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New *ex vivo* approaches distinguish effective and ineffective single agents for reversing HIV-1 latency *in vivo*

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HIV-1 persists in a latent reservoir despite antiretroviral therapy (ART)[1–5]. This reservoir is the major barrier to HIV-1 eradication[6,7]. Current approaches to purging the latent reservoir involve pharmacologic induction of HIV-1 transcription and subsequent killing of infected cells by cytolytic T lymphocytes (CTLs) or viral cytopathic effects[8–10]. Agents that reverse latency without activating T cells have been identified using *in vitro* models of latency. However, their effects on latently infected cells from infected individuals remain largely unknown. Using a new *ex vivo* assay, we demonstrate that none of the latency-reversing agents (LRAs) tested induced outgrowth of HIV-1 from the latent reservoir of patients on ART. Using a quantitative reverse transcription PCR assay specific for all HIV-1 mRNAs, we demonstrate that LRAs that do not cause T cell activation do not induce substantial increases in intracellular HIV-1 mRNA in patient cells; only the protein kinase C agonist bryostatin-1 caused significant increases. These findings demonstrate that current *in vitro* models do not fully recapitulate mechanisms governing HIV-1 latency *in vivo*. Further, our data indicate that non-activating LRAs are unlikely to drive the elimination of the latent reservoir *in vivo* when administered individually.

HIV-1 cure is hindered by viral persistence in a small fraction (~1 in 1×10^6) of resting CD4+ T cells that harbor latent but replication-competent proviruses[1–3]. Upon cellular activation, latency is reversed and replication-competent virus is produced. Although T cell activation reverses latency, global T cell activation is toxic, generating interest in small-molecule LRAs that do not activate T cells. Owing to the low frequency of latently infected resting CD4+ T cells *in vivo*, cell models have been used to identify a number of mechanistically distinct LRAs. These include: (i) histone deacetylase (HDAC) inhibitors, thought to function through epigenetic and other mechanisms[11–14]; (ii) disulfiram, postulated to involve nuclear factor-κB[15,16]; and (iii) the bromodomain-containing protein 4 inhibitor JQ1, which elicits effects through positive transcription elongation factor[17–20]. Acting through signaling pathways associated with T cell activation, protein kinase C (PKC) agonists such as phorbol esters, prostratin[21–23] and bryostatin-1 (refs. 12,24–26) also reverse latency in cell models.

Evidence that putative LRAs reverse latency *ex vivo* in primary resting CD4+ T cells from HIV-1–infected individuals is limited; disulfiram and the HDAC inhibitor vorinostat have been tested in patient cells with inconsistent results[11,13,16,27,28]. Clinical trials in patients on ART are ongoing with disulfiram and the HDAC inhibitors vorinostat, romidepsin and panobinostat[27,29]. A recent trial of disulfiram showed no consistent evidence of latency reversal[30]. In another clinical trial, a single dose of vorinostat modestly increased intracellular RNAs containing HIV-1 *gag* sequences in resting CD4+ T cells of patients on ART[27]. *Ex vivo* treatment of patient cells with vorinostat induced outgrowth in some studies[11,13] but no virion production in another study[28]. Importantly, no LRA has been shown to reduce the size of the latent reservoir in infected individuals.

A consistent *ex vivo* validation strategy has not been employed to compare putative LRAs. Given the costs and risks associated with clinical trials, such a strategy is important for HIV-1 eradication research. Therefore, we used three independent assays to evaluate the efficacy of LRAs in cells from HIV-1–infected individuals on suppressive ART (participant characteristics in **Supplementary Table 1**).

We first tested LRAs in a modified viral outgrowth assay[1]. In the original assay, patient-derived resting CD4+ T cells were activated and cultured with CD4+ T lymphoblasts from healthy donors to expand released virus. Induction of outgrowth provides conclusive evidence of latency reversal. In the modified assay, we replaced T cell activation with LRA treatment. The subsequent co-culture of patient resting CD4+ T cells with healthy donor lymphoblasts constitutes a mixed lymphocyte reaction, which induces background reactivation of latent HIV-1 (ref. 31) and complicates LRA evaluation. Therefore, we treated resting CD4+ T cells with LRAs and then cultured the cells with a transformed CD4+ T cell line (MOLT-4/CCR5) (**Fig. 1a**) that supports robust HIV-1 replication but does not induce allogeneic stimulation of resting CD4+ T cells (**Supplementary Fig. 1a–c**). We treated 5×10^6 purified resting CD4+ T cells from infected individuals on ART with single LRAs for 18 h and then cultured the cells with MOLT-4/CCR5 cells for 14 d to permit viral outgrowth. T cell activation with phorbol 12-myristate 13-acetate plus ionomycin (PMA/I) served as a positive control. We concurrently measured the frequency of latently infected cells[32]. We evaluated vorinostat, romidepsin, panobinostat, disulfiram and bryostatin-1 at clinically relevant concentrations that effectively reversed latency in a primary cell model (see below) and

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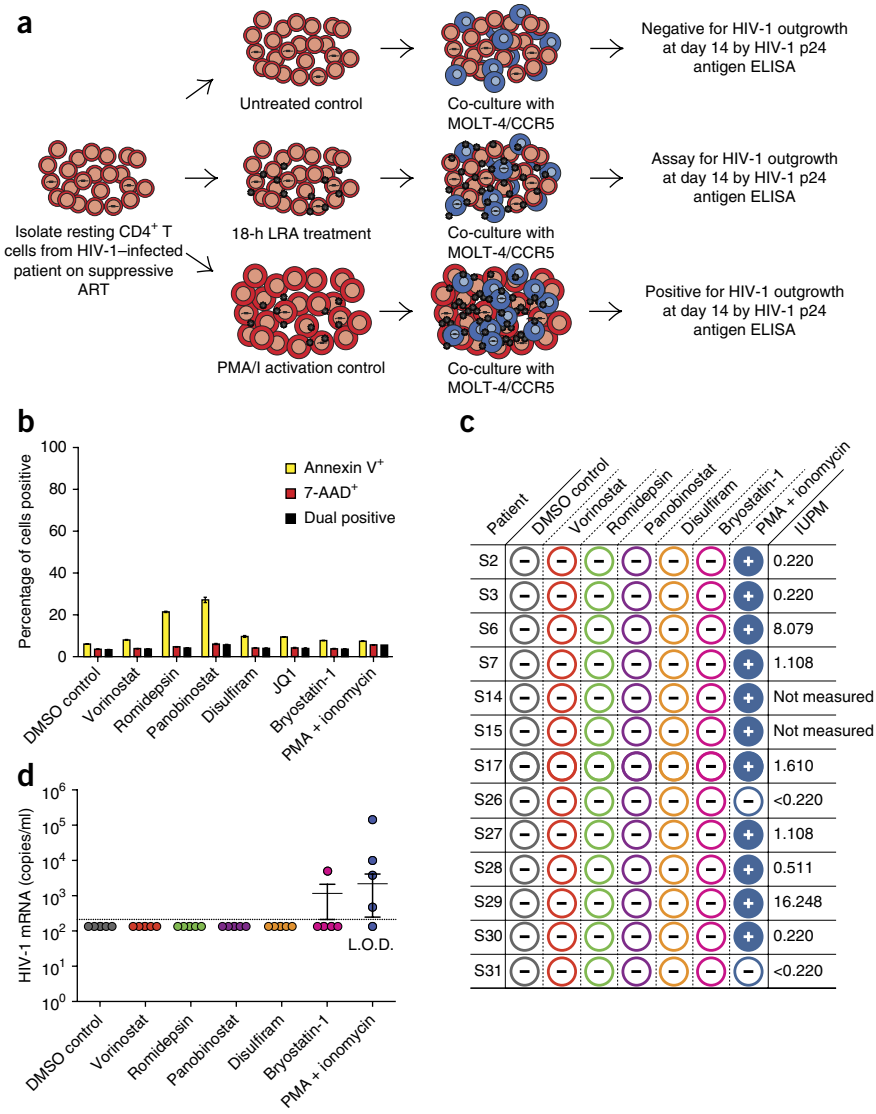

Figure 1 LRAs do not induce outgrowth of latent HIV-1. (**a**) Schematic of LRA outgrowth assay. (**b**) LRA-treated resting CD4+ T cells were stained for annexin V and with 7-AAD. Toxicity was defined as percentage positivity by flow cytometry. Data are representative of 6 independent experiments. (**c**) Viral outgrowth from LRA-treated resting CD4+ T cells from infected individuals (n = 13). Wells positive by enzyme-linked immunosorbent assay (ELISA) for HIV-1 p24 antigen at 14 d are depicted with a plus sign. Negative wells are depicted with a minus sign. When sufficient cells were available, a standard viral outgrowth assay was also carried out. Results are indicated in the form of infectious units per million (IUPM) resting CD4+ T cells. (**d**) HIV-1 mRNA (copies per ml) in the culture supernatant of LRA-treated resting CD4+ T cells obtained from five infected individuals (S26–S30). Dotted line indicates limit of detection (208.3 copies per ml). Error bars indicate mean ± s.e.m.

that were not toxic to resting CD4+ T cells. No drug treatment induced cell death, as shown by the lack of 7-AAD staining (**Fig. 1b**). Unexpectedly, none of the LRAs induced viral outgrowth from cells from any individual tested, whereas PMA/I-treated cultures were positive for every patient from whom latently infected cells could be quantified in a standard viral outgrowth assay (**Fig. 1c**).

We next asked whether LRA treatment induced rapid virus release. We collected culture supernatants from resting CD4+ T cells from five infected individuals (S26–S30) after 18 h of LRA treatment and before addition of MOLT-4/CCR5 cells for measurement of viral outgrowth. PMA/I induced virus release, as detected by HIV-1 mRNA in the supernatant, from four out of five individuals (S26–S29) (**Fig. 1d**). Bryostatin-1 treatment induced detectable HIV-1 mRNA in the supernatant of cells from one infected individual (S27), whereas no other LRA had a measurable effect (**Fig. 1d**). After LRA treatment and measurement of virus release, the treated cells were carried forward into the modified outgrowth assay described above. None of the LRAs induced subsequent viral outgrowth from these treated cells, including the cells from the single individual (S27) that released HIV-1 mRNA after bryostatin-1 treatment (**Fig. 1c**).

The most widely used method to detect induction of HIV-1 transcription[16,27] in cells from infected individuals involves the measurement of RNAs containing HIV-1 *gag* sequences. Because this method lacks a stringent selection for polyadenylated RNAs, it does not exclusively detect fully elongated and correctly processed HIV-1 mRNAs. Therefore, we devised a new assay specific for intracellular HIV-1 mRNA using a primer/probe set that detects the 3′ sequence common to all correctly terminated HIV-1 mRNAs (**Fig. 2a**). We detected baseline intracellular HIV-1 mRNA levels in resting CD4+ T cells from 10 out of 11 infected individuals. Stimulation with PMA/I for 18 h markedly increased intracellular HIV-1 mRNA (mean increase of 115.5-fold, **Fig. 2b**). However, at clinically relevant concentrations, vorinostat, romidepsin, panobinostat, disulfiram and JQ1 failed to increase intracellular HIV-1 mRNA levels at 18 h in resting CD4+ T cells from infected individuals when used as single agents (**Fig. 2b**,**c**). Bryostatin-1 caused significant increases in cells from some infected individuals (**Fig. 2c**). We observed similar results after 6 h of LRA treatment (**Supplementary Fig. 2**).

Although we saw no effect in latently infected cells from infected individuals, LRA treatment increased intracellular HIV-1 mRNA levels in a B cell lymphoma 2 (*BCL2*)-transduced primary resting CD4+ T cell model of latency (**Fig. 3a**). LRA-induced increases in HIV-1 mRNA were consistent with measurements of the fraction of cells that had upregulated HIV-1 gene expression, as assessed by GFP reporter assay (**Fig. 3b**). The frequency of latent infection in this model is substantially higher than that observed *in vivo*[4]. To confirm that our assay effectively detects intracellular HIV-1 mRNA increases at frequencies of latent infection seen *in vivo*, we treated *BCL2*-transduced cells with a known percentage of latent infection with DMSO only, vorinostat or PMA/I and then serially diluted these cells into resting CD4+ T cells from uninfected individuals immediately before RNA isolation. We detected proportionate increases in intracellular HIV-1 mRNA in vorinostat-treated cells as infected cells were diluted down to a frequency of 1 in 1×10^6 cells (**Fig. 3c**,**d**). Therefore,

