Abstract

Despite effective antiretroviral therapy, human immunodeficiency virus type 1 (HIV-1) persists in all infected individuals as a quiescent provirus within resting CD4+ T lymphocytes. These transcriptionally silent proviruses, which exist as integrated DNA within the genome of the infected cell, are not targeted by antiretroviral therapy nor by the host immune response. While the majority of these latent HIV-1 proviruses are defective, a fraction of these proviruses retains the ability to replicate. Upon the interruption or cessation of antiretroviral therapy, stochastic reactivation of these replication-competent latent proviruses leads to rebound in viremia within weeks. The population of latently infected, resting CD4+ T lymphocytes is very stable, with a half-life estimated at 44 months. Therefore, this stably persistent latent reservoir of replication competent HIV-1 is the major barrier to curing HIV-1 infection. HIV-1 latency reversal has been proposed as a curative strategy. Under this approach, latency reversing agents would reignite HIV-1 gene expression, driving the elimination of latently infected CD4+ lymphocytes via viral cytopathic effects or clearance by the immune system. To advance this approach, I have developed a number of new assays for measuring the latency reversing potential of candidate latency reversing agents in lymphocytes taken directly from HIV-1 infected individuals. Using these assays, I have identified effective combinations of HIV-1 latency reversing agents, which are predicted to potently reverse latency \textit{in vivo}. Furthermore, the quantitative approach described herein will facilitate future identification and characterization of candidate latency reversing agents with the goal of eliminating the HIV-1 latent reservoir.
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Preface

In the early days of the HIV/AIDS epidemic, diagnosis with HIV-1 infection was considered a death sentence. Without effective antiretrovirals, HIV-1 replication went unchecked in infected individuals, causing CD4+ T cell counts to plummet. The resulting immunodeficiency left HIV-1 infected individuals susceptible to opportunistic infections, which combined with other AIDS associated co-morbidities led to the death of nearly all infected individuals. With the introduction of the first antiretroviral, the nucleoside reverse transcriptase inhibitor azidothymidine\textsuperscript{1-4}, came the hope that HIV-1 infection could one day be controlled. It was not, however, until the advent of combination antiretroviral therapy, dubbed HAART, that pharmacological suppression of HIV-1 replication became possible.\textsuperscript{5-7}

The dramatic data from early HAART studies suggested that complete suppression of HIV-1 replication by HAART in treated individuals would lead to a cure\textsuperscript{7}. However, the war against HIV-1 would not be so easily won. Groundbreaking studies by the Siliciano lab at Johns Hopkins\textsuperscript{8-10}, the Richman lab at UCSF\textsuperscript{11}, and then the Fauci lab at NIAID\textsuperscript{12} established that HIV-1 persisted in a subset of resting CD4+ T cells despite effective antiretroviral therapy. Discouragingly, these groups demonstrated that although quiescent, the virus lurking within these resting CD4+ T cells was capable of stochastically reemerging and rekindling HIV-1 replication even after years of effective antiretroviral therapy. This latent reservoir of HIV-1, then, represents the major barrier to curing HIV-1 infection.

Our understanding of the biology of latently infected CD4+ T cells has grown immensely in the years since its initial discovery and characterization. For instance,
Chomont et al. have defined the distribution of latent HIV-1 across the subsets of resting, memory CD4+ T cells13. We have measured the half-life of the latent reservoir (44 months)14, and uncovered the diverse genetic composition of the latent reservoir within each HIV-1 infected individual15-18. Recently, work from our lab (Ho et al.) has demonstrated that only a small fraction of the HIV-1 proviral DNA sequence contained within resting CD4+ T cells is intact and capable of replication19. In addition, new studies by our lab have more completely defined the various defects found within this non-replication competent HIV-1 proviral DNA, as well as determined that the quantitative viral outgrowth assay likely underestimates the true size of the latent reservoir19. A central feature of latently infected cells is an absence of HIV-1 gene expression and/or virion production of intact integrated HIV-1. Importantly, we have gained a better understanding of what transcriptional and translational blocks are present in resting CD4+ T cells that hinder latent HIV-1 expression and virion production.

This deeper understanding of HIV-1 latency not only highlights the complex virus-host relationship that gives rise to the latent state, but also reveals avenues available to target and eventually eliminate this barrier to a cure. The scientific data indicate that reawakening latent virus from its quiescent state will be a necessary first step in eliminating the latent reservoir. As such, the HIV-1 cure field is developing a “kick and kill” approach for curing HIV-1 infection: first, reactivate latent HIV-1 expression with small molecule latency reversing agents under the cover of antiretroviral therapy, then kill the cells harboring reactivated HIV-1 either by host immune responses or from cytopathic effects of virion production.
My research has focused primarily on developing assays to measure latency reversal and identifying effective means of reactivating latent HIV-1 in cells from infected individuals. I believe that the work described in this dissertation has aided the fight to cure HIV-1 infection and hope that it may serve as a foundation for the identification, development, and clinical testing of latency reversing agents.
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Chapter 1: Rapid Quantification of the Latent Reservoir for HIV-1 using a Viral Outgrowth Assay

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Introduction

Highly active antiretroviral therapy (HAART) has significantly reduced the morbidity and mortality associated with HIV-1 infection. However, while HAART can reduce plasma viral load to below the clinical limit of detection (50 copies HIV-1 RNA/mL) in adherent patients\textsuperscript{5-7}, this treatment is not curative. Even in individuals on prolonged suppressive HAART, HIV-1 persists as a latent but replication-competent provirus integrated in the genomes of a small percentage of resting memory CD4\textsuperscript{+} T cells\textsuperscript{8-11,20}. These latently infected cells are extremely long lived as a consequence of the biology of memory T cells, with an estimated half-life of 44 months\textsuperