention (22), a process previously observed for topotecan, another latency promoting agent (23, 24). Alternatively, HIV can be silenced epigenetically (25–27), including RNA-induced epigenetic silencing (28–30), or by retargeting the provirus out of active chromatin into transcriptionally silent regions (31, 32).

In this study, we examined both previously reported drugs and new drug candidates for their ability to act as "latency promoting/silencing agents (LPAs)" that can reduce or

prevent HIV expression after T cell activation. We screened 26 molecules from various categories, including: 1) agents previously reported to inhibit HIV transcription (such as inhibitors of Tat-dependent HIV transcription, Rev, HSF-1/PTEF-b, and HSP90) and/or T cell activation (Jak/Stat inhibitors); 2) drugs with mechanisms opposite of previously reported "latency reversing agents" (PKC inhibitors); and 3) drugs predicted to inhibit HIV transcription initiation (inhibitors of NF-κB, SP-1, or histone acetyltransferase; NR2F1 agonists), elongation (inhibitors of CDK9/PTEF-b or SIRT1/Tat deacetylation), completion (inhibitors of PolyA-polymerase), or splicing (inhibitors of human splice factors) [overview in **Table S1**]. Many of the selected drugs are either in clinical use or human trials.

The aims of this study were to use drugs as mechanistic probes to inform us about cellular pathways involved in HIV latency/reactivation, to identify new LPAs, and to compare the efficacy of various LPAs in cells from PWH. We hypothesized that drugs acting through different mechanisms would act selectively to impair the activation-induced reversibility of blocks at various stages of HIV transcription (or enhance baseline blocks in unstimulated cells). To investigate this hypothesis, we measured levels of different HIV RNA regions/transcripts in PBMC from ART-suppressed individuals following ex vivo treatment with different drugs (or control) with or without subsequent T cell activation. Various drugs selectively blocked activation-induced increases in HIV transcriptional initiation, elongation, mid-transcription, completion, and/or splicing. We discovered new LPAs, such as CDK inhibitors and splicing inhibitors, with nanomolar potency. Some drugs (including the CDK9 inhibitor AZD4573, the splicing inhibitor pladienolide B, the HSP90-inhibitor tanespimycin, and triptolide) inhibited multiple stages of HIV transcription and blocked the production of supernatant viral RNA (schematic overview in **Figure S1**). These drugs and combinations should be investigated further as latency silencing/promoting agents aimed to curb immune activation or towards a functional cure.

Results

Latency reversal assay in cells from PWH reveals candidate latency promoting agents (LPAs)

We selected 26 small molecules targeting pathways implicated in HIV transcription and/or the blocks to HIV transcription underlying latency **(Table S1)**. As positive controls, we included drugs such as ruxolitinib (21, 33) and KRIBB11 (34) for their well-characterized inhibition of HIV expression (Didehydro-Cortistatin A (15) could not be obtained). Initial drug concentrations were chosen based on levels attainable in plasma (when known) or set at twice the IC_{50} for the target of the drug and subsequently adjusted to the lowest effective and nontoxic dose. Initial screening of all drugs was performed in freshly isolated PBMCs from two ART-suppressed individuals **(Figure 1; Figure S2)**. PBMCs were cultured in the presence of individual drugs in DMSO or DMSO alone as a control, subsequently activated (anti-CD3/CD28), and harvested after 24h. Most drugs had no impact on cell viability **(Figure 1A)**, except triptolide, pladienolide B, and isoginkgetin. As a result, the doses of those three drugs were subsequently reduced. The progression through different blocks to HIV transcription was quantified by measuring the levels of initiated (TAR), 5' elongated (LLTR), mid-transcribed/unspliced (Pol), completed (PolyA), and multiply spliced (TatRev) HIV transcripts. The levels of those HIV transcripts were normalized to 1 µg of total cellular RNA (to normalize for the effect of the drug on global cellular transcription) and expressed as a percent of the activated DMSO control.

Triptolide profoundly reduced elongated (median relative to activated DMSO: 21%), mid-transcribed (9%), and multiply spliced (0%) HIV transcripts **(Figure S2A)**. CDK inhibitors reduced elongated HIV transcripts (median relative to activated DMSO between 67-76%) and further reduced mid-transcribed (median 20-43%) and multiply spliced HIV transcripts (8-13%), except for atuveciclib (46%, **Figure S2C**). Splicing inhibitors induced a

modest reduction in mid-transcribed HIV RNA (52-77%) but further reduced completed (2% for isoginkgetin, 17% for pladienolide B,) and multiply spliced HIV transcripts (23% for herboxidiene, **Figure S2D**). PKC inhibitors were most effective in reducing mid-transcribed HIV transcripts (51-68%), although staurosporine and sotrastaurin also reduced completed HIV transcripts (both 41%, **Figure S2E**).

Of the signal transduction inhibitors, ruxolitinib (Jak/Stat inhibitor), KRIBB11 (HSF1/P-TEFb inhibitor), and tanespimycin (HSP90 inhibitor) reduced elongated HIV transcripts (medians relative to activated DMSO: 39%, 60%, and 49%, respectively) and mid-transcribed HIV transcripts (14%, 16%, and 31%, respectively), but no additional decrease was observed in completed or multiply spliced HIV transcripts **(Figure S2H)**. In contrast, quercetin (SIRT1 activator/PI3K inhibitor) had no effect on elongated transcripts but induced progressive reductions in mid-transcribed (57%), completed (41%), and multiply spliced HIV transcripts (21%, **Figure S2H**). No substantial reductions in HIV transcripts were observed for inhibitors of NF-κB, vRNA processing, SP-1, or histone acetyltransferase **(Figure S2B, S2F and S2I)**. Since multiply spliced HIV transcripts are used as a predictive marker for productive infection after latency reversal (35, 36), drugs that reduced multiply spliced and/or completed HIV transcripts by more than 50% were selected from our drug screen as candidate LPAs for further experiments (i.e., ruxolitinib, triptolide, alvocidib, dinaciclib, AZD4573, quercetin, pladienolide B, isoginkgetin, herboxidiene, KRIBB11, and tanespimycin; **Figure 1B**).

Dose-response curves demonstrate LPAs with nanomolar potency

We examined dose-response curves for ruxolitinib, CDK inhibitors (alvocidib, dinaciclib, and AZD4573), quercetin, and pladienolide B **(Figure 2)**. The 50% maximum inhibitory concentration (IC_{50}) was determined based on the reduction in completed (PolyA) as well as multiply spliced (TatRev) HIV transcripts relative to activated DMSO **(Table 1)**.

We obtained low micromolar IC_{50} for ruxolitinib (0.27 and 0.26 μM for PolyA and TatRev transcripts, respectively). For CDK inhibitors, we obtained nanomolar IC_{50} for alvocidib (3.41 and 4.23 nM, respectively), and low nanomolar IC_{50} for AZD4573 (0.28 and 0.23 nM) and dinaciclib (0.55 nM). The most potent IC_{50} was observed for pladienolide B (0.18 and 0.06 nM for PolyA and TatRev transcripts, respectively). Quercetin demonstrated an IC_{50} of 1.41 μM and 1.13 μM for PolyA and TatRev transcripts, respectively. Due to possible decreases in viability at 10 μM, its concentration was reduced to 5 μM for subsequent experiments.

Various LPAs impact distinct blocks to HIV transcription after activation

The ability of our candidate LPAs to inhibit HIV transcriptional progression after activation was examined in PBMCs from a minimum of seven PWH per drug tested **(Figure 3)**. PBMCs were cultured in the presence of individual drugs in DMSO or in DMSO alone as a control, subsequently activated (anti-CD3/CD28), and harvested after 24h. Only ruxolitinib and triptolide reduced initiated HIV transcripts (**Figure 3A and Table 2**; median relative to activated DMSO: 34% and 2%, respectively; P=0.02 for triptolide). Ruxolitinib further reduced mid-transcribed HIV RNA (16%, P=0.02), but no additional reductions were observed in completed or multiply spliced HIV transcripts, in line with previous data (33). The CDK inhibitors alvocidib, dinaciclib, and AZD4573 potently reduced elongated (37%, 31%, and 25%, respectively; all P=0.02, except P=0.03 for AZD4573), mid-transcribed (20%, 16%, and 13%; all P=0.02), and multiply spliced HIV RNA (7%, 15%, and 3%; all P=0.02, except P=0.03 for alvocidib). Quercetin had little effect on initiated or elongated HIV RNA, but significantly reduced mid-transcribed (54%; P=0.02) and multiply spliced HIV RNA (29%; P=0.03).

Of the splicing inhibitors, pladienolide B and herboxidiene reduced elongated HIV RNA (**Figure 3A and Table 2**; median relative to activated DMSO: 32% and 44%, respectively; P=0.02 for herboxidiene), while isoginkgetin had less effect on elongated HIV RNA but instead reduced mid-transcribed HIV RNA (28%; P=0.02). Pladienolide B and isoginkgetin both potently reduced completed HIV RNA (8 and 6%, respectively; both P=0.02), while pladienolide B and herboxidiene further reduced multiply spliced HIV transcripts (0%, P=0.02 and 18%, P=0.03, respectively). KRIBB11 and tanespimycin both reduced elongated HIV RNA (36% and 34%, respectively; both P=0.02) and mid-transcribed HIV RNA (14% and 16%; both P=0.02), but only KRIBB11 was able to further reduce multiply spliced HIV transcripts (9%; P=0.02). Most of the effects on 5'elongation, mid-transcription, completion, and splicing remained significant even after correcting for multiple comparisons (Benjamini–Hochberg method; **Table 2, File S1**). When analyzing the effects of the drugs in individual study participants, we observed consistent reductions in 5'elongated, mid-transcribed, completed, and multiply spliced HIV transcripts, with only a few exceptions where specific drugs did not show an effect in a certain participant **(Figure S3)**.

We also calculated the ratio of one HIV RNA to another, allowing to evaluate the progression through HIV transcriptional elongation, completion, and splicing independent of effects at prior stages of HIV transcription, and independent of infection frequency or normalization to cell numbers. HIV transcriptional elongation (ratio of 5' elongated to initiated HIV transcripts [LLTR/TAR]; **Figure 3B**) was inhibited by CDK-inhibitors, splicing inhibitors, KRIBB11, and tanespimycin as compared to the activated DMSO. Significant reductions were obtained for herboxidiene and tanespimycin (both P=0.03), and trends were observed for alvocidib and isoginkgetin (both P=0.06). Interestingly, although ruxolitinib and triptolide dramatically reduced HIV transcriptional initiation **(Figure 3A, S3A and Table 2),** they had no impact on the subsequent elongation phase **(Figure 3B)**.

HIV transcriptional completion (ratio of polyadenylated to 5'elongated HIV RNA [PolyA/LLTR]; **Figure 3C**) was reduced for the CDK inhibitors alvocidib and AZD4573 (P=0.008 and P=0.02, respectively), but not for dinaciclib. Completion was also reduced for the splicing inhibitors pladienolide B (P=0.02) and isoginkgetin (P=0.03), but not for herboxidiene. KRIBB11 and triptolide also reduced completion (both P=0.02). Finally, only a few drugs were able to reduce HIV multiple splicing (ratio of multiply spliced to completed HIV RNA [TatRev/PolyA]; **Figure 3D**), including alvocidib (P=0.02), triptolide (P=0.03), and pladienolide B (P=0.046). After correcting for multiple testing, only the effects on completed transcripts remained significant for all the drugs, except for isoginkgetin **(Figure 3C, File S1)**.

Some LPAs sustain reduced HIV transcription for six days, depending on the study participant

Next, we evaluated the ability of the candidate LPAs to sustain the inhibitory effects during a prolonged ex vivo culture. To this end, PBMCs were cultured in the presence of individual drugs in DMSO or in DMSO alone as control, activated (anti-CD3/CD28), and harvested after six days. None of the drugs significantly reduced viability compared to activated DMSO **(Figure S4A)**. Ruxolitinib and triptolide caused sustained reductions in initiated HIV transcripts (medians relative to activated DMSO: 27%, P=NS and 1%, P=0.03, respectively; **Figure 4A and Table 3**). Mithramycin A was not selected from our drug screen initially due to the lack of an effect at 24h **(Figure 1B, Figure S2G).** However, we decided to test the drug on day six because extra cells were available from some study participants.

Although dinaciclib, tanespimycin, and mithramycin A showed no effects on transcriptional initiation after 24h **(Figure 3 and Table 2)**, they reduced initiated HIV transcripts after six days (48%, 15%, and 13%, respectively; all P=0.02; **Figure 4A and Table 3**). Compared to 24h, tanespimycin induced greater reductions in completed and

multiply spliced HIV transcripts at day six (3% and 0%, respectively; both P=0.02). On day six, the CDK inhibitors alvocidib, dinaciclib, and AZD4573 reduced elongated HIV transcripts (**Figure 4A and Table 3**; median relative to activated DMSO between 50-72%; all P=0.02 except P=0.06 for AZD4573), but the reduction was less than at 24h (**Figure 3A and Table 2**; median relative to activated DMSO at 24h between 25-37%). The CDK inhibitors also reduced completed and multiply spliced HIV RNA at day six, albeit to a lesser extent than the reductions observed after 24h. The effects of quercetin on mid-transcribed and multiply spliced HIV RNA at 24h were no longer observed at day six.

In contrast to 24h, the splicing inhibitors had little impact on elongated HIV RNA at day six, but the effect on multiply spliced HIV RNA was partially sustained (23%, 0%, and 3% for pladienolide B, isoginkgetin and herboxidiene, respectively; P=0.03 for isoginkgetin; **Figure 4A and Table 3**). Compared to 24h, KRIBB11 tended to show less effect on elongated (72%) and multiply spliced HIV RNA (38%). After correcting for multiple comparisons, we only obtained significant reductions in 5' elongated HIV transcripts for ruxolitinib, alvocidib, dinaciclib, tanespimycin, and mithramycin A **(Table 3, File S1)**, indicating a reduced ability of the drugs to maintain their effectiveness until day six compared to the initial 24h. In general, we observed greater variability in the effect of the drugs among study participants after six days **(Figure S4B-F)**. Some drugs had no effect or showed non-significant increases in the levels of different HIV transcripts compared to activated DMSO in certain study participants, which was very rare after 24h **(Figure S3A-E)**.

When calculating the ratio of one RNA to another on day six, we observed little effect of the drugs on HIV transcriptional elongation and completion **(Figure 4B and 4C)**. However, the ratio of multiply spliced transcripts to completed HIV RNA (TatRev/PolyA), was significantly reduced for both alvocidib (P=0.02) and isoginkgetin (P=0.03; **Figure 4D**), suggesting a sustained inhibition of those drugs on HIV splicing. After correcting for multiple

testing, these P values were no longer significant. However, the power was limited due to some participants who had undetectable TatRev or PolyA transcripts, resulting in values of zero or not defined (0/0 or x/0), especially for tanespimycin (2 out of 7) and triptolide (5 out of 6).

CDK-inhibitors appear to decrease baseline HIV splicing

For a selection of the compounds, we evaluated the ability to reduce baseline HIV transcription without activation. PBMCs were cultured in the presence of individual drugs in DMSO or DMSO alone as a control and harvested after 24 hours. For each drug, we tested PBMCs from at least three PWH, as indicated in **FigureS5B**. In line with previous studies (33), ruxolitinib and KRIBB11 (34) had little effect on the HIV transcription levels in the absence of T cell activation **(Figure S5A and B)**. The CDK-inhibitors dinaciclib and AZD4573 did not reduce the levels of 5'elongated or completed HIV transcripts compared to DMSO, but they eliminated multiply spliced HIV transcripts in all study participants (**Figure S5C**; median relative to DMSO: both 0%). Likewise, pladienolide B appeared to reduce the level of multiply spliced transcripts (33%, **Figure S5A and B**).

When evaluating the ratio of one RNA transcript to another **(Figure S5D)**, all tested drugs seemed to cause a modest reduction in HIV transcriptional elongation (LLTR/TAR). However, dinaciclib, AZD4573, and pladienolide B appeared to severely reduce HIV splicing (TatRev/PolyA). Due to the limited number of participants tested, these effects did not reach statistical significance.

Several LPAs block the production of supernatant viral RNA after T cell activation

Next, we evaluated if the drugs were able to limit the production of supernatant viral RNA after stimulation. PBMCs were cultured in the presence of individual drugs in DMSO or DMSO alone as control, activated (anti-CD3/CD28), and assessed for levels of PolyA HIV RNA in the supernatant at day six. Ruxolitinib, triptolide, and tanespimycin inhibited the

release of viral RNA in supernatant almost entirely (**Figure 5**; median relative to activated DMSO: all 0.0%; P=0.008, P=0.06, and P=0.03, respectively). The CDK-inhibitor AZD4573 and the splicing inhibitor pladienolide B also reduced the production of viral RNA in the supernatant (1.9% and 3.8%, respectively; P=0.008 for both). Ruxolitinib, AZD4573, and pladienolide B significantly reduced viral RNA production in supernatant even after correcting for multiple comparisons **(File S1)**. Dinaciclib, quercetin, isoginkgetin, herboxidiene, and mithramycin A also tended to reduce the median levels of supernatant HIV RNA, but the effects were not consistent enough to reach statistical significance.

Lower levels of HIV transcripts are not attributed to reduced infection frequency

Some of our candidate LPAs can block the effects of T cell activation, which could affect T cell proliferation. Therefore, we evaluated the extent to which variations in the level of infected cells may contribute to reductions in different HIV RNA transcripts. We measured the total number of viable cells (as a measure of proliferation) and the conserved U3-U5 long terminal repeat (LTR) HIV DNA region (as a measure of total infection frequency) at day six. Total live cell numbers were significantly lower for PBMCs treated with ruxolitinib, pladienolide B, isoginkgetin, tanespimycin, mithramycin A, and triptolide compared to the activated DMSO control, indicating reduced proliferation after T cell stimulation (all $P \leq 0.03$; **Figure 6A**). In contrast, CDK-inhibitors, quercetin, herboxidiene, and KRIBB11 did not change proliferation rates compared to the activated DMSO control. Despite differences in cell proliferation, we found no significant differences in total LTR HIV DNA after treatment with any of the drugs **(Figure 6B)**, indicating that differences in cell proliferation rates have no impact on infection frequency (median infection frequency among study participants was 600 HIV copies/~10^6 cells, or 0.06%, at day 6). This result implies that the observed decreases in HIV transcripts are not attributed to reduced proliferation or killing of infected cells, but instead due to enhancement of blocks at the different stages of HIV transcription.

Discussion

The goals of this study were to discover new latency promoting/silencing agents that reduce or prevent HIV expression after T cell activation, compare them to drugs previously reported to reduce HIV transcription, and investigate the mechanisms underlying HIV latency/reactivation. We screened 26 small molecules, of which most are being tested in human trials and/or FDA-approved (mostly for the treatment of cancer; **Table S1**). Our study demonstrated that certain drugs inhibit specific stages of HIV transcription in cells obtained from PWH ex vivo without impacting cellular viability. We identified new candidate LPAs, including CDK inhibitors (dinaciclib and AZD4573) and splicing inhibitors (pladienolide B, isoginkgetin, and herboxidiene). Some drugs (CDK inhibitors, splicing inhibitors, tanespimycin, and triptolide) inhibited multiple stages of HIV transcription (schematic overview in **Figure S1**) and blocked the production of supernatant viral RNA. Additionally, the CDK-inhibitors (dinaciclib and AZD4573) and pladienolide B appeared to inhibit the baseline expression of multiply spliced HIV transcripts in unstimulated PBMC **(Figure S5)**.

HIV transcriptional 5’ elongation and splicing were most sensitive for HIV suppression at six days following drug exposure. The CDK-inhibitors and mithramycin A durably reduced 5’ elongation for six days. The splicing inhibitors, in contrast, did not sustain the inhibitory effects on 5’ elongation after a six-day culture, but the effect on splicing was partially sustained **(Figure 4)**. It should be noted that we had less statistical power at day six compared to 24h, due to a smaller number of individuals tested per drug, and that there was an amplification failure of Pol and TatRev transcripts in participant 2461. Of note, there was also an amplification failure of TatRev in participant 2027 at 24h. In addition, it is possible that some drugs degraded over six days in the cell culture medium, since we did not add fresh drugs after day 0.

None of the tested drugs increased the infection frequency **(Figure 6B)**, which is promising since we aim to avoid expanding the reservoir, but at the same time, none of the drugs reduced the infection frequency. Previous studies have shown that ex vivo treatment of CD4+ T cells from viremic individuals with ruxolitinib decreased the frequencies of infected cells with integrated HIV (21). The effect was attributed to reduced anti-apoptotic Bcl-2 expression, a downstream target of STAT5 signaling. The absence of this effect in the current study could be attributed to the differential expression of anti-apoptotic genes between viremic and ART-suppressed study participants (37). We also used PBMCs, which are a more representative cell type than CD4+ T cells but have lower infection frequencies and could also show the effects of other cell types (for example, CD8+ T cells) on HIV transcription.

Triptolide

The most dramatic inhibition of HIV transcription was observed after treatment with triptolide, with a more than 95% decrease in initiated TAR transcripts. Triptolide is a general RNA polymerase inhibitor (38, 39), induces proteasomal degradation of Tat (40), and also inhibits NF-κB signaling (41). Furthermore, triptolide has been investigated to modulate cancer gene expression via epigenetic downregulation of super-enhancer-associated genes (e.g., BRD4, MYC, RNA Pol II) (42). Although both initiated (TAR) and 5' elongated (LLTR) transcripts were decreased relatively to activated DMSO, the high ratio of LLTR/TAR transcripts **(Figure 3B)** indicates that the predominant mechanism is likely through inhibition of cellular (or perhaps Tat-mediated) HIV transcriptional initiation, or epigenetic mechanisms that prevent initiation of HIV transcription, and not at the level of Tat-mediated transcriptional elongation. Although promising, triptolide has limitations regarding bioavailability and toxicity, limiting its clinical potential (43). However, there is promise in the analog LLDT-8, which has shown less toxicity (43, 44).

CDK-inhibitors

Our data suggest that CDKs (including CDK9) or their downstream targets appear to be involved in activation-induced reversal of the baseline block to HIV transcriptional completion and multiple splicing. In addition, CDKs may contribute to the baseline block to HIV splicing in unstimulated cells. Only a few studies have investigated the effect of CDK-inhibitors on HIV expression in cells from PWH. One study showed a potent reduction of HIV expression in cell lines after treatment with FIT-039 (45). However, FIT-039 had no effect on *in vitro* infected primary cells. In addition, the CDK inhibitor flavopiridol (alvocidib) was shown to reduce HIV RNA in supernatant from infected cells from PWH even after drug withdrawal (46). Recently, the selective CDK9 inhibitor LDC000067 has been shown to reduce HIV expression in cell lines (47). LDC000067 also reduced multiply spliced TatRev transcripts in cells from PWH, which is congruent with our findings using other CDK inhibitors **(Figure 3A)**, although alvocidib, dinaciclib, and AZD4573 reduced the levels of multiply spliced HIV transcripts more profoundly than LDC000067 in cells from PWH. The same study showed that LDC000067 maintained decreased levels of HIV expression after drug removal *in vitro*, but only in combination with an inhibitor of CDK8/19 (47). A limitation of the current study is that we did not investigate the effects after drug removal.

Splicing inhibitors

Control of HIV splicing is vital for HIV replication, as it allows the expression of different mRNAs and proteins at particular stages in the viral life cycle. Previously, we have shown that blocks to HIV splicing represent a conserved mechanism of HIV latency in multiple primary cell models using infectious viruses (48), as well as cells from blood and tissues of HIV-suppressed PWH (8, 10). In addition, we have also identified human splice factors that are differentially expressed upon activation (48). Consequently, interfering with

HIV splicing represents an interesting drug target to silence HIV expression. To our knowledge, this is the first time that the splicing inhibitors pladienolide B, isoginkgetin, or herboxidiene have been investigated for their effects on HIV transcription and reactivation. We expected most of the effect to occur on the level of multiply spliced transcripts, as was previously observed for other splicing inhibitors (49). However, pladienolide B and herboxidiene also greatly reduced 5'elongated HIV transcripts at 24h, while isoginkgetin reduced mid-transcribed/unspliced HIV transcripts. Moreover, pladienolide B and isoginketin caused additional reductions in completed HIV transcripts and reduced the ratio of completed to 5'elongated HIV transcripts at 24h. Our findings indicate that the human splice factors (such as SF3b1) and/or their downstream targets may be involved in activation-induced reversal of the baseline block to HIV transcriptional elongation, completion, and splicing.

These inhibitors likely reduce the splicing of multiple human transcripts, some of which may encode proteins that normally promote various stages of HIV transcription. Pladienolide B and herboxidiene inhibit the splicing factor SF3b1, which is also known to interact with HIV Tat and the P-TEFb complex (50). As a result, inhibition of SF3b1 reduces RNAPII associated with HIV-1 promoter and elongation sites. In line with our data, inhibition of SF3b1 has been associated with reductions in both unspliced and multiply spliced HIV RNA (50), indicating that splicing inhibitors profoundly inhibit HIV expression at multiple stages of HIV transcription.

Quercetin

There is conflicting evidence on whether the flavonoid quercetin activates (51) or suppresses (52) HIV gene expression, which may be the result of its multimodal mechanism of action. Quercetin enhances the function of SIRT1 deacetylase, which is responsible for the deactivation of Tat and other factors that induce HIV transcription in T cells (e.g., NF-κB) (53). Secondly, quercetin inhibits the PI3K/Akt pathway, which favors HIV latency but also

HIV reactivation (54). In this study, we observed a gradual decrease in HIV transcriptional progression after 24h treatment. Unfortunately, this effect was not sustained after six days of culture, which may be due to the rapid oxidation of quercetin in the cell culture medium (55).