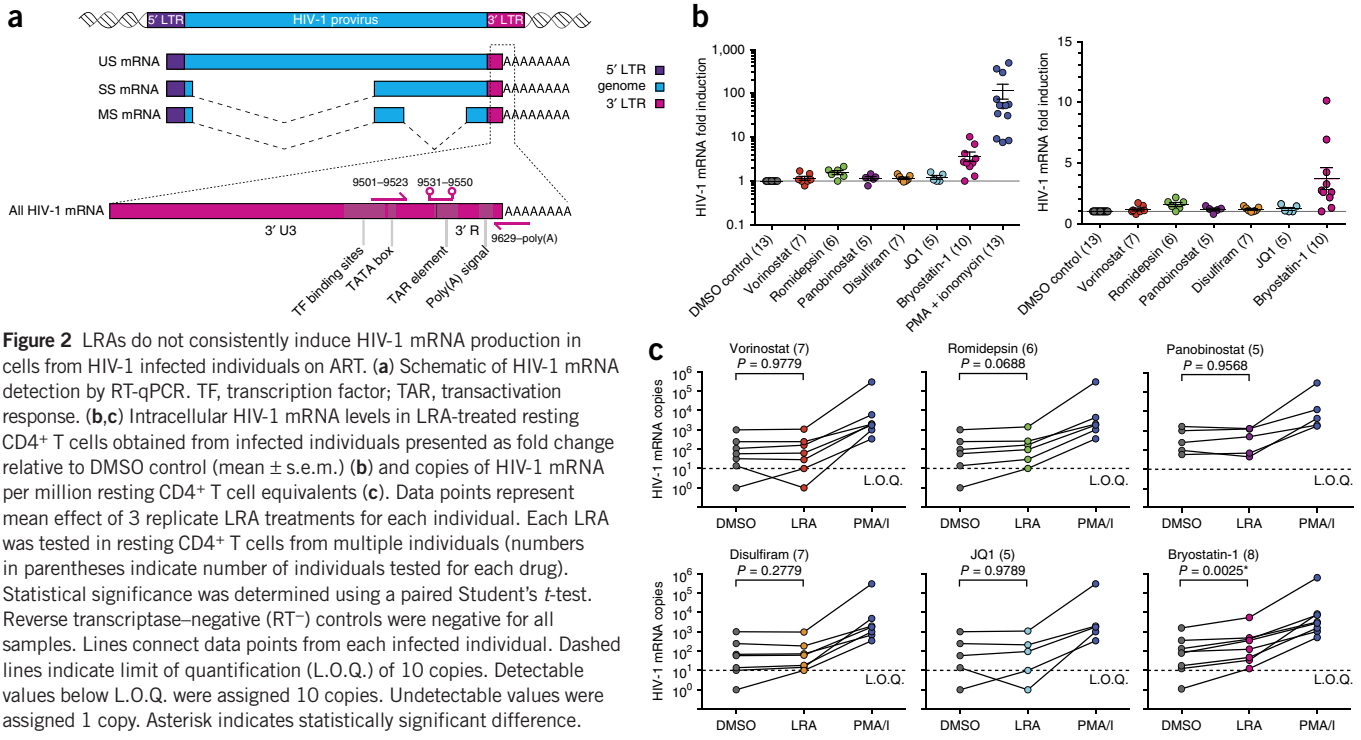

Figure 2 LRAs do not consistently induce HIV-1 mRNA production in cells from HIV-1 infected individuals on ART. (**a**) Schematic of HIV-1 mRNA detection by RT-qPCR. TF, transcription factor; TAR, transactivation response. (**b**,**c**) Intracellular HIV-1 mRNA levels in LRA-treated resting CD4+ T cells obtained from infected individuals presented as fold change relative to DMSO control (mean ± s.e.m.) (**b**) and copies of HIV-1 mRNA per million resting CD4+ T cell equivalents (**c**). Data points represent mean effect of 3 replicate LRA treatments for each individual. Each LRA was tested in resting CD4+ T cells from multiple individuals (numbers in parentheses indicate number of individuals tested for each drug). Statistical significance was determined using a paired Student's *t*-test. Reverse transcriptase–negative (RT−) controls were negative for all samples. Lines connect data points from each infected individual. Dashed lines indicate limit of quantification (L.O.Q.) of 10 copies. Detectable values below L.O.Q. were assigned 10 copies. Undetectable values were assigned 1 copy. Asterisk indicates statistically significant difference.

the lack of LRA efficacy in cells from HIV-1–infected individuals is not a result of assay insensitivity. Rather, our findings demonstrate that freshly isolated latently infected cells from infected individuals responded differently to LRAs than latency model cells.

Quantitative reverse transcription PCR (RT-qPCR) assays that detect *gag*-containing sequences in total RNA are frequently used to detect latency reversal. These sequences do not necessarily represent bona fide unspliced HIV-1 mRNA. HIV-1 integrates into host genes that are actively transcribed in resting CD4+ T cells[33,34], allowing for the production of chimeric host–HIV-1 primary transcripts. Such transcripts, initiated at host promoters, could contain *gag* sequence and would be indistinguishable from HIV-1 long terminal repeat (LTR)-initiated transcripts by conventional *gag* RT-qPCR assays (**Fig. 4a**). We therefore designed a primer-probe set that amplifies a region of the LTR that is not transcribed during LTR-initiated and correctly terminated HIV-1 transcription. This primer-probe set is specific for transcripts containing readthrough of the 5′ LTR or 3′ LTR, independent of proviral orientation (**Fig. 4a**). We treated 1×10^7 resting CD4+ T cells from infected individuals on ART with vorinostat or PMA/I for 6 h and compared the levels of HIV-1 mRNA, readthrough transcripts and transcripts containing *gag* sequence (**Fig. 4a**,**b**). We detected a small increase (approximately twofold) in transcripts containing *gag* sequence in vorinostat-treated resting CD4+ T cells from four out of five infected individuals, consistent with previous reports[27] (**Fig. 4b**). Vorinostat treatment also induced increases in readthrough transcripts (**Fig. 4b**) comparable to the increases in transcripts containing *gag* sequence but had no effect on levels of HIV-1 mRNA (**Fig. 4b**).

To prove that the readthrough signal is amplified from a transcript that initiated upstream of the 5′ LTR and contains *gag* sequence,

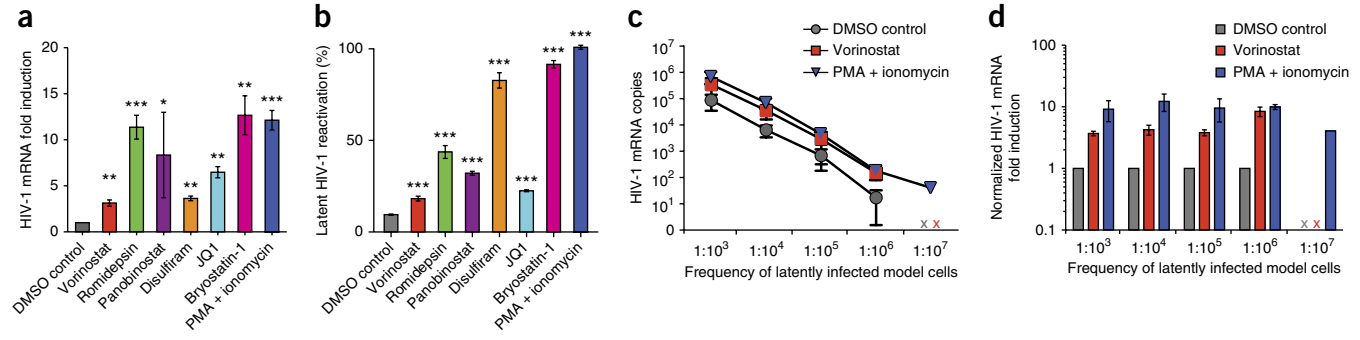

Figure 3 A primary CD4+ T cell model of HIV-1 latency is responsive to LRAs. (**a**) Intracellular HIV-1 mRNA from LRA-treated *BCL2*-transduced primary CD4+ T latency model cells. Changes are presented as fold induction relative to DMSO control (mean ± s.d., $n = 4$). (**b**) LRA-induced reactivation in latency models cells, defined as the percentage of GFP+ cells normalized to the effect of PMA/I treatment (mean ± s.d., $n = 3$) as measured by flow cytometry. (**c**,**d**) Intracellular HIV-1 mRNA in serially diluted latency model cells, presented as copies of HIV-1 mRNA per million resting CD4+ T cell equivalents (mean ± s.d., $n = 3$) (**c**) and fold change relative to DMSO control (mean ± s.d., $n = 3$) (**d**). An x indicates the sample was below the limit of detection. RT− controls were negative for all samples. For **a** and **b**, statistical significance was determined using unpaired Student's *t*-test. $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$. Statistical significance was determined using underlying values for mRNA copies/ml.

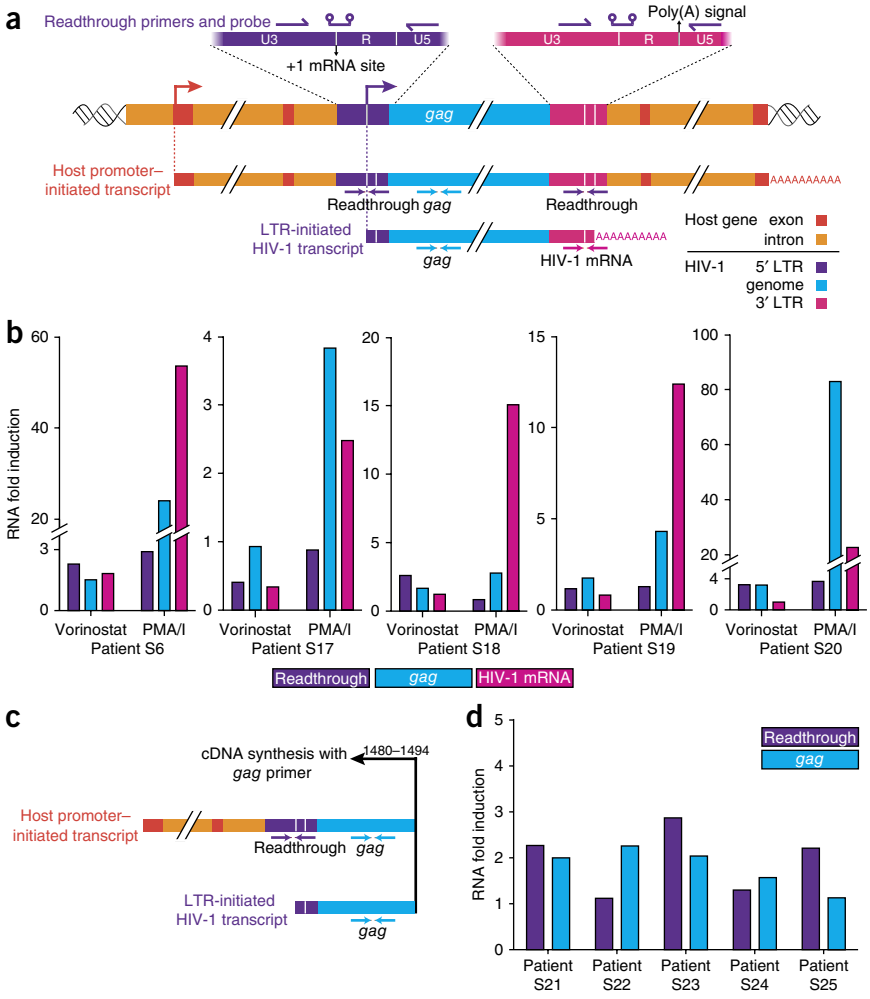

Figure 4 Vorinostat induces transcripts containing HIV-1 *gag* sequence but not HIV-1 mRNA in cells from HIV-1–infected individuals on ART. (**a**) Schematic of RT-qPCR detection of host–HIV-1 readthrough transcripts (purple arrows), transcripts containing HIV-1 *gag* sequence (blue arrows) and HIV-1 mRNA (pink arrows). (**b**) Effect of vorinostat and PMA/I on intracellular HIV-1 readthrough, *gag*-containing and mRNA transcripts in resting CD4⁺ T cells from five infected individuals, presented as fold change relative to DMSO control. (**c**) Schematic of *gag*-specific cDNA synthesis and qPCR detection of readthrough transcripts. (**d**) Effect of vorinostat on readthrough transcripts containing *gag* from five infected individuals, presented as fold change relative to DMSO control. RT(–) controls were negative for all samples.

we primed cDNA synthesis with a *gag* primer (**Fig. 4c**). We detected comparable, statistically significant inductions of readthrough and *gag* transcripts after 6 h of vorinostat treatment (**Fig. 4d**) ($P = 0.027$, $P = 0.011$, respectively; ratio paired Student's *t*-test of transcript copies), which is indicative of readthrough transcription. PMA/I induction of *gag* transcripts greatly exceeded that of readthrough transcripts, indicative of LTR-initiated transcription (**Supplementary Fig. 3**). Although not every potential LRA will induce readthrough transcription by activating a host gene, our data show that chimeric host–HIV-1 transcripts can have a confounding effect on the RT-qPCR signal obtained with standard *gag* primers. Such an effect should be taken into consideration when evaluating LRAs using conventional *gag* RT-qPCR assays.

