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Research Article

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Ex vivo analysis identifies effective HIV-1 latency-reversing drug combinations

Gregory M. Laird,¹ C. Korin Bullen,¹ Daniel I.S. Rosenbloom,² Alyssa R. Martin,³ Alison L. Hill,⁴ Christine M. Durand,¹ Janet D. Siliciano,¹ and Robert F. Siliciano^{1,5}

¹Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA. ²Department of Biomedical Informatics, Columbia University Medical Center, New York, New York, USA.

³Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA. ⁴Program for Evolutionary Dynamics, Harvard University, Cambridge, Massachusetts, USA. ⁵Howard Hughes Medical Institute, Baltimore, Maryland, USA.

Reversal of HIV-1 latency by small molecules is a potential cure strategy. This approach will likely require effective drug combinations to achieve high levels of latency reversal. Using resting CD4⁺ T cells (rCD4s) from infected individuals, we developed an experimental and theoretical framework to identify effective latency-reversing agent (LRA) combinations. Utilizing ex vivo assays for intracellular HIV-1 mRNA and virion production, we compared 2-drug combinations of leading candidate LRAs and identified multiple combinations that effectively reverse latency. We showed that protein kinase C agonists in combination with bromodomain inhibitor JQ1 or histone deacetylase inhibitors robustly induce HIV-1 transcription and virus production when directly compared with maximum reactivation by T cell activation. Using the Bliss independence model to quantitate combined drug effects, we demonstrated that these combinations synergize to induce HIV-1 transcription. This robust latency reversal occurred without release of proinflammatory cytokines by rCD4s. To extend the clinical utility of our findings, we applied a mathematical model that estimates in vivo changes in plasma HIV-1 RNA from ex vivo measurements of virus production. Our study reconciles diverse findings from previous studies, establishes a quantitative experimental approach to evaluate combinatorial LRA efficacy, and presents a model to predict in vivo responses to LRAs.

Introduction

HIV-1 persists in a latent reservoir despite suppressive antiretroviral therapy (ART) (1–5). Resting CD4⁺ T cells (rCD4s) that harbor latent proviruses allow little to no HIV-1 gene expression (6), thereby rendering the virus imperceptible to the host immune response. However, cellular activation reverses this latent state, allowing HIV-1 transcription and subsequent production of replication-competent virus (1–5). This small but stable latent reservoir necessitates life-long ART (7–9) and is a major barrier to curing HIV-1 infection. One proposed strategy for eliminating the latent reservoir is to pharmacologically stimulate HIV-1 gene expression in latently infected cells, rendering these cells susceptible to cytolytic T lymphocytes or viral cytopathic effects (10). While global T cell activation effectively reverses latency, toxicity due to cytokine release precludes its clinical use (11). This has fueled the search for small molecule latency-reversing agents (LRAs) that do not induce T cell activation and cytokine release (reviewed in ref. 12).

Given the low frequency of latently infected rCD4s in vivo, in vitro models of latency have played a central role in the search for compounds that reactivate latent HIV-1 (compared in ref. 13). Many LRAs have been identified using these models (13–27). Histone deacetylase (HDAC) inhibitors in particular have shown high latency-reversing potential in in vitro models. Pioneering

studies by Archin and colleagues have provided some evidence that the HDAC inhibitor vorinostat can perturb HIV-1 latency in vivo (28, 29), and similar results have recently been reported by Rasmussen and colleagues with another HDAC inhibitor, panobinostat (30). However, the magnitude of these effects relative to the total size of the latent reservoir is unclear. When tested in ex vivo assays — which use primary rCD4s recovered directly from HIV-1-infected individuals — these drugs exhibit minimal to modest latency-reversing activity relative to global T cell activation (31–34). These results emphasize that LRAs should be validated by studies using rCD4s from infected individuals. In addition to providing greater physiological relevance than in vitro latency models, primary rCD4s from infected individuals are routinely used in ex vivo viral outgrowth assays that define the size of the latent reservoir in vivo (4, 33, 35).

We recently demonstrated that candidate LRAs, including (a) HDAC inhibitors (vorinostat, panobinostat, romidepsin), (b) disulfiram, which is believed to activate NF- κ B, and (c) JQ1, which is a bromo and extra terminal (BET) bromodomain inhibitor, were only minimally active at reversing latency in rCD4s from infected individuals (32). The PKC agonist bryostatin-1 was the only single LRA to significantly induce intracellular HIV-1 mRNA production ex vivo (32). This effect, however, was a mere 4% of the maximum reactivation elicited by T cell activation. To assess the activity of LRAs, it is essential to compare their activity relative to both trace baseline levels of HIV-1 gene expression in rCD4s (which vary from individual to individual) and to maximal T cell activation, which serves as a positive control. Maximal T cell activation, used in the viral outgrowth assays with which the latent reservoir

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Table 1. Characteristics of HIV-1–infected study participants

Patient ID	Age	Sex	Race	Duration of infection (months)	ART regimen	Time on ART (months)	Time on suppressive ART (months)	Peak reported viral load (copies ml ⁻¹)
S1	45	M	W	89	EFV/FTC/TDF	87	40	60,070
S2	42	M	B	236	EFV/FTC/TDF	129	65	50,7612
S3	48	M	B	293	RAL/DRV/r	149	28	66
S4	52	M	B	221	ABC/3TC/ATV/r	197	8	67,555
S5	54	M	B	149	RAL/FTC/DRV/v	96	9	>750,000
S6	47	F	B	197	FTC/TDF/ATV/r	118	106	36,276
S7	49	M	W	136	ABC/3TC/RAL	135	134	10,414
S8	60	M	B	89	RAL/3TC/DRV/r	56	49	151,114
S9	52	M	B	137	RAL/3TC/DRV/r	135	41	739,349
S10	56	M	B	136	FTC/TDF/EVG/c	135	32	10,485
S11	55	F	B	136	FTC/TDF/EVG/c	136	108	158,523
S12	31	M	W	100	FTC/TDF/EFV	78	65	74,934
S13	52	M	B	216	EFV/DRV/r/RAL	192	16	NA
S14	62	F	B	193	ABC/3TC/ATV/r	129	21	53,327

M, male; F, female; W, mixed European/white, B, African American/black; ABC, abacavir; FTC, emtricitabine; 3TC, lamivudine; TDF, tenofovir; EFV, efavirenz; ETR, etravirine; NVP, nevirapine; ATV/r, atazanavir boosted with ritonavir; DRV/r, darunavir boosted with ritonavir; FPV/r, fosamprenavir boosted with ritonavir; LPV/r, lopinavir boosted with ritonavir; EVG/c, elvitegravir boosted with cobicistat; RAL, raltegravir; MVC, maraviroc.

was identified, provides an upper bound for latency reversal. LRA regimens that substantially reverse latency *ex vivo* compared with the benchmark of maximal T cell activation (typically ~100-fold induction; ref. 32) have not yet been identified. New approaches for latency reversal beyond the use of single LRAs will likely be required for reservoir clearance and a potential cure.

Combinations of mechanistically distinct LRAs may be necessary to overcome the multiple mechanisms governing HIV-1 latency *in vivo* (36–40). While some combinations have previously been tested in CD4⁺ T cells from infected individuals (37, 41), no comparative *ex vivo* study has been performed to assess the efficacy of multiple 2-drug combinations of leading candidate LRAs. We therefore measured intracellular HIV-1 mRNA levels and supernatant virion production following LRA treatment *ex vivo* in rCD4s collected from infected individuals on suppressive ART. We identified synergistic drug combinations that reverse latency to levels approaching those of maximal T cell activation. Strikingly, we show here that these robust levels of latency reversal can be achieved without causing functional CD4⁺ T cell activation.

Several clinical trials testing latency reversal by disulfiram or the HDAC inhibitors vorinostat, romidepsin, or panobinostat are ongoing or have been completed in patients on ART (28–30, 42–44). One indication of successful latency reversal *in vivo* is a transient increase in plasma HIV-1 RNA, reflecting the release of virus from the latent reservoir. Thus far, only romidepsin has been shown to induce detectable increases in plasma HIV-1 RNA using quantitative clinical assays (43). Currently, no quantitative framework exists to predict *in vivo* responses to LRA treatment using data collected *ex vivo*. To aid in selecting optimal LRA treatments, we designed a mathematical model to estimate the impact of LRA treatment on *in vivo* plasma HIV-1 RNA levels based on *ex vivo* measurements of LRA-induced viral production. With this model, we reconcile the diverse findings of previous *in vitro* and *ex vivo*

studies and recently reported clinical trial results, highlighting that quantitative analysis of LRA efficacy *ex vivo* is a useful resource for the design of latency-reversing strategies.