HSP90 inhibition by tanespimycin

Our data illustrate that HSP90 inhibition is a promising pathway to block HIV expression and reactivation. HSP90 localizes to the HIV LTR and upregulates NF-κB, NFAT, and STAT5-induced gene expression (56). In our study, the HSP90 inhibitor tanespimycin emerged as one of the most promising LPAs. Tanespimycin reduced HIV transcriptional elongation at 24h and reduced initiated HIV transcripts after six days of culture. Second, tanespimycin completely blocked the production of supernatant viral RNA after T cell activation in all six study participants tested. Its potential has been demonstrated previously in a study where tanespimycin durably prevented viral rebound in vivo in a humanized mouse model, even after removal of the drug (20). In our study, we showed that tanespimycin potently blocks HIV expression and reactivation in cells from PWH, and that the inhibition mainly occurs at the level of HIV transcriptional initiation and elongation, consistent with its proposed mechanism (56).

The effect of some, but not all, previously studied LPAs was confirmed in PBMCs from PWH

Digoxin and 8-azaguanine have been reported to interfere with the Rev-mediated export of unspliced and single-spliced HIV mRNAs (57), leading to over-splicing and an abundance of multiply spliced HIV transcripts relative to single-spliced and unspliced transcripts. We observed an increase in multiply spliced transcripts after 8-azaguanine but not after digoxin treatment **(Figure 1B and S2F)**, perhaps because our digoxin concentration (which was chosen based on therapeutic plasma levels) was lower than that used previously.

Moreover, we did not observe decreases in elongated, unspliced, or completed HIV transcripts with either digoxin or 8-azaguanine **(Figure S2F)**.

Spironolactone has been reported to inhibit HIV transcription via TFIIH inhibition (58, 59). In our study, we observed little reduction in HIV transcription with spironolactone, perhaps because we used a lower concentration (chosen based on plasma levels in humans). Other differences in the methods may also contribute to discrepancies from prior studies using 8-azaguanine, digoxin, and spironolactone. However, given that these drugs showed little effects on HIV transcription in the first two study participants **(Figure 1B)**, we did not pursue further studies with these drugs.

In agreement with other studies, ruxolitinib (21, 22) and triptolide (40, 44, 60) profoundly reduced HIV transcription and reactivation. While KRIBB11 significantly decreased elongated HIV transcripts at 24h, this effect was not maintained after six days, and KRIBB11 did not inhibit the production of viral RNA in the supernatant. It has been shown previously that the ability of KRIBB11 to prevent latency reversal depends on the type of reactivating agent (34). In contrast to other studies, we did not observe much reduction in our initial drug screen for aspirin (61) or 53425191 **(Figure 1B and S2B and S2H)** (62). For 53425191, which has been shown to alter HIV splicing (62), we did observe a strong reduction in multiply spliced HIV transcripts in cells from two study participants at day six (**Figure S6A**) but only a 50% reduction in viral RNA in the supernatant **(Figure S6B)**. Likewise, the SP-1 inhibitor mithramycin A showed no effect after 24h but strongly reduced initiated HIV transcripts after six days **(Figure 4A)**. Furthermore, mithramycin A inhibited the production of viral RNA in supernatant in five out of six study participants **(Figure 5)**, corroborating a prior study in which it reduced latency reactivation (63).

Mesalamine, cordycepin, SPV106, and C26 have not been previously studied for their effects on HIV transcription. The compound C26 was reported to induce cancer cell

dormancy by increasing NR2F1 activity (64). Since NR2F1 recruitment by the chromatin remodeling factor RBBP4 was shown to repress LTR-mediated HIV transcription (65) and RBBP4 was shown to be differentially expressed between transcriptionally active and silent proviruses in cells from PWH (66), we tested C26 in this study. However, C26 had no impact on HIV transcription at 24h **(Figure 1B, S2H)**, nor did it drastically inhibit HIV transcription or the production of viral RNA in supernatant after six days in two tested participants (**Figure S6A and B**). Similarly, for mesalamine, cordycepin, and SPV106, we did not observe any reduction in different HIV transcripts after 24h compared to activated DMSO **(Figure 1B and S2B, S2F, S2I)**.

Transcriptional silencing as a strategy to block immune activation

Chronic immune activation in PWH on ART is linked to non-AIDS morbidities, such as cardiovascular disease, neurocognitive impairment, type 2 diabetes, and cancer (67). The most obvious causes of immune activation are the innate and adaptive immune responses against the virus and its antigens (67). While ART suppresses viral replication to undetectable levels in most PWH, we and others have detected cell-associated HIV RNA in the vast majority of PWH on prolonged ART (8). A considerable portion of these HIV RNAs may be transcribed from defective proviruses; nonetheless, some may activate intracellular defenses or express viral proteins (68–70). Recent evidence shows that those sequences contribute as much, if not more, to immune activation and inflammation in PWH on ART. Both innate and adaptive immunity have been shown to be driven by the expression of mostly defective HIV RNAs and proteins (71, 72). Moreover, multiple studies found no correlation between immune activation or inflammation and the level of intact proviruses (73, 74). Therefore, HIV studies and interventions aimed at reducing immune activation will have to consider both intact and defective proviruses, or at least those that are transcriptionally and translationally active and/or inducible. An advantage of HIV transcription inhibitors as antiviral or cure strategy is their

capacity to target all infected cells, without requiring the provirus to be intact or replication competent. Furthermore, transcriptional inhibition can be obtained across different types of infected cells and does not require an immune response. It is still unclear if LPAs will need to be administered as a one-time treatment, intermittently, or for life. The answer to this question depends on the degree to which the effects on HIV transcription are sustained, which will require testing the drugs over longer timespans, potentially with washout periods. Nonetheless, transcriptional inhibitors, even if used as permanent adjuvant to ART, might be beneficial to suppress the residual HIV pathogenesis caused by HIV expression from intact and defective proviruses in PWH.

Transcriptional silencing in a block-and-lock cure strategy

The most clinical benefit will likely be obtained from drugs that not only impair HIV transcription but also block the production of viral RNA in supernatant after T cell activation. We observed this effect with ruxolitinib, triptolide, AZD4573, pladienolide B, and tanespimycin. Each of those drugs works by a different mechanism, suggesting the potential for single or combination therapies to prevent reactivation of latent HIV as a proof of concept for a “block-and-lock” approach to a functional cure. Ruxolitinib has shown promise in clinical trials in PWH for its ability to decrease specific markers of inflammation and immune activation (75). However, no reduction was observed in cell-associated HIV RNA, indicating that the immunomodulatory effect of ruxolitinib in vivo may not be related to reducing HIV transcription. To our knowledge, only one drug (ABX464, an anti-inflammatory agent and putative Rev inhibitor) has been shown to reduce HIV transcription in ART-suppressed PWH, and the effect was reversed after withdrawing ABX464 (76). One explanation is that many LPAs can inhibit HIV transcription (block), but not all of them can permanently put the provirus in a deep latent state (lock) (24). The determinants of durable HIV suppression

remain unclear, but epigenetics may play a major role [reviewed in (77)]. LPAs eliciting a successful block-and-lock often induce altered chromatin organization (22, 47, 78–81).

In addition, while there are multiple host pathways to target for HIV silencing, inhibiting these cellular factors/pathways may lead to off-target effects. Our data suggest some specificity of the drugs to block HIV transcription, since all HIV transcripts were normalized to 1 µg of total cellular RNA to account for the effect of the drug on global cellular transcription before further normalization to the DMSO control. Targeting the host transcription or splicing machinery may induce cellular toxicity by interfering with the expression of essential cellular genes. However, HIV transcription may be more dependent on particular cellular genes, while cellular toxicity may be avoided by redundant mechanisms. For example, blocking the expression of certain SR proteins needed for splicing can be compensated by other pathways in the host cell without a significant loss of function, but not for HIV splicing (82). To best approximate clinical practice, we selected low drug concentrations, and if known, concentrations that are currently tested in clinical trials for other indications. We did not observe reduced cell viability, which may indicate that clinically acceptable concentrations may not induce cellular off-target effects.

In summary, we have validated existing drugs but also identified new drugs and druggable targets to inhibit HIV transcription and/or latency reactivation ex vivo. Our study gives new insights into the cellular factors governing HIV expression, such as: 1) CDKs (including CDK9) or their downstream targets may contribute to the baseline block to HIV splicing in unstimulated cells; 2) in addition to CDKs, the targets of splice factor 3b1 seem to be involved in activation-induced reversal of the baseline block to HIV transcriptional completion; 3) in addition to SF3b1, CDKs or their downstream targets appear to be involved in activation-induced reversal of the baseline block to HIV multiple splicing. Targeting HIV transcription as part of ART may provide virological benefits for individuals with drug-

resistant HIV strains or non-suppressible viremia (83). The effects of cell-associated HIV RNA on chronic immune activation and inflammation in suppressed PWH are not well studied. Therefore, future studies should examine the effects of various HIV transcription inhibitors, alone or in combination, on HIV expression, immune activation, and rebound after stopping ART.

Materials and Methods

Sex as a Biologic Variable

De-identified blood samples were supplied by our collaborators from study participants recruited from UCSF's Scope/Options Cohort or the San Francisco VA Medical Center. The demographics of the study participants reflect those of people living with HIV who are enrolled in Scope/Options or receive care at the San Francisco VA. Given the demographics of the study participants, we were not able to explore sex as a biologic variable.

Cell culture and treatments

Peripheral blood mononuclear cells (PBMC) were isolated from fresh venous blood from 16 ART-suppressed HIV-infected study participants using Ficoll density gradient centrifugation. The cells were seeded at $6x10^6$ cells/well and cultured with ARVs (nevirapine and indinavir) to prevent new infection. On the next day, PBMCs were activated with anti-CD3/CD28 coated beads (Invitrogen Inc.) in the presence of 20U/ml IL-2 and individual drugs in DMSO or DMSO alone as a control. Since we were limited in the PBMC yield per study participant, different study participants were used for different experiments (such as the initial screen, dose response, different time points, and studies in activated vs. unstimulated cells). In addition, the yields of PBMCs were sometimes insufficient to test all drugs for a given question or experiment.

For quantification of cell-associated HIV transcripts, PBMCs were harvested either after 24 hours or after six days. The cell viabilities were measured by trypan blue staining, then the cells were pelleted by centrifuging at 300*g* for 6 min and stored at -80℃. For quantification of virion-associated HIV RNA, cell culture supernatant was collected from $6x10^6$ PBMCs that were treated and activated for six days as described above. Residual cells and cellular debris were subsequently removed from the supernatant by differential centrifugation (300*g* for 6 min, then 20,000*g* for 10 min), and the supernatant was stored at -80℃.

Nucleic acid isolation, reverse transcription, and ddPCR

Total cellular DNA and RNA were extracted in parallel using Trireagent (Molecular Research Center) according to the manufacturer's instructions, except for study participants 2147 and 2461 **(Figure 4)**, for whom DNA and RNA were extracted using the Qiagen AllPrep DNA/RNA/miRNA Universal Kit with on-column DNaseI treatment of the RNA and the manufacturer's modification to enhance recovery of short transcripts. RNA and DNA concentrations were measured using UV spectrophotometry (NanoDrop One, Thermo Fisher). Total initiated HIV (TAR) transcripts were quantified by a 3-step polyadenylation-RT-ddPCR as described previously (8, 84). HIV 5' elongated (LLTR), mid-transcribed (Pol), completed (PolyA), and multiply spliced (TatRev) transcripts were quantified by a 2-step RT-ddPCR as previously described (8). The levels of each HIV transcript were normalized to 1 µg of total cellular RNA and expressed as a percent of the (activated) DMSO control. To quantify virion-associated RNA in supernatant, RNA was extracted from 400 µL supernatant using Trireagent-LS (Molecular Research Center) according to the manufacturer's instructions. Levels of U3-polyadenylated HIV RNA were measured in the supernatant by RT-ddPCR, normalized to copies per ml, and expressed as a percent of the activated DMSO. For measuring the HIV infection frequency at day six, the HIV DNA U3-U5 long terminal

repeat region was quantified by using ddPCR as described previously (8). HIV DNA copies were normalized to cell numbers according to the mass of DNA input per well (calculated from the DNA concentration and input volume)(85).

Statistical analysis

The Wilcoxon signed-rank test (two-tailed) was used to compare the relative measures of different HIV RNA transcripts to the (activated) DMSO. A P-value less than 0.05 was considered significant. The wells with no positive droplets (observed after LPA treatment) were assigned a value of zero to calculate the median and P-values (8, 10). All statistics were performed using GraphPad Prism (Version 9.5.1). P-values were corrected for multiple comparisons using the Benjamini–Hochberg method as outlined in **File S1**.

Study approval

HIV-infected study participants were recruited sequentially from the San Francisco VA or the University of California, San Francisco (UCSF) Scope/Options cohort. The study was approved by the local Institutional Review Board of UCSF and the San Francisco Veterans Affairs Medical Centre. All participants provided written informed consent.

Data availability

Data are available in the "Supporting data values" XLS file or from the corresponding author upon request.

Author contributions

SAY and JKW designed the study; SGD and JKW provided samples; SAY, PK and JJ designed experiments; JJ, PK, SJK, AW, and GNK conducted experiments; JJ, PK, and SAY analyzed data; JJ and SAY wrote the original draft; all authors reviewed and edited the manuscript; SAY, JKW, and PWH provided supervision and funding. All authors read and approved the final manuscript.

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Figures and legends

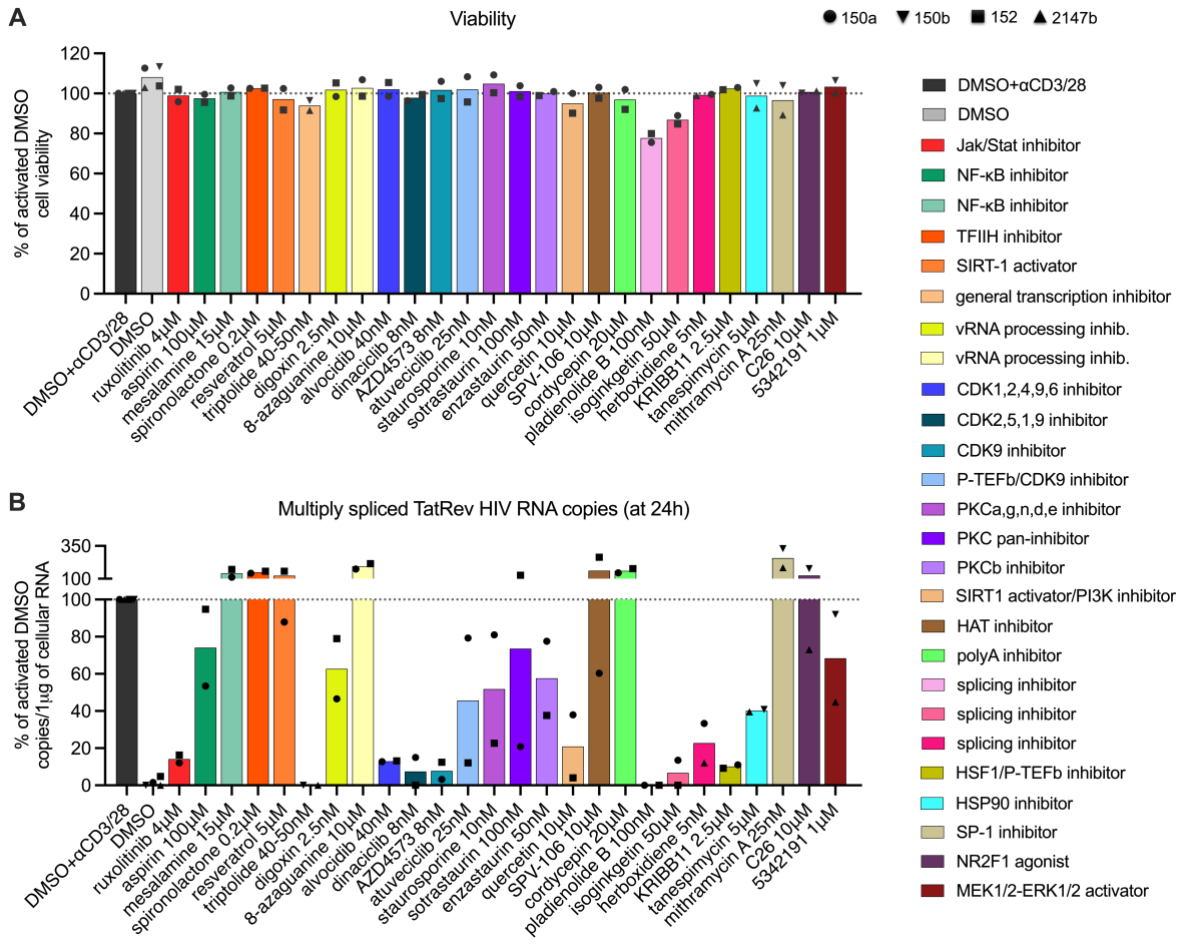


Figure 1. Screening of candidate latency promoting agents by measuring the change in multiply spliced HIV transcripts after activation. Each drug was tested in PBMCs from two ART-suppressed study participants (denoted in the legend by varying symbol shapes). PBMCs were aliquoted into wells at $6x10^6$ cells/well. After activation, the cells were cultured with antiretrovirals in the presence of individual drugs in DMSO or DMSO alone as control. All conditions were tested in the presence of CD3/28 T cell activating beads, except for the unactivated 'DMSO' condition. **A)** After 24h, the viability was measured by trypan blue staining and then normalized to the levels of the activated DMSO (% of activated DMSO). Bars indicate medians. **B)** Total cellular RNA was extracted, and the levels of multiply spliced HIV transcripts (TatRev) were measured by RT-ddPCR, normalized to 1 μg of total cellular RNA, and expressed as a percent of the activated DMSO control (% of activated DMSO).

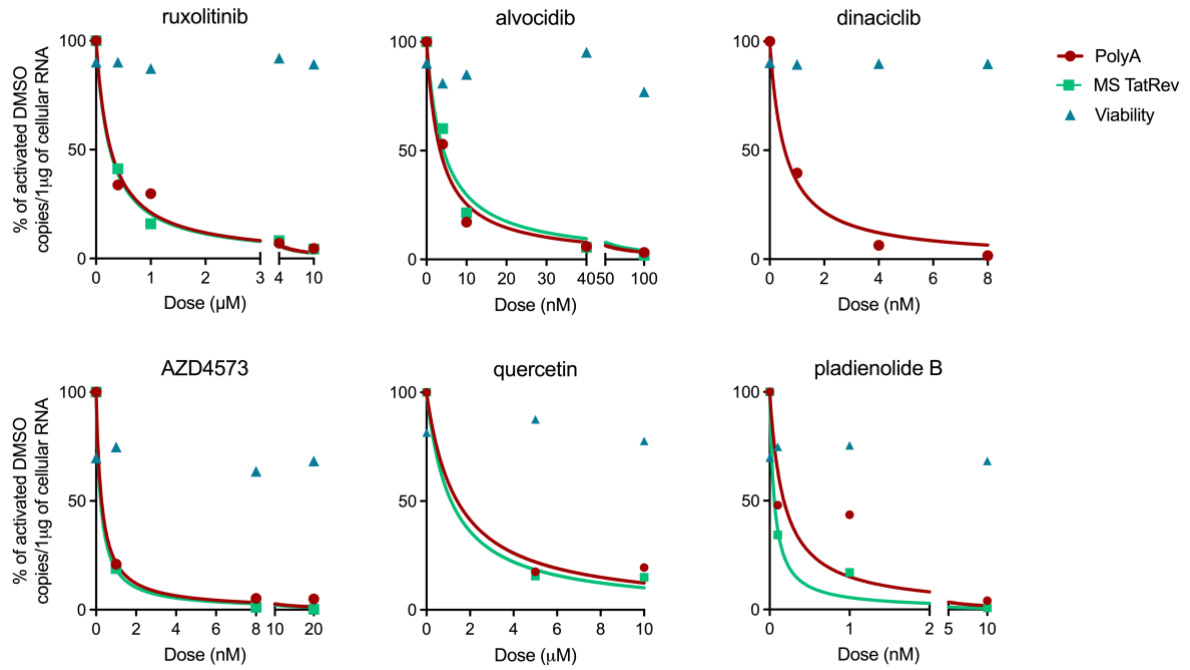


Figure 2. Dose response curves of latency promoting agents. Dose response curves for each drug were generated in PBMCs from one ART-suppressed study participant per drug. PBMCs were aliquoted into wells at $6x10^6$ cells/well, activated, and cultured with antiretrovirals in the presence of DMSO alone (no drug) or varying concentrations of individual drugs in DMSO. After 24h, the levels of polyadenylated and multiply spliced HIV transcripts were quantified by RT-ddPCR, normalized to 1 µg of total cellular RNA, and expressed as a percent of the activated DMSO control (% of activated DMSO). The 50% maximum inhibitory concentration (IC_{50}) was determined for ruxolitinib, alvocidib, dinaciclib, AZD4573, quercetin and pladienolide B using nonlinear regression in Prism.

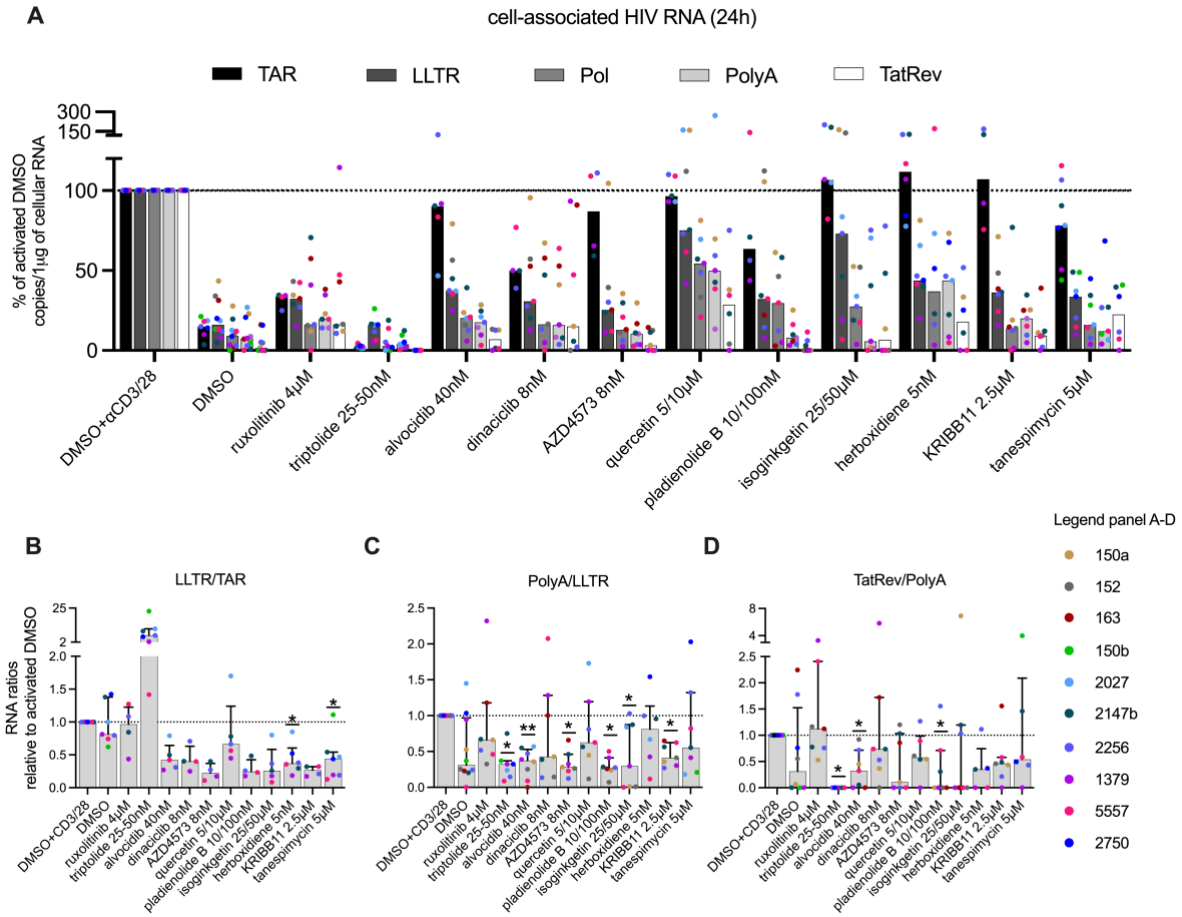


Figure 3. LPAs block HIV reactivation in freshly isolated PBMCs from PWH by reducing transcriptional initiation, elongation, completion, or splicing after activation. Each drug was tested in PBMCs from seven ART-suppressed study participants. The PBMCs were aliquoted into wells at $6x10^6$ cells/well. After activation, the cells were cultured with antiretrovirals in the presence of individual drugs in DMSO or DMSO alone as control. All conditions were tested in the presence of CD3/28 T cell activating beads, except for the unactivated 'DMSO' condition. After 24h, total RNA was extracted and the progression through different stages of HIV transcription was quantified by measuring the levels of initiated (TAR), 5' elongated (LLTR), mid-transcribed/unspliced (Pol), completed (PolyA), and multiply spliced (TatRev) HIV transcripts. **A)** The levels of all HIV transcripts were normalized to 1 µg of total cellular RNA and expressed as a percent of the activated DMSO control (% of activated DMSO). Medians are shown as well as the individual values per study participant in different colors. **B-D)** The effect of each drug on HIV transcriptional progression after activation was analyzed by the ratio of one HIV transcript to another. Ratios are independent of HIV infection frequency or normalization to cell numbers. Shown are the proportion of **B)** all HIV transcripts that were elongated [LLTR/TAR]; **C)** elongated HIV transcripts that were completed [PolyA/LLTR]; and **D)** completed transcripts that were multiply spliced [TatRev/PolyA]. Medians and IQR are presented, as well as the individual values per study participant in different colors. P-values were calculated using the Wilcoxon signed-rank test: *P<0.05; **P<0.01.

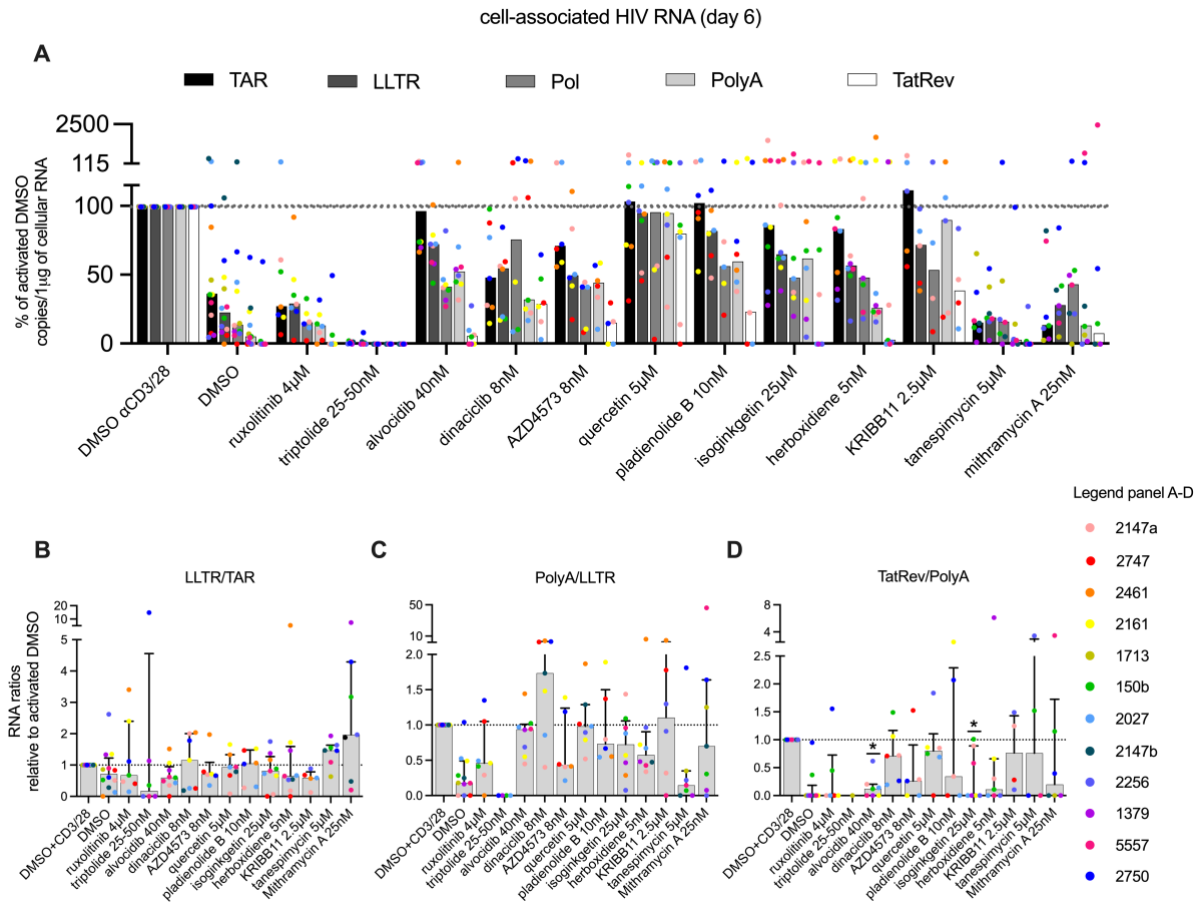
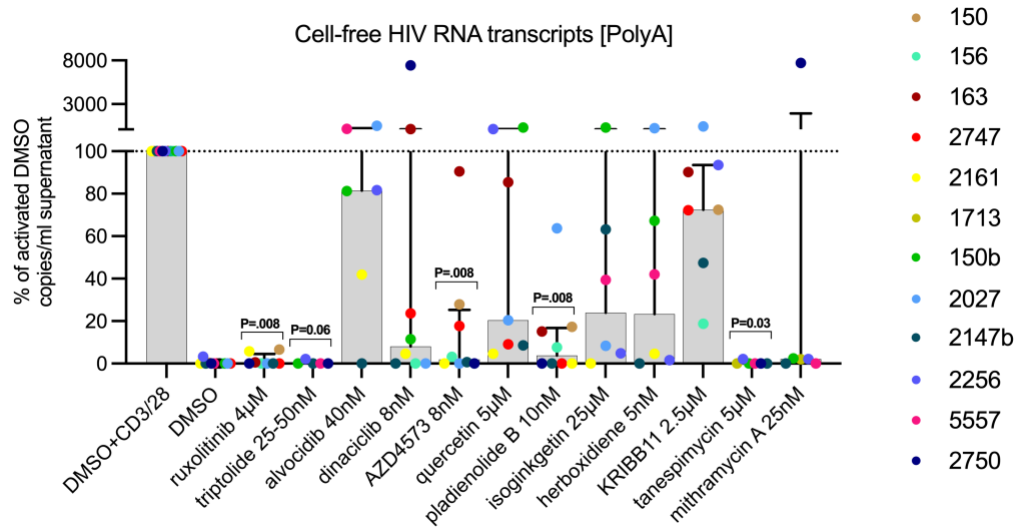


Figure 4. Some LPAs sustain reduced HIV transcription for six days, depending on the study participant. Each drug was tested in PBMCs from at least six ART-suppressed study participants (except for KRIBB11, n=5). PBMCs were aliquoted into wells at $6x10^6$ cells/well. After activation, the cells were cultured with antiretrovirals in the presence of individual drugs in DMSO or DMSO alone as control. All conditions were tested in the presence of CD3/28 T cell activating beads, except for the unactivated 'DMSO' condition. After six days, total cellular RNA was extracted and the progression through different stages of HIV transcription was quantified by measuring the levels of initiated (TAR), 5' elongated (LLTR), mid-transcribed/unspliced (Pol), completed (PolyA), and multiply spliced (TatRev) HIV transcripts. **A)** The levels of all HIV transcripts were normalized to 1 µg of total cellular RNA and expressed as a percent of the activated DMSO control (% of activated DMSO). Medians are shown as well as the individual values per study participant in different colors. **B-D)** The effect of each drug on HIV transcriptional progression after activation was analyzed by the ratio of one HIV transcript to another. Ratios are independent of HIV infection frequency or normalization to cell numbers. Shown are the proportion of **B)** all HIV transcripts that are elongated [LLTR/TAR]; **C)** elongated HIV transcripts that are completed [PolyA/LLTR]; and **D)** completed transcripts that are multiply spliced [TatRev/PolyA]. Medians and IQR are presented, along with the individual values per study participant in different colors. P-values were calculated using the Wilcoxon signed-rank test: *P<0.05.



883

884 **Figure 5. Several LPAs block the production of viral RNA in supernatant after T cell**
885 **activation.** Each drug was tested in PBMCs from six ART-suppressed study participants
886 (except for triptolide, n=5). PBMCs were aliquoted into wells at $6x10^6$ cells/well. After
887 activation, the cells were cultured with antiretrovirals in the presence of individual drugs in
888 DMSO or DMSO alone as control. All conditions were tested in the presence of CD3/28 T
889 cell activating beads, except for the unactivated 'DMSO' condition. After six days, RNA was
890 extracted from the culture supernatant. Polyadenylated HIV RNA in the supernatant was
891 quantified by RT-ddPCR, expressed as copies/mL, and then normalized to the levels of the
892 activated DMSO (% of activated DMSO). Medians and IQR are presented, as well as the
893 individual values per study participant in different colors. Comparisons were performed using
894 the Wilcoxon signed-rank test.