The new assays presented herein facilitated what is to our knowledge the first comparative *ex vivo* evaluation of candidate LRAs. Our data demonstrate that none of the leading candidate non–T cell–activating LRAs tested significantly disrupted the latent reservoir *ex vivo*. The discordance between the effects of nonstimulating LRAs in *in vitro* models of HIV-1 latency and their effects *ex vivo* in resting CD4⁺ T cells from infected individuals on ART indicates that these models do not fully capture all mechanisms governing HIV-1 latency *in vivo*. These compounds are unlikely to drive the elimination of the latent reservoir *in vivo* when administered individually. The only effective single agent was the PKC agonist bryostatin-1, which is probably too toxic for clinical use. Whether other PKC agonists or other compounds that stimulate signaling pathways associated with T cell activation can be safely administered to patients remains to be seen, and further progress may depend on finding safe and active combinations of LRAs.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

G.M.L., C.K.B. and R.F.S. designed experiments; G.M.L. and C.K.B. performed experiments; C.M.D. obtained institutional review board approval and managed study participant recruitment; G.M.L., C.K.B., J.D.S. and R.F.S. analyzed the data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Cell isolation and culture. The Johns Hopkins Institutional Review Board approved this study, and all research participants in this study gave written informed consent. HIV-1–infected individuals were enrolled under the criteria of suppression of viremia to undetectable levels (<50 copies per ml) on ART for at least 6 months. Peripheral blood mononuclear cells were purified using density centrifugation from whole blood or continuous-flow centrifugation leukapheresis product. $CD4^+$ T lymphocytes were enriched by negative depletion ($CD4^+$ T cell Isolation Kit, Miltenyi Biotec). Resting $CD4^+$ T lymphocytes were further enriched by depletion of cells expressing CD69, CD25 or human leukocyte antigen DR (HLA-DR) (CD69 MicroBead Kit II, Miltenyi Biotec; CD25 MicroBeads, Miltenyi Biotec; Anti–HLA-DR MicroBeads, Miltenyi Biotec). Purity of resting $CD4^+$ lymphocytes was verified by flow cytometry and was typically greater than 95%. With the exception of experiments designed to detect viral outgrowth, cells were cultured with 10 μM T20 to prevent new infection events.

Treatment of resting $CD4^+$ T cells with LRAs. Resting $CD4^+$ T cells were treated with the following concentrations: 335 nM vorinostat, 40 nM romidepsin, 30 nM panobinostat, 500 nM disulfiram, 1 μM JQ1, 10 nM bryostatin-1 or 50 ng ml^{-1} PMA plus 1 μM ionomycin.

MOLT-4/CCR5 outgrowth assay. 5 million purified resting $CD4^+$ T cells were treated with LRA for 18 h in a volume of 1 ml RPMI + 10% FBS. Cells were then resuspended, transferred to a microcentrifuge tube and pelleted. Cells were washed with 1 ml sterile PBS to remove residual drug and pelleted. Resting $CD4^+$ T cells were then cultured with MOLT-4/CCR5 cells in 8 ml RPMI + 10% FBS in individual wells in six-well plates. After 4 d of culture, cells were resuspended and split into two wells of a six-well plate with the volume of medium adjusted to 8 ml per well. After 7 d of culture, wells were resuspended and split 1:2 with the medium volume adjusted to 8 ml per well. Viral outgrowth was assessed at 14 d using the Alliance HIV-1 p24 antigen ELISA kit (PerkinElmer).

Cell lines. MOLT-4/CCR5 cells were obtained from the NIH AIDS Reagent Program, NIAID, NIH[35].

Generation of latently HIV-1–infected *BCL2*–transduced cells. Latently HIV-1–infected *BCL2*–transduced cells were generated as described previously[36]. Briefly, primary $CD4^+$ lymphoblasts were transduced with *BCL2* and allowed to return to a resting state in the absence of exogenous cytokines. *BCL2*–transduced cells were then activated and expanded in the presence of exogenous IL-2. After expansion, cells were activated again and infected with a recombinant HIV-1 containing *GFP* in place of the *env* gene. After infection, cells were allowed to return to a resting state, and GFP-negative cells were isolated via cell sorting. This population of cells includes the fraction of cells that are *in vitro* latently infected. Reversal of latency is assessed by flow cytometry analysis of GFP expression.

Measurement of intracellular HIV-1 RNA transcripts. Cells were treated with each LRA in triplicate in the presence of 10 μM T20 (5×10^6 cells for experiments measuring only HIV mRNA and 10×10^6 cells for experiments measuring multiple transcripts). Cells were pelleted in RNase-free low-binding microcentrifuge tubes and subsequently lysed with 1 ml of TRIzol reagent (Invitrogen). RNA was isolated using the manufacturer's protocol. For experiments in which multiple transcripts were measured, a DNase digest was performed using TURBO DNase (Ambion). RNA was subsequently reextracted using acid-phenol:choloroform, pH 4.5 (Ambion) per the manufacturer's protocol. cDNA synthesis was performed using qScript cDNA Supermix containing random hexamers and oligo-dT primers (Quanta Biosciences). *Gag*-specific cDNA synthesis was performed using Superscript III First-Strand Synthesis (Invitrogen) using only a *gag* primer (sequence listed below). A fraction of the RNA was retained for RT(−) control reactions.

Real-time PCR was performed in triplicate using TaqMan Universal PCR Master Mix (Applied Biosystems) on an ABI7900 Real-Time PCR machine. Approximately 1 million cell equivalents of cDNA or RNA (for no-RT control reactions) template was used in each PCR reaction. Primers and probes are listed below. The cycling parameters were as follows: (i) 2 min at 50 °C; (ii) 10 min at 95 °C; and (iii) 45–50 cycles at 95 °C for 15 s and then 60 °C for 60 s. Molecular standard curves were generated using serial dilutions of a TOPO plasmid containing the 5′ LTR, *gag* or the last 352 nucleotides of viral genomic RNA plus 30 deoxyadenosines.

Results from the triplicate samples for each drug treatment were averaged and presented as fold change relative to DMSO control (mean ± s.e.m.) or copies of HIV-1 mRNA per million resting $CD4^+$ T cell equivalents. The limit of quantification was set as the dilution point at which the Ct of the plasmid molecular standard replicates had an s.d. > 0.5. We determined that the limit of quantification for all transcripts was 10 copies. A PCR signal of less than 10 copies (1–9 copies) was treated as 10 copies in calculations of fold change and marked as 10 copies on graphs depicting RNA copies. Undetectable PCR signal was treated as 10 copies in calculations of fold change and marked as 1 copy on graphs depicting RNA copies. Levels of RNA polymerase II (Pol2) and glucose-6-phosphate dehydrogenase (G6PD) RNA were also measured for each sample to use as an endogenous control. Voronistat, romidepsin, panobinostat, JQ1 and PMA/I treatment consistently increased expression of Pol2 and G6PD. Samples treated with the same drug had even levels of Pol2 and G6PD, indicating that the template inputs were approximately equal.

Measurement of supernatant HIV-1 mRNA. HIV-1 mRNA was extracted from 0.2 ml of supernatant from 5 million cultured resting $CD4^+$ T cells after 18 h of LRA treatment using the ZR-96 Viral RNA kit (Zymo Research). cDNA synthesis was performed using qScript cDNA Supermix (Quanta Biosciences). Real-time PCR was performed using TaqMan Fast Advanced mastermix (Applied Biosystems) on an ABI Viia 7 Real-Time PCR machine. Primers and probes are listed below. Manufacturer's thermal cycling conditions were used. Molecular standard curve was generated as described above.

Primer and probe sequences. Nucleotide coordinates are indicated relative to HXB2 consensus sequence. HIV-1 mRNAs were detected using the following primers and probe, modified from Shan *et al.*[37]: forward (5′→3′) CAGATGCTGCATATAAGCAGCTG (9501–9523), reverse (5′→3′) TTTTT TTTTTTTTTTTTTTTTTTTTGAAGCAC (9629-poly(A)) and probe (5′→3′) FAM-CCTGTACTGGGTCTCTCTGG-MGB (9531–9550).

Transcripts containing HIV-1 *gag* sequence were detected using the following primers and probe, described previously[27]: forward (5′→3′) ACATCAAGCAGCCATGCAAAT (1368–1388), reverse (5′→3′) TCTGGCCT GGTGCAATAGG (1453–1471) and probe (5′→3′) VIC-CTATCCCATTCTGC AGCTTCCTCATTGATG-TAMRA (1401–1430).

Chimeric host–HIV-1 readthrough transcripts were detected using the following primers and probe: forward (5′→3′) CAGATGCTGCATATAAGCAGCTG (416–438, 9501–9523), reverse (5′→3′) CACAACAGACGGGCACACAC (556–575, 9641–9660) and probe (5′→3′) FAM-CCTGTACTGGGTCTCTCTGG-MGB (446–465, 9531–9550).

cDNA synthesis reaction with *gag* primer sequence: reverse (5′→3′) GTCACTTCCCCTTGG (1480–1494).

Statistical analyses. Student's *t*-test was used to determine statistical significance where indicated. We considered $P < 0.05$ to be statistically significant. HIV-1-infected adult volunteers meeting criteria for viral load suppression were enrolled. As samples from all patients were handled in the same way for a particular set of experiments, there was no randomization or blinding. No statistical method was used to predetermine sample size.

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