Results

Quantifying the combined effects of 2 or more LRAs requires first understanding the effect of each drug alone. Therefore, we treated 5 million purified rCD4s from infected individuals on suppressive ART (participant characteristics in Table 1) with single LRAs or vehicle alone for 24 hours and then measured levels of intracellular HIV-1 mRNA using a primer/probe set that detects the 3' sequence common to all correctly terminated HIV-1 mRNAs (32, 45). Drugs were used at concentrations previously shown to be effective at reversing latency in model systems. Of the LRAs tested individually, only the HDAC inhibitor romidepsin and the PKC agonists bryostatin-1 and prostratin caused statistically significant increases in intracellular HIV-1 mRNA (mean increases of 2.2-, 12.8-, and 7.7-fold, respectively, Figure 1A and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI80142DS1). In contrast, the T cell activation control of PMA plus ionomycin (PMA/I) dramatically elevated levels of intracellular HIV-1 mRNA (mean increase of 148.8-fold, Figure 1A). Treatment of CD4 T cells with PMA/I caused a dramatic upregulation of numerous signaling pathways downstream of the T cell receptor, many of which promote efficient HIV-1 transcription. When LRA-induced increases in HIV-1 mRNA were normalized as a percentage of the effect elicited by T cell activation with PMA/I, it became apparent that individual LRAs generally show limited efficacy *ex vivo* (Figure 1B).

To identify effective 2-drug combinations of LRAs, we treated rCD4s from infected individuals on suppressive ART with bryostatin-1, prostratin, or disulfiram in combination with a mechanistically distinct LRA. Of the 11 combinations tested, 10 caused a significant increase in intracellular HIV-1 mRNA rela-

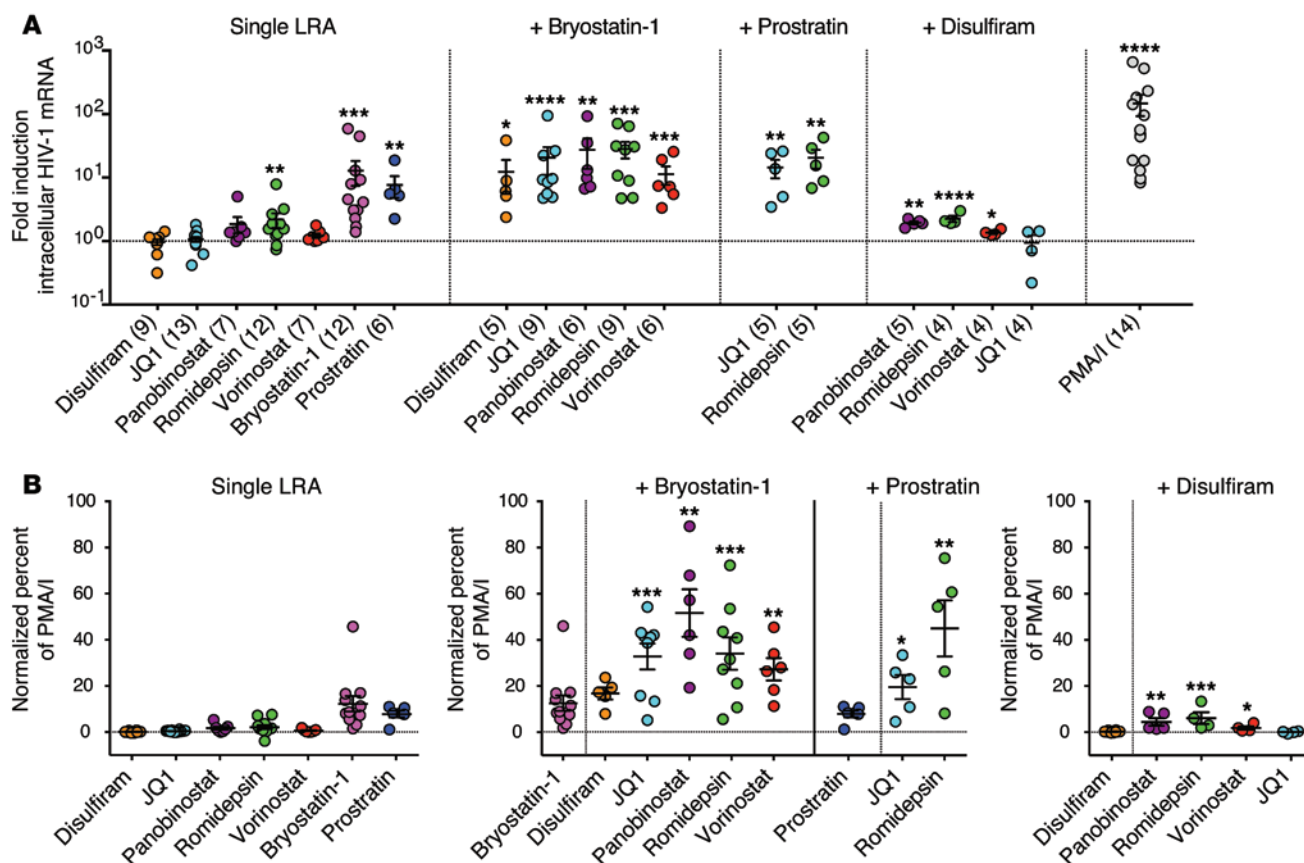


Figure 1. Combination LRA treatment robustly increases HIV-1 mRNA expression in rCD4s from infected individuals on ART. (A) Intracellular HIV-1 mRNA levels in rCD4s, obtained from infected individuals and treated ex vivo with a single LRA or a combination of 2 LRAs, presented as fold induction relative to DMSO control. Numbers in parentheses indicate number of individuals used for each treatment. (B) Induction of intracellular HIV-1 mRNA by single LRAs, PKC-agonist-containing LRA combinations, and disulfiram-containing LRA combinations presented as a percentage of the effect of maximal reactivation with PMA/I. Data points represent the mean effect of 2 or 3 replicate LRA treatments of 5 million cells for each individual. For A and B, statistical significance was calculated from the HIV-1 mRNA copy number values using a ratio paired *t* test compared with (A) the DMSO control, (B) bryostatatin-1 or prostratin alone, or disulfiram alone. **P* < 0.05; ***P* < 0.005; ****P* < 0.0005; *****P* < 0.00005. Error bars represent SEM.

tive to the DMSO control (Figure 1A and Supplemental Figure 1). To compare the efficacy of these combinations, we plotted increases in intracellular HIV-1 mRNA levels as a percentage of the effect of the T cell activation control, PMA/I. Combinations of the PKC agonist bryostatatin-1 with JQ1 or with each of 3 different HDAC inhibitors were significantly more effective than bryostatatin-1 alone (Figure 1B), with some combinations approaching the magnitude of induction stimulated by T cell activation with PMA/I. For example, treatment with a combination of bryostatatin-1 and panobinostat caused increases in intracellular HIV-1 mRNA that were on average 51.5% of those seen with the PMA/I control, with increases of 89.1% seen in some infected individuals. Similarly, treatment with a combination of bryostatatin-1 and JQ1 caused increases in intracellular HIV-1 mRNA that were on average 32.6% of those seen with the PMA/I control. Combinations of the PKC agonist prostratin with JQ1 or romidepsin produced increases in HIV-1 RNA that were significantly greater than those seen with prostratin alone. Two-drug combinations containing disulfiram and an HDAC inhibitor were significantly more active than either compound alone. However, the observed induction of intracellular HIV-1 mRNA did not exceed 14% of the PMA/I response (Figure 1B).

Commonly used models for determining whether drugs act synergistically are based on the assumption that the drugs act through the same mechanism, an assumption that does not apply to combinations of LRAs (46). To quantitate interactions between LRAs, we compared the experimentally observed combined effects to the effects predicted under the Bliss independence model for combined drug effects (ref. 47 and Figure 2). This model assumes that compounds act through different mechanisms, such that their effects multiply when administered in combination. A drug combination whose effect significantly exceeds that predicted by the Bliss model can be said to exhibit synergy. We found that the PKC agonists synergize significantly with JQ1 and the HDAC inhibitors to induce intracellular HIV-1 mRNA ex vivo (Figure 2). Disulfiram-containing combinations did not exhibit synergy, but rather conformed to the predictions of the Bliss independence model (Figure 2).

To further explore the synergistic relationship between bryostatatin-1 and the HDAC inhibitors, we tested a 10-fold lower concentration of bryostatatin-1 alone and in combination with the HDAC inhibitor romidepsin. Treatment with 1 nM bryostatatin-1 did not induce significant intracellular HIV-1 mRNA. However, when 1 nM bryostatatin-1 was combined with romidepsin, we

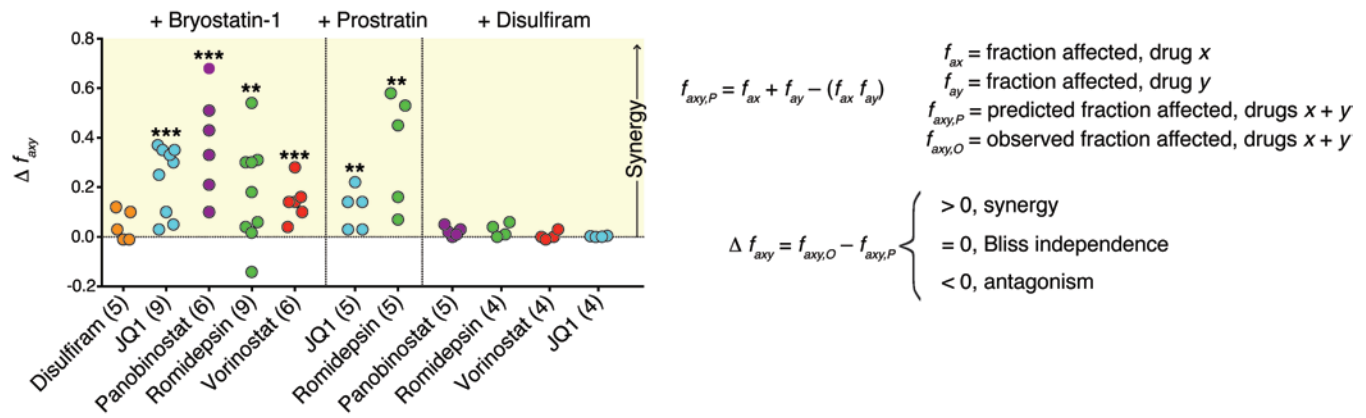


Figure 2. PKC agonists synergize with JQ1 and with HDAC inhibitors to significantly increase HIV-1 mRNA expression in rCD4s from infected individuals on ART. Calculation of synergy for LRA combinations using the Bliss independence model. Data are presented as the difference between the observed and predicted fractional response relative to PMA/I (fraction affected, f_o) presented in Figure 1. See Methods for more details. Numbers in parentheses indicate number of individuals used for each treatment. Data points represent the mean effect of 2 or 3 replicate LRA treatments of 5 million cells for each individual. Statistical significance for the experimental f_o was calculated using ratio paired t test compared with the predicted f_o for each combination. $**P < 0.005$; $***P < 0.0005$.

observed significant induction of intracellular HIV-1 mRNA (Figure 3A, mean 20.2-fold induction), and this combination was synergistic (Figure 3B).

Production and release of HIV-1 virions by LRA-treated rCD4s indicates complete reversal of latency in those cells. To assess whether combinations of LRAs induced rapid virus release, we measured HIV-1 mRNA in the culture supernatants of LRA-treated rCD4s from infected individuals on suppressive ART using a quantitative reverse-transcriptase PCR (RT-qPCR) assay previously shown to provide sensitive and accurate quantitation of HIV-1 virion production (45). We focused on LRAs showing synergistic effects, particularly JQ1, romidepsin, and the PKC agonists bryostatatin-1 and prostratin. No virus production was observed after 24 hours of treatment with the DMSO control in any of the individuals tested (limit of detection = 150 copies HIV-1 RNA/ml supernatant), whereas treatment with PMA/I induced an average of 2.6×10^5 HIV-1 mRNA copies/ml supernatant. Of the LRAs tested, only bryostatatin-1 and prostratin induced significant virus release as single agents (Figure 4, A and B). Combinations of bryostatatin-1 or prostratin with JQ1 or romidepsin also caused significant virus release (Figure 4, A and B), but the combined effects did not significantly exceed those of bryostatatin-1 or prostratin alone (Figure 4B). Surprisingly, combination LRA treatment exceeded the effect seen with maximal T cell activation by PMA/I in some instances (Figure 4B). We again applied the Bliss independence model to quantitate interactions between LRAs. While synergy was observed in some individuals, collectively, the combined LRA effects on virus production did not significantly exceed those predicted by the Bliss independence model (Figure 4C).

Next, we examined the relationship between intracellular HIV-1 mRNA levels and HIV-1 virion production by LRA-treated rCD4s (Figure 5). Treatments including the PKC agonists bryostatatin-1 or prostratin clustered with PMA/I (where PMA is also a PKC agonist), while treatments lacking a PKC agonist showed much lower activity, especially with regard to virion production. Tobit regression analysis of only the treatments containing a PKC agonist yielded a significant correlation between increases in intra-

cellular HIV-1 mRNA and virion release (Figure 5, $P = 0.008$ for χ^2 test). Thus, with respect to inducing virion production from latently infected cells, PKC agonists appear to be of particular importance.

We then asked whether robust induction of latent HIV-1 by treatments containing a PKC agonist was coupled with T cell activation or toxicity. rCD4s stimulated with PKC agonists alone or in combination with another LRA exhibited increased surface expression of the early activation marker CD69 (Figure 6A), consistent with previous studies (14, 48). While some induction of CD25 surface expression on rCD4s occurred after treatment with PKC agonists alone, this expression was reduced with the addition of another LRA (Figure 6A). Treatments containing a PKC agonist caused minimal decreases in rCD4 cell viability, as assessed by annexin V and 7-AAD staining (Figure 6B). Importantly, combination LRA treatment did not cause cellular toxicity exceeding that caused by single LRA treatment (Figure 6B). Although activation marker expression is a useful indication of drug activity, the production and release of proinflammatory cytokines provides a more direct measurement of functional T cell activation, especially with regard to potential toxic effects. Global activation by PMA/I treatment induced the production and release of high levels of multiple cytokines from both rCD4s and PBMCs, while treatment with PKC agonists alone or in combination with other LRAs caused little or no cytokine production by rCD4s (Figure 7A). Similarly, treatment of unfractionated PBMCs with PKC agonists alone or in combination with other LRAs caused little or no cytokine production (Figure 7B).

To date, no latency-reversing strategy has been shown to reduce the latent reservoir in infected individuals. One potential indication of LRA efficacy in vivo would be a transient increase in plasma HIV-1 RNA levels following LRA administration. To place our results in a broader clinical context, we used a mathematical model of viral dynamics (Figure 8A; complete description of model in Supplemental Materials) to predict the in vivo changes in plasma HIV-1 levels following LRA treatment from our ex vivo measurements of virus production in response to LRAs. This model assumes that patients are being treated with suppressive ART

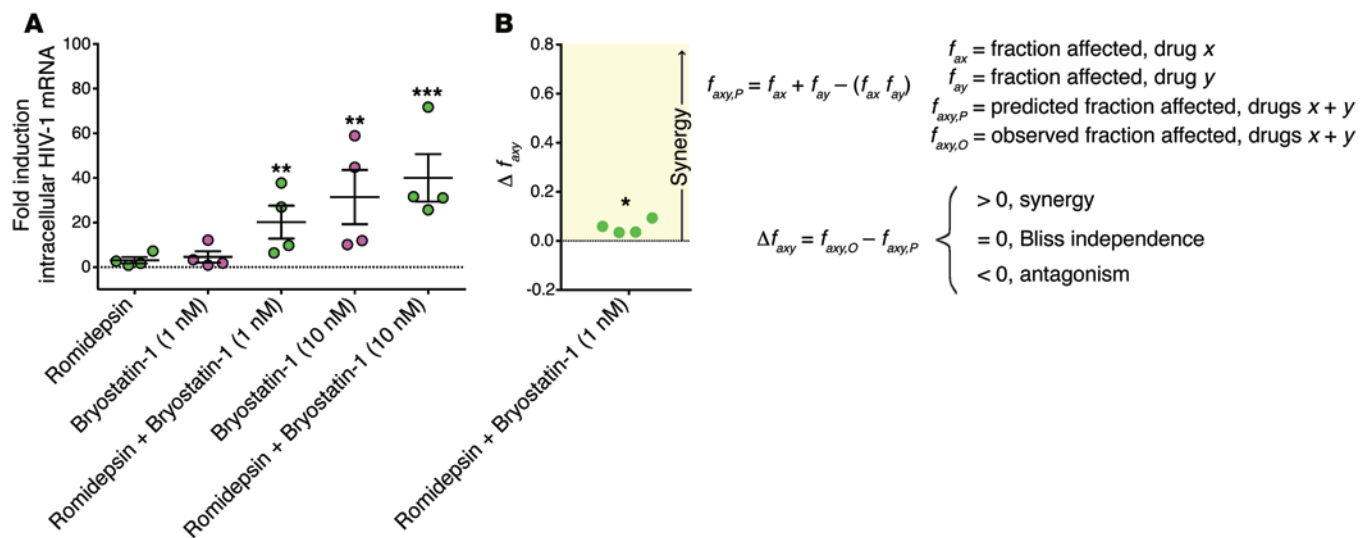


Figure 3. Lower doses of bryostatin-1 synergize with romidepsin to reverse latency. (A) Intracellular HIV-1 mRNA levels in rCD4s, obtained from infected individuals and treated ex vivo with bryostatin-1 (1 nM or 10 nM) alone or in combination with romidepsin, presented as fold induction relative to DMSO control. Statistical significance was calculated from the HIV-1 mRNA copy number values using a ratio paired *t* test compared with the DMSO control. ***P* < 0.005; ****P* < 0.0005. (B) Calculation of synergy for bryostatin-1 (1 nM) and romidepsin using the Bliss independence model. Data are presented as the difference between the observed and predicted fractional response relative to PMA/1 (f_o). See Methods for more details. Statistical significance for the experimental f_o was calculated using paired *t* test compared with the predicted f_o for each combination. **P* < 0.05. rCD4s from 4 HIV-1-infected individuals were tested per condition.

regimens and have baseline plasma HIV-1 RNA levels below the limit of detection prior to LRA administration. Figure 8B relates the ex vivo fold change in supernatant mRNA caused by LRA treatment to the predicted peak plasma HIV-1 RNA that would occur in vivo if the LRA were administered continuously with activating potential comparable to that in the ex vivo assay and if the latent reservoir were not replenished by an alternate source (e.g., cryptic viral replication or cellular compartments not affected by the LRA). Combinations including PKC agonists are predicted to cause increases in plasma HIV-1 RNA that are readily measurable with clinical assays (limit of detection of 50 copies/ml). Note that the fold change for each treatment reported in Figure 8B is a lower bound for the true value, as no detectable HIV-1 virion production occurred ex vivo for the DMSO control. The actual peak may therefore exceed the prediction shown.

More realistic clinical scenarios involve multiple doses separated by several days or weeks, with each dose active for a short period of time. Under such conditions, the peak plasma HIV-1 RNA level would be expected to decay immediately after LRA activity ceased, and the theoretical peak described in Figure 8B would not be achieved. In the most conservative scenario considered by this model, LRA-activated cells survive no longer than cells functionally activated by antigenic stimulation, LRA activity lasts for only 24 hours, and no viral replication occurs. Even in this conservative model, plasma HIV-1 RNA levels of more than 100 copies/ml are predicted for all treatments investigated, except for romidepsin (Figure 8C), which results in detectable plasma HIV-1 RNA levels only if LRA-activated cells are assumed to survive 3 times as long as functionally activated cells (Figure 8D). Thus, the results predicted by this model are consistent with clinical trials in which HDAC inhibitors alone produce increases in HIV-1 RNA that are close to or below the limit of detection of clinical assays.

Fortunately, regimens with stronger latency-reversing activity, comparable to the synergistic combinations studied here, should produce readily measurable increases in plasma HIV-1 RNA.

Discussion

The “shock and kill” strategy for elimination of the HIV-1 latent reservoir in rCD4s requires robust latency reversal. However, given the multifactorial nature of HIV-1 latency, no single drug may be capable of effectively reversing all blocks to proviral gene expression. Indeed, previous studies by our group and others have demonstrated that single LRAs are relatively ineffective at reversing latency ex vivo (32–34). These studies suggested that combination therapy comprising mechanistically distinct LRAs may be required to robustly reverse latency. In this study, we employed 2 distinct measures of latency reversal to evaluate the efficacy of 2-drug LRA combinations in rCD4s from infected individuals.

We report here a number of new 2-drug LRA combinations that effectively reverse HIV-1 latency. We show that PKC agonists, when combined with JQ1 or a variety of HDAC inhibitors, dramatically induced viral transcription in rCD4s from patients on ART (Figure 1). This upstream measure of latency reversal revealed drug synergy in these combinations as formally revealed by our analysis based on the Bliss independence model (Figure 2), which predicts the combined drug effects of drugs with distinct and independent mechanisms. Thus, our finding of synergy for these drug combinations suggests a mechanistically complex interaction. Unraveling the mechanism of these combined effects will further our understanding of HIV-1 latency and aid in the design of new LRAs. To this end, a recent study by the Peterlin group suggests that positive transcription elongation factor b (P-TEFb) may play a central role in the combined effects of PKC agonists and HDAC inhibitors in reversing latency (49). P-TEFb,

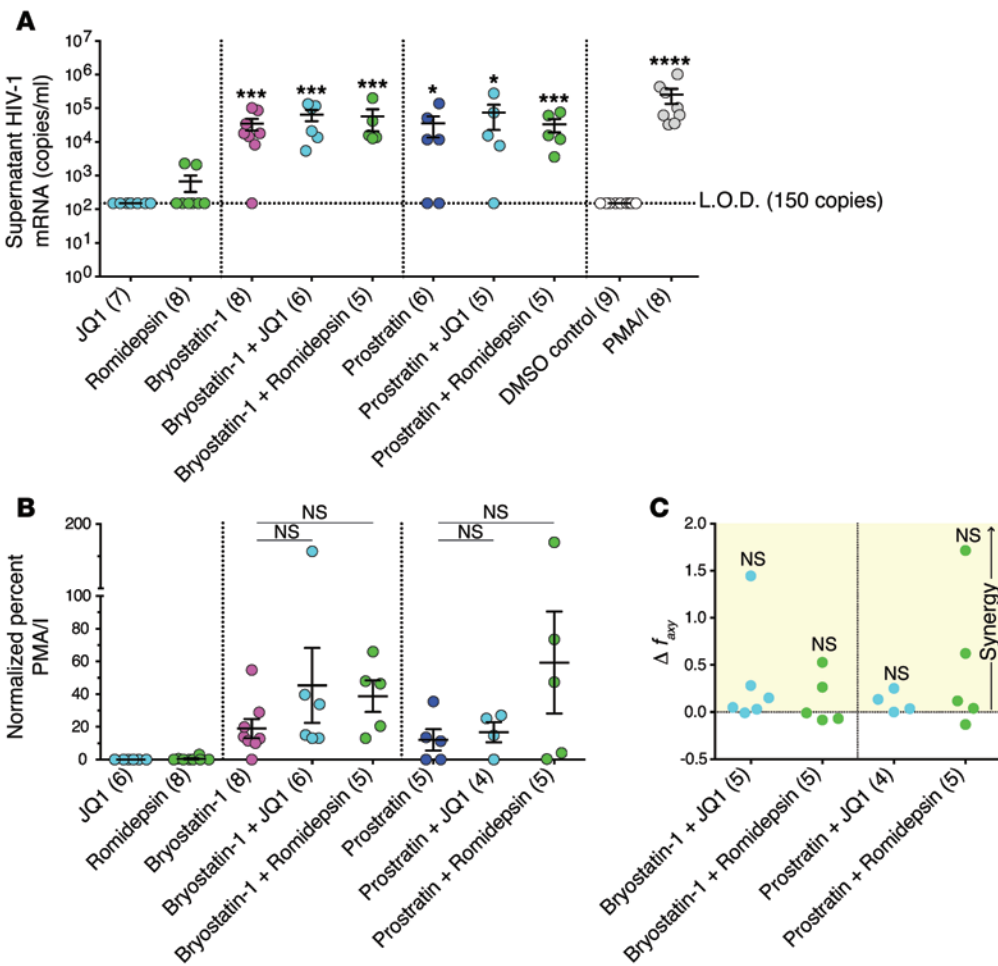


Figure 4. PKC agonists alone or in combination with other LRAs induce HIV-1 virus release by rCD4s from infected individuals on ART. HIV-1 virion levels in the culture supernatant of rCD4s from infected individuals 24 hours after addition of a single LRA or a combination of 2 LRAs, presented as (A) HIV-1 mRNA copies/ml supernatant and (B) as a percentage of the effect of maximal reactivation with PMA/I. Dotted line indicates limit of detection (150 copies per ml). Numbers in parentheses indicate number of individuals used for each treatment. Error bars indicate mean \pm SEM. Statistical significance was calculated from the HIV-1 mRNA copy number values using a ratio paired *t* test compared with (A) DMSO control, or (B) bryostatatin-1 or prostratin alone. (C) Calculation of synergy for LRA combinations using the Bliss independence model. Data are presented as the difference between the observed and predicted fraction of supernatant HIV-1 mRNA levels in copies/ml induced by LRA combinations relative to PMA/I (f_o). See Methods for more detail. Statistical significance for the experimental f_o was calculated using a ratio paired *t* test compared with the predicted f_o for each combination. **P* < 0.05; ****P* < 0.0005; *****P* < 0.00005.

which is required for efficient HIV-1 transcription, is typically present at very low levels in rCD4s. The study by the Peterlin group suggests that the combined effects of PKC agonists and HDAC inhibitors are a result of the induction of P-TEFb production by PKC agonists and the release of this P-TEFb from the inhibitory 7SK-snRNP by HDAC inhibitors.

Notably, we also observed statistically significant inductions of intracellular HIV-1 mRNA production when disulfiram was combined with an HDAC inhibitor (Figure 1). By the rigorous Bliss independence criterion, we did not observe synergy for the disulfiram combinations we tested (Figure 2), suggesting that disulfiram and the HDAC inhibitors reverse latency by independent mechanisms. This conclusion is consistent with the proposed mechanisms of latency reversal by disulfiram (50) and the HDAC inhibitors (49, 51, 52). Our findings support further study of disulfiram combinations and consideration of future clinical testing.

To extend our assessment of latency reversal, we also measured virion release induced by LRA treatment. In our study, treatments including a PKC agonist induced substantial virion release ex vivo, approaching the levels seen with full T cell activation (Figures 4 and 5). However, an effective LRA regimen need not induce significant virion production. Viral protein production following latency reversal may be sufficient to drive elimination of these cells by viral cytopathic effects or immune-mediated clearance. Measurement of virion production after ex vivo treatment

of rCD4s with LRAs serves as a proxy for viral protein production. Thus, our results suggest that inclusion of PKC agonists in an LRA regimen would be sufficient to induce viral protein production that may lead to the elimination of reactivated cells.

In this study, we observed robust latency reversal in rCD4s from infected individuals with several different combinations of a PKC agonist and an HDAC inhibitor. These results are consistent with a previous report that demonstrated the combined effects of prostratin and vorinostat (37). Our findings indicate that HDAC inhibitors may be effective as a part of a combination LRA regimen despite relatively limited activity as single agents. Unexpectedly, a recent study demonstrated that certain HDAC inhibitors impair the ability of HIV-1-specific cytotoxic T lymphocytes (CTLs) to kill HIV-1-infected cells, both ex vivo and in in vitro models (53). This impairment of the HIV-1 CTL response by HDAC inhibitors may limit their clinical utility in eradication trials. Importantly, our finding that PKC agonists also synergize with JQ1 to robustly reverse latency indicates that HDAC inhibitors are not necessary for robust latency reversal.

Our findings highlight the potential importance of PKC agonists for latency reversal and provide a rationale for the detailed analysis of the safety profiles of LRA combination therapies containing PKC agonists. While prostratin has not yet been tested in humans, dozens of phase I and phase II clinical trials of bryostatatin-1 efficacy in the treatment of a variety of cancers have been

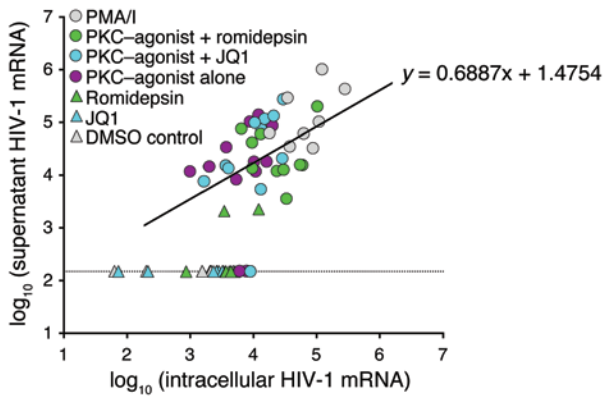


Figure 5. Correlation between intracellular and extracellular HIV-1 mRNA after ex vivo LRA treatment. Plot of intracellular HIV-1 mRNA copy number against supernatant HIV-1 mRNA copy number after exposure of rCD4s from the same infected individual to treatments containing (circles) or lacking (triangles) a PKC agonist. For PKC agonist-containing treatments, a statistically significant correlation was demonstrated by Tobit regression analysis. $P = 0.008$, χ^2 test. See Methods.

safely completed. Lower doses of bryostatin-1 were well tolerated, but dose-limiting toxicities of grade 3/4 myalgia, arthralgia, and weakness have been observed in patients receiving high doses. While this clinical toxicity has been postulated to result from a cytokine storm induced by bryostatin-1, we did not observe the induction of proinflammatory cytokine release by PKC agonists at concentrations that effectively reversed HIV-1 latency ex vivo (Figure 7). Nevertheless, it is possible that these drugs may

have toxic effects unrelated to cytokine production by cells in the peripheral blood. One important question remains: can effective concentrations of bryostatin-1 be achieved in HIV-1-infected individuals? In a recent clinical study of bryostatin-1 in patients with myeloid malignancies, plasma levels of bryostatin-1 were determined using an liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) assay in patients receiving bryostatin-1 in combination with granulocyte-macrophage CSF (GM-CSF) (54). Plasma steady-state concentrations of bryostatin-1 ranging from roughly 0.2 nM to 1 nM were achieved in patients receiving the approximated maximally tolerated dose of 16 $\mu\text{g}/\text{m}^2/\text{d}$ continuously infused for 14 or 21 days, and these concentrations were maintained over the course of the infusion. As presented in Figure 3, we found that 1 nM bryostatin-1 induced significant

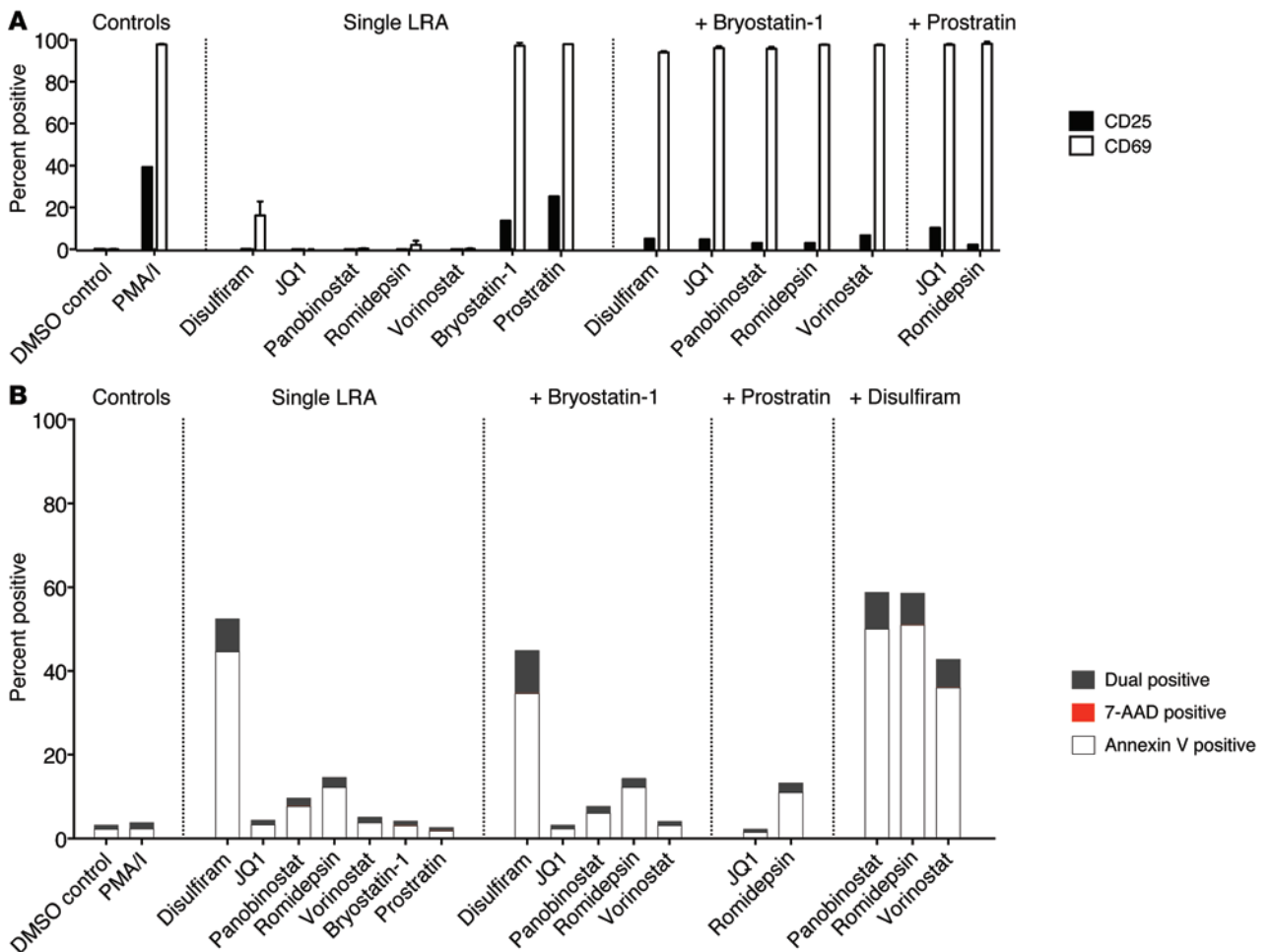


Figure 6. Effect of LRA treatment on T cell activation-associated surface markers and toxicity. Primary rCD4s treated with a single LRA or a combination of 2 LRAs were assayed for (A) surface expression of CD25 and CD69 and (B) positivity for annexin V and 7-AAD staining. Data are the mean effect of 2 or 3 independent experiments. Error bars represent SEM.

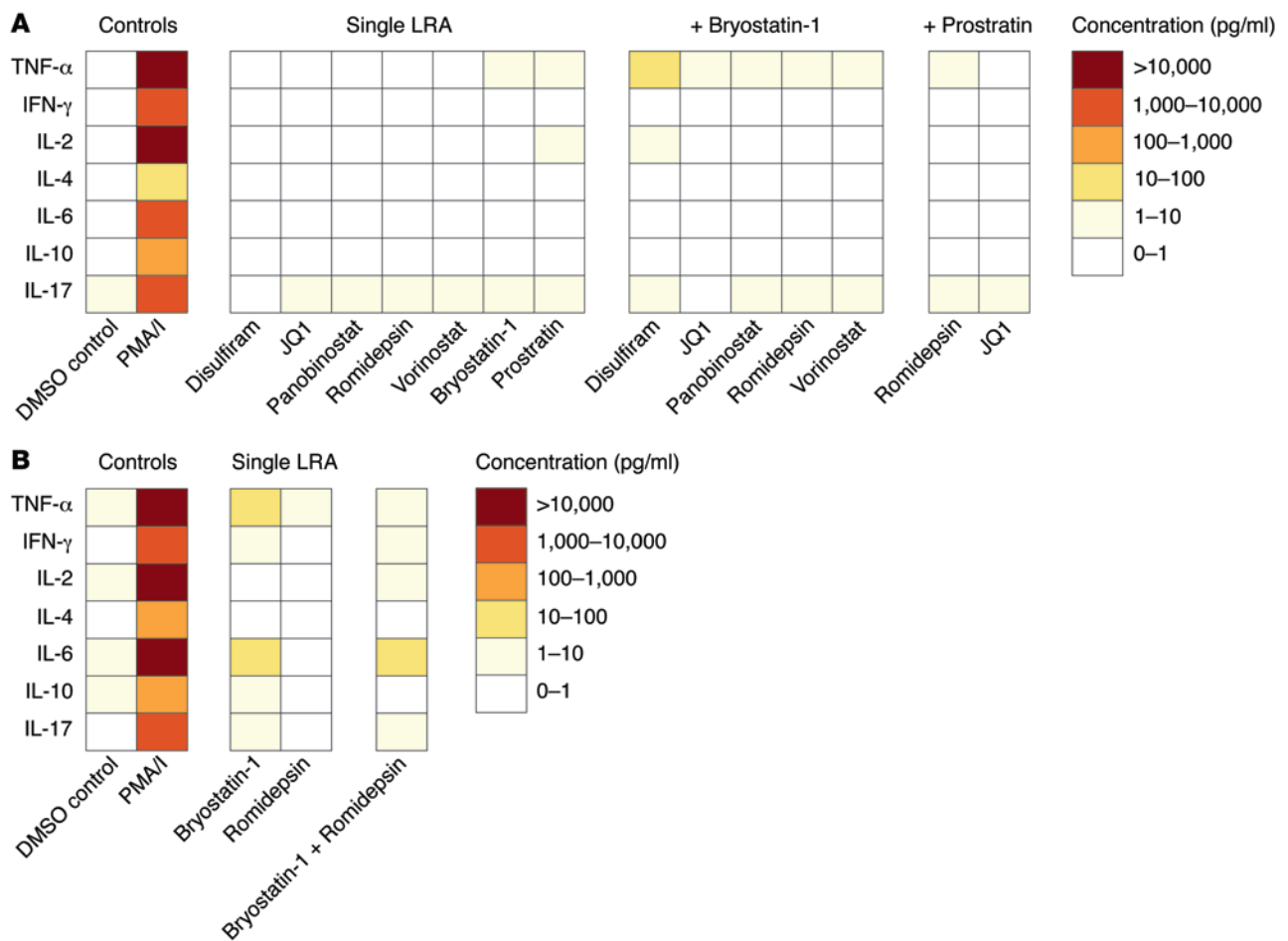


Figure 7. PKC agonists alone or in combination with another LRA do not induce substantial cytokine production. Primary (A) rCD4s or (B) PBMCs were treated with a single LRA, or a combination of 2 LRAs were assayed for supernatant cytokine concentrations (pg/ml). Data are the mean effect of 2 or 3 independent experiments.

intracellular HIV-1 mRNA production *ex vivo* when combined with an HDAC inhibitor. Thus, synergies of the kind described here may allow the use of lower, safer doses of PKC agonists. On the basis of the available clinical data and our *ex vivo* findings, we cautiously suggest that it may be possible to achieve effective concentrations of bryostatatin-1 *in vivo* by taking advantage of synergies of the kind described here. In light of the unpredictable toxicities observed in animal models, such an approach would require extreme caution and very careful patient monitoring. Bryostatatin-1 is a natural product available only in small amounts. Several synthetic analogs of both bryostatatin-1 and prostratin have recently been developed (48, 55). However, the clinical utility of these analogs remains to be established.

Previous studies of LRAs have given divergent results that can be summarized as follows. Multiple classes of LRAs show high activity in T cell-line and primary T cell models of latency. However, each LRA has different levels of activity in different model systems, indicating the need for caution in using these models to define which agents should be advanced into nonhuman primate studies and clinical trials. Some LRAs also increase HIV-1 RNA production in *ex vivo* assays using cells from patients on ART (32–34). However, in general, the activity of individual LRAs in these

systems is weak compared with maximal T cell activation (32). In clinical trials, HDAC inhibitors have been shown to cause modest increases in cell-associated HIV-1 RNA in some studies (28–30, 42, 43), but clear changes in plasma HIV-1 RNA have been seen in only one study to date (43), and no study has demonstrated a decrease in the size of the reservoir or a delay in rebound.

In order to reconcile these diverse outcomes, we have measured both increases in intracellular HIV-1 RNA and the production of virus particles following LRA treatment of rCD4s from patients on ART. Quantitating virus production allowed us to make predictions about how LRA therapy would affect a readily measurable clinical parameter, plasma HIV-1 RNA, using an established model of viral dynamics. Consistent with previous results, individual LRAs induced only minimal increases in cell-associated HIV-1 RNA, while substantial increases in HIV-1 RNA were seen with some combinations of LRAs that included a PKC agonist, and only treatments including PKC agonists induced significant virus production. Our model predicts that this level of virus production would result in transient increases in plasma HIV-1 RNA that are readily measurable with standard clinical assays in the context of a clinical trial. However, the predicted levels of HIV-1 induced by single LRAs are generally at or below the detection limit. The estimates generated

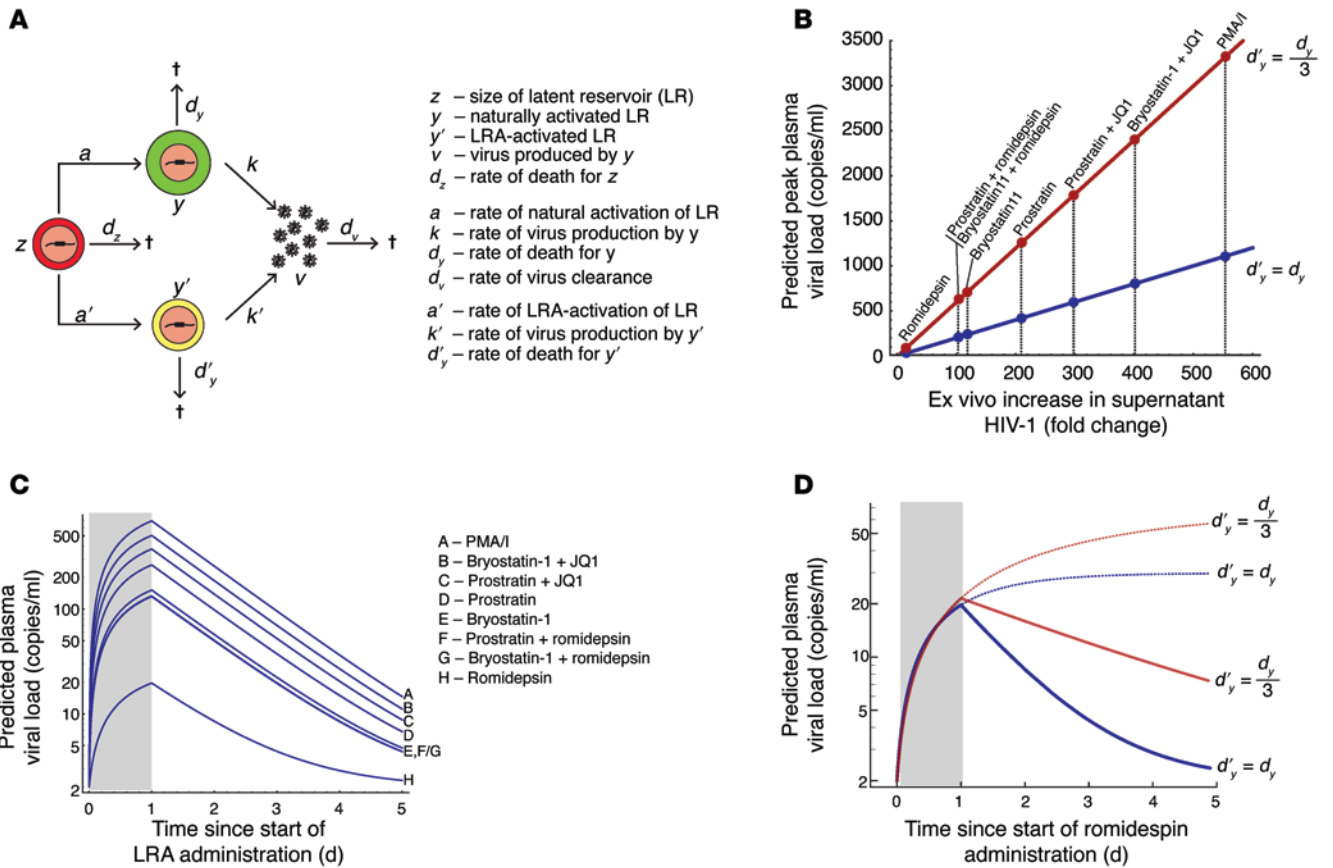


Figure 8. Mathematical model relating ex vivo virus release to predicted increases in plasma HIV-1 RNA levels in vivo. A viral dynamic model (A, detailed in Supplemental Materials) was used to estimate changes in plasma HIV-1 RNA levels in response to the LRA treatments for which ex vivo data on virus release was available. Arrows depict routes from latently infected cells to productively infected cells after exposure to antigen or LRAs. Crosses indicate elimination/death. (B) Predicted peak plasma HIV-1 RNA levels during LRA treatment. For each LRA treatment, median fold change in supernatant HIV-1 versus the DMSO control (x axis) was used to estimate LRA-driven activation rate a' ; this parameter estimate was used to predict peak plasma viral load following continuous administration of the LRA (y axis). (C) Predicted time course of viral load (y axis, log scale) following administration of single-dose LRA treatment that remains active for 1 day. (D) Predicted time course of viral load (y axis, log scale) following administration of single-dose romidepsin that remains active for 1 day (solid lines) or that continues indefinitely (dotted lines). Gray shading in C and D indicates duration of LRA activity. Parameters: $d_y = 1/\text{day}$; $d'_{y'} = 1 \text{ day}^{-1}$ (blue curves in B and D, all curves in C) or one-third day^{-1} (red curves in B and D); $a + d_z = 5.2 \times 10^{-4} \text{ day}^{-1}$ (reservoir half-life of 44 months), initial viral load = 2 copies/ml. For other parameters, see Supplemental Materials.

by our mathematical model are in line with a recently reported clinical trial in which the administration of multiple doses of romidepsin produced detectable plasma HIV-1 RNA levels, ranging from 43 to 103 copies/ml in 5 of 6 patients (43). Clinical trials of disulfiram (44), vorinostat (28, 29), and panobinostat (30) found no increases in plasma HIV-1 RNA using quantitative clinical assays, consistent with our observations that these drugs fail to stimulate detectable viral production ex vivo (32) and consistent with the predictions of this mathematical model. As plasma HIV-1 RNA is predicted to change rapidly following LRA administration (Figure 8C), multiple measurements in the first few hours and days of an LRA trial may be needed to measure latency reversal precisely. This ex vivo analysis of LRA efficacy coupled with modeling of the clinical response to LRA therapy will likely aid in both the selection of candidate LRAs for translation to the clinic and in clinical trial design.

We caution that predicting in vivo viral load changes — a proxy measure for LRA effectiveness — is not the same as predicting the overall decay rate in the latent reservoir over long-term administration. In particular, certain latently infected cells may

be resistant to induction by any LRA (56), an effect that is neither measured in our experiments nor included in our model. The LRA-induced changes in cell-associated HIV-1 RNA and virion release described here may represent increases in the magnitude of HIV-1 gene expression by a fixed number of cells, increases in the number of cells expressing HIV-1 genes, or a combination of both. Our group and others are exploring single-cell methods to resolve the frequency and amplitude of latency reversal, but with in vivo frequencies on the order of 1 per million, the quantitation of infected cells by such methods is extremely challenging. Flow cytometry-based methods are readily applied to primary cell models of HIV-1 latency in which the frequency of latently infected cells is several orders of magnitude higher, and in those models, latency-reversing agents clearly increase the number of cells expressing HIV-1 genes (13, 19, 57). Further studies of the fraction of cells induced ex vivo as well as the life span of newly induced cells may address these questions. It is also important to note that following reversal of latency, infected cells may not die without additional interventions to enhance HIV-1 immunity (58).

There is an increased interest in developing clinical assays that are capable of quantifying the latent reservoir using measures of intracellular or extracellular HIV-1 RNA. However, it is not certain whether either of these HIV-1 RNA measures can be used to accurately measure the frequency of replication-competent latent HIV-1 in cells from infected individuals. The data we present in Figure 5 indicate that intracellular HIV-1 mRNA can be detected in cells that fail to release virions into the supernatant under certain conditions. Recent work by Cillo et al. examined the fraction of proviruses that could be induced by CD3/CD28 costimulation to produce intracellular HIV-1 RNA or virions (33). They found that roughly 7.5% of proviruses produced intracellular HIV-1 RNA, while only 1.5% produced virions after costimulation. This is consistent with the data we present here (Figure 5), in which we fail to see a correlation between intracellular and supernatant HIV-1 mRNA measures. While the underlying cause of this discrepancy is not established, these data suggest that intracellular HIV-1 RNA measures may not directly relate to the frequency of replication-competent latent HIV-1.

In conclusion, using multiple assays for latency reversal ex vivo in rCD4s from infected individuals, we have carried out a comparative study to identify highly effective LRA combinations. Although individual LRAs may cause detectable increases in cell-associated HIV-1 RNA, these increases are small in comparison with the effect of T cell activation and are not expected to cause measurable increases in plasma HIV-1 RNA or significant decreases in the latent reservoir. We identified multiple new 2-drug combinations that reverse latency ex vivo. We demonstrated that PKC agonists combine with JQ1 and with HDAC inhibitors to induce robust reversal of latency to a degree that is comparable to the benchmark of maximal T cell activation. This degree of latency reversal is expected to produce readily measurable transient increases in plasma HIV-1 RNA and, it is hoped, some long-term decrease in the size of the latent reservoir. We demonstrate that this degree of latency reversal can be achieved without inducing proinflammatory cytokine production, although it remains unclear whether agents such as PKC agonists can be safely used in this setting. We suggest that the experimental and mathematical framework developed here to predict in vivo responses to LRAs will inform the design of future eradication clinical trials.

Methods

Study subjects. HIV-1-infected individuals were enrolled in the study at Johns Hopkins Hospital based on the criteria of suppressive ART and undetectable plasma HIV-1 RNA levels (<50 copies per ml) for a minimum of 6 months. Characteristics of study participants are presented in Table 1.

Isolation and culture of resting CD4⁺ T lymphocytes. PBMCs from whole blood or continuous-flow centrifugation leukapheresis product were purified using density centrifugation on a Ficoll-Hypaque gradient. Resting CD4⁺ lymphocytes (CD4⁺, CD69⁻, CD25⁻, and HLA-DR⁻) were enriched by negative depletion as described (32). Cells were cultured in RPMI medium supplemented with 10% fetal bovine serum at a concentration of 5×10^6 cells/ml for all experiments.

Latency-reversing agent treatment conditions. rCD4s were stimulated with latency-reversing agents at the following concentrations

for all single and combination treatments unless otherwise indicated: 10 nM bryostatin-1, 300 nM prostratin, 500 nM disulfiram, 1 μ M JQ1, 30 nM panobinostat, 40 nM romidepsin, 335 nM vorinostat, 50 ng ml⁻¹ PMA plus 1 μ M ionomycin, or media alone plus DMSO. The final DMSO percentage was 0.2% (v/v) for all single and combination treatments. Concentrations were chosen based on previous ex vivo studies with rCD4s from infected individuals as well as studies using in vitro latency models (13, 28, 29, 32–34) with the aim of selecting clinically relevant concentrations.

Measurement of intracellular HIV-1 mRNA. Five million rCD4s isolated from HIV-1-infected individuals on suppressive ART were treated with each LRA alone or with the indicated LRA combination in triplicate (single or duplicate if cell number was limiting) for 6 or 24 hours in a volume of 1 ml RPMI plus 10% FBS. Total RNA was isolated, and cDNA synthesis and RT-qPCR were performed as described (32). Briefly, each PCR reaction contained template from approximately 1 million cell equivalents of cDNA or RNA (for no-RT control reactions). Serial dilutions of a TOPO plasmid containing the last 352 nucleotides of viral genomic RNA plus 30 deoxyadenosines were used for a molecular standard curve (pVQA, catalog 12666, AIDS Reagent Program). No-RT control reactions were performed on every treatment sample from only 1 individual to confirm the absence of signal from contaminating nucleotides, but were not done for every individual, since the primer/probe set used to detect the 3' polyadenylated sequence for correctly terminated HIV-1 mRNAs does not amplify HIV-1 proviral DNA (45).

Results from the triplicate samples for each drug treatment were averaged and presented as copies of HIV-1 mRNA per million rCD4 equivalents, fold change relative to DMSO control, and normalized percentage of the effect of PMA/I: $([\text{copies}_{\text{LRA}} - \text{copies}_{\text{DMSO control}}] / [\text{copies}_{\text{PMA/I}} - \text{copies}_{\text{DMSO control}}])$. The limit of quantification was 10 copies, as described (32). Some samples from 1 individual yielded a PCR signal of less than 10 copies (undetectable to 9 copies) and were assumed to have 10 copies in calculations of both fold change and normalized percentage of PMA/I, and these samples were marked as 10 copies on graphs depicting RNA copies.

Levels of RNA polymerase II (Pol2) and glucose-6-phosphate dehydrogenase (G6PD) RNA were also measured for each sample as an endogenous control (TaqMan Gene Expression Assays Hs00172187_m1 and Hs00166169_m1, respectively). The relative fold change for each transcript was determined using the comparative Ct quantification method (relative fold change = $2^{-\Delta\text{Ct}}$, $\Delta\text{Ct} = \text{Ct}_{\text{LRA}} - \text{Ct}_{\text{DMSO control}}$). Particular LRA treatments consistently changed expression of Pol2 and/or G6PD (Supplemental Figure 2). Samples treated with the same LRA regimen had nearly similar levels of Pol2 or G6PD, indicating that the inputs were approximately equal.

Measurement of HIV-1 mRNA in culture supernatants. HIV-1 mRNA was extracted from 0.25 ml of supernatant from the LRA-treated cell cultures described above with 0.75 ml of TRIzol LS Reagent (Invitrogen) according to the manufacturer's protocol. cDNA synthesis and real-time quantitative PCR were performed as described (32). Results were presented as copies of HIV-1 mRNA per ml supernatant and normalized percentage of the effect of PMA/I: $([\text{copies}_{\text{LRA}} - \text{copies}_{\text{DMSO control}}] / [\text{copies}_{\text{PMA/I}} - \text{copies}_{\text{DMSO control}}])$. The limit of detection for each qPCR was 10 copies per reaction, which scaled to a limit of detection of 150 copies/ml of culture supernatant. Primers and probes are listed below. Molecular standard curve was generated as described above.

Quantitative analysis of latency-reversing agent combinations. We used the Bliss independence model, one method for predicting the expected combined effects of multiple drugs, assuming the drugs act through independent mechanisms, as a metric by which to evaluate the latency-reversing activity of drug combinations. The Bliss independence model is defined by the equation $fa_{xy,P} = fa_x + fa_y - (fa_x)(fa_y)$, where $fa_{xy,P}$ is the predicted fraction affected by a combination of drug x and drug y given the experimentally observed fraction affected for drug x (fa_x) and drug y (fa_y) individually. The experimentally observed fraction affected by a combination of drug x and drug y ($fa_{xy,O}$) can be compared with the predicted fraction affected, which is computed using the Bliss model ($fa_{xy,P}$) as follows: $\Delta fa_{xy} = fa_{xy,O} - fa_{xy,P}$. If $\Delta fa_{xy} < 0$ with statistical significance, then the combined effect of the 2 drugs exceeds that predicted by the Bliss model and the drug combination displays synergy. If $\Delta fa_{xy} = 0$, then the drug combination follows the Bliss model for independent action. If $\Delta fa_{xy} > 0$ with statistical significance, then the combined effect of the 2 drugs is less than that predicted by the Bliss model and the drug combination displays antagonism.

In our analysis, the fraction affected was calculated as follows for intracellular HIV-1 mRNA and for supernatant HIV-1 virion quantitation: $fa_x = (\text{copies drug } x - \text{copies DMSO control}) / (\text{copies PMA/I} - \text{copies DMSO control})$.

Flow cytometry. rCD4s isolated from 3 healthy individuals were incubated with each LRA alone or with the indicated LRA combination in duplicate for 24 hours. The cells were subsequently used to measure the expression levels of T cell activation markers or the frequency of viable cells. For surface-receptor analysis, cells were stained with FITC-conjugated anti-human CD69 antibody and PE-conjugated anti-human CD25 antibody (BD Biosciences — Pharmingen). For toxicity analysis, cells were stained for PE-conjugated annexin V and with 7-AAD using the PE Annexin V Apoptosis Detection Kit I (BD Biosciences — Pharmingen). Samples were analyzed using a FACSCalibur flow cytometer and Cell Quest software (BD). Live-cell gating in forward versus side scatter plots was performed for T cell activation analysis. Toxicity was defined by the total percentage of annexin V positivity.

Cytokine release assay. Supernatant was collected from the LRA-treated cell cultures described above and stored at -80°C for later analysis. Supernatant cytokine levels were determined using Human Th1/Th2/Th17 Cytometric Bead Array (CBA) according to the manu-

facturer's protocol (BD Biosciences). Briefly, 50 μl supernatant or kit standards were mixed with 50 μl mixed-capture beads and 50 μl PE-conjugated detection antibodies and incubated for 3 hours. Then samples were washed to remove unbound PE antibodies and analyzed using a FACSCanto cytometer (BD Biosciences) and FCAP Array software (Soft Flow).

Primer and probe sequences. Nucleotide coordinates are indicated relative to HXB2 consensus sequence. Primers and probe used for HIV-1 mRNA measurement were as described (32): forward (5'→3') CAGATGCTGCATATAAGCAGCTG (9501–9523), reverse (5'→3') TTTTTTTTTTTTTTTTTTTTTTTTGAAGCAC (9629–poly A), probe (5'→3') FAM-CCTGTACTGGGTCTCTCTGG-MGB (9531–9550).

Statistics. Ratio paired Student's t test was used to determine statistical significance where indicated. $P < 0.05$ was considered statistically significant. Approximately a quarter of the experiments measuring intracellular and supernatant HIV-1 mRNA were blinded. All samples were handled and LRA treated in the same way for each set of experiments and were not randomized. No statistical method was used to predetermine sample size.

Study approval. The Johns Hopkins Institutional Review Board granted approval for this study. All research participants enrolled in this study provided written, informed consent prior to inclusion in this study.

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Address correspondence to: Robert F. Siliciano, Johns Hopkins University School of Medicine, Department of Medicine, 733 North Broadway, BRB 879, Baltimore, Maryland 21205, USA. Phone: 410.955.2958; E-mail: rsiliciano@jhmi.edu.

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