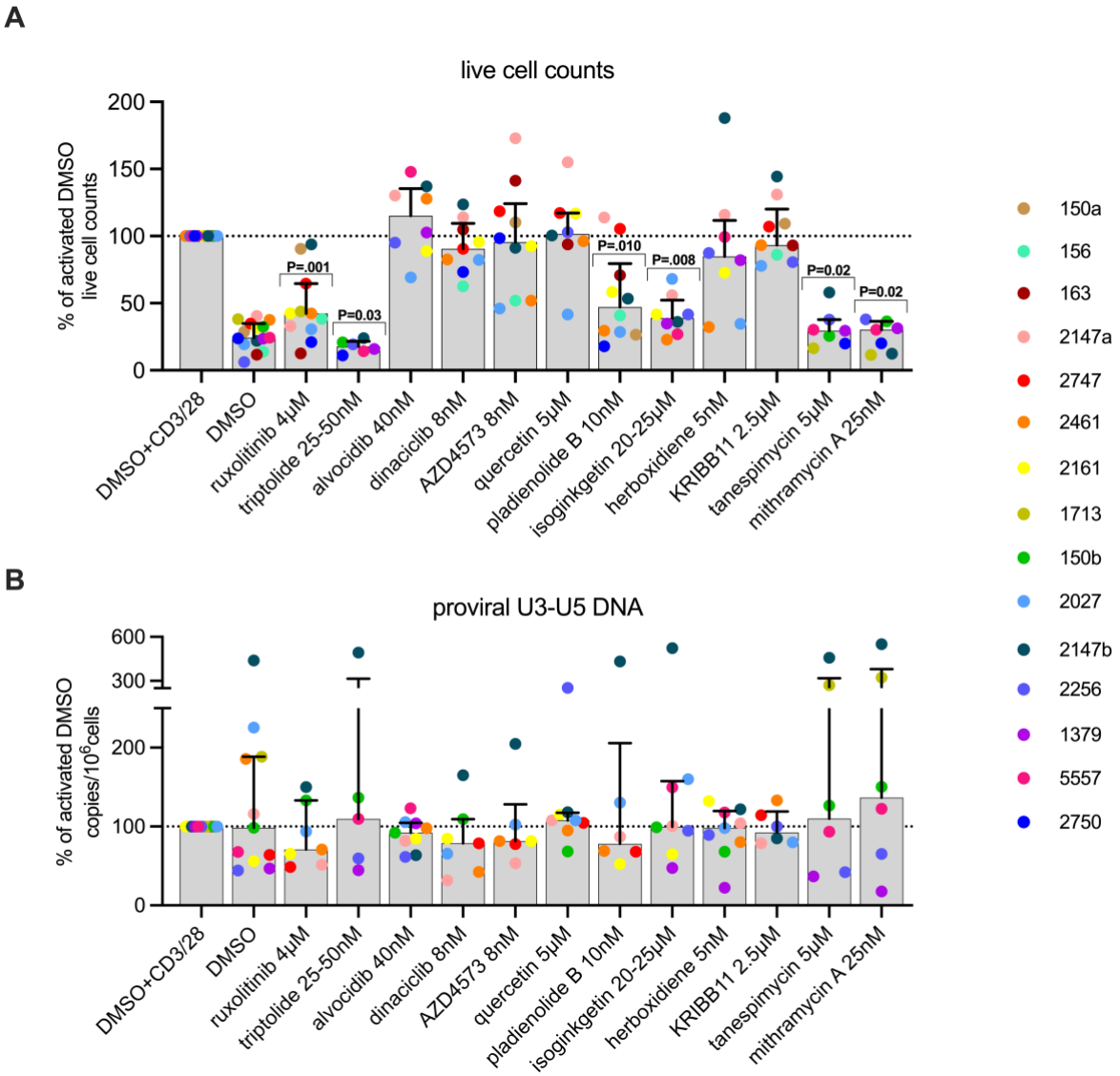


Figure 6. Some LPAs block cell proliferation but do not affect infection frequency. A) After six days, the total number of live cells was measured by trypan blue staining and then normalized to the levels of the activated DMSO (% of activated DMSO). All conditions were tested in the presence of CD3/28 T cell activating beads, except for the unactivated 'DMSO' condition. Bars indicate medians, and different colors indicate individual study participants. Comparisons were performed using the Wilcoxon signed-rank test. **B)** Total cellular DNA was extracted, and the levels of U3-U5 HIV DNA were measured to quantify infection frequency at six days. HIV DNA levels were normalized to copies per 10^6 cells using the input of cellular DNA (assuming 1 μg of total DNA corresponds to 160,000 cells) and then normalized to the activated DMSO control (% of activated DMSO). Bars indicate medians, and different colors indicate individual study participants.

	Completed (PolyA)	Multiply Spliced (TatRev)
	IC50 + CI95	IC50 + CI95
ruxolitinib	0.27 µM [0.15 ; 0.44]	0.26 µM [0.19 ; 0.33]
alvocidib	3.41 nM [2.08 ; 5.27]	4.23 nM [2.46 ; 6.88]
dinaciclib	0.55 nM [0.28 ; 0.94]	/
AZD4573	0.28 nM [0.16 ; 0.41]	0.23 nM [0.18 ; 0.28]
quercetin	1.41 µM [* ; 3.79]	1.13 µM [0.17 ; 2.46]
pladienolide B	0.18 nM [* ; 0.65]	0.06 nM [0.02 ; 0.44]

Table 1. IC_{50} of latency promoting agents. The 50% inhibitory concentration (IC_{50}) and 95% confidence intervals for each of the drugs. * Indicates that the Prism software was unable to accurately estimate the lower confidence interval.

cell-associated HIV RNA (24h)		Median (% of activated DMSO) and P values				
Compound	Exp (n)	TAR	LLTR	Pol	PolyA	TatRev
ruxolitinib 4μM	7	34 (4)	32 0.02	16 0.02	20 0.02	16 0.03
triptolide 25-50nM	7	2 0.02	16 0.02	3 0.02	4 0.02	0 0.03 (6)
alvocidib 40nM	7	90 (5)	37 0.02	20 0.02	18 0.02	7 0.03 (6)
dinaciclib 8nM	7	50 (4)	31 0.02	16 0.02	16 0.02	15 0.02
AZD4573 8nM	7	87 (4)	25 0.03	13 0.02	10 0.02	3 0.02
quercetin 5/10μM	7	97 (5)	75 0.81	54 0.02	50 0.30	29 0.03 (6)
pladienolide B 10/100nM	7	64 (4)	32 0.08	30 0.02	8 0.02	0 0.02
isoginkgetin 25/50μM	7	107 (5)	73 0.37	28 0.02	6 0.02	7 0.03 (6)
herboxidiene 5nM	7	112 (6)	44 0.02	37 0.16 (6)	44 0.02	18 0.03 (6)
KRIBB11 2.5μM	7	107 (4)	36 0.02	14 0.02	20 0.02	9 0.02
tanespimycin 5μM	7	78	34 0.02	16 0.02	12 0.02	23 0.03 (6)

Table 2. LPAs block HIV reactivation in freshly isolated PBMCs from PWH by reducing transcriptional initiation, elongation, completion, or splicing after activation. The medians from Figure 3A are presented along with the P-values calculated using the Wilcoxon signed rank test. The effect of each drug on the different transcripts was studied in seven study participants, unless specified otherwise in parentheses. A green background indicates a significant P-value even after adjusting for FDR using the Benjamini-Hochberg method.

cell-associated HIV RNA (day6)		Median (% of activated DMSO) and P values				
Compound	Exp (n)	TAR	LLTR	Pol	PolyA	TatRev
ruxolitinib 4μM	7	27	29 0.02	15 0.03 (6)	13 0.02	0 0.03 (6)
triptolide 25-50nM	6	1 0.03	0.4 0.03	0 0.03	0 0.03	0 0.03
alvocidib 40nM	8	96	72 0.02	41 0.02 (7)	52 0.05 (7)	5 0.02 (7)
dinaciclib 8nM	7	48 0.02	55 0.02	76 0.84 (6)	32 >0.99	29 0.03 (6)
AZD4573 8nM	6	71	50 0.06	41 0.06 (5)	44 0.03	15 0.06 (5)
quercetin 5μM	7	103	95 0.47	95 >0.99(6)	95 0.94	80 0.21 (6)
pladienolide B 10nM	6	102	82 0.09	56 0.62 (5)	60 0.22	23 0.75 (5)
isoginkgetin 25μM	8	86	65 0.84	48 0.37 (7)	62 0.46 (7)	0 0.03 (7)
herboxidiene 5nM	8	83	57 0.74	48 0.37 (7)	26 0.74 (7)	3 0.11 (7)
KRIBB11 2.5μM	5	111	72 0.06	54 0.62 (4)	90 0.44 (4)	38 0.12 (4)
tanespimycin 5μM	7	15 0.02	19 0.02	16 0.03	3 0.02 (5)	0 0.02
mithramycin A 25nM	7	13 0.02	28 0.02	43 0.30	13 0.37 (5)	7 0.44 (6)

Table 3. Some LPAs sustain reduced HIV transcription for six days, depending on the study participant. The medians from Figure 4A are presented along with the P-values calculated using the Wilcoxon signed rank test. The effect of each drug on the different transcripts was studied in five to eight study participants as indicated in the second column, unless specified otherwise in parentheses. A green background indicates a significant P-value even after adjusting for FDR using the Benjamini-Hochberg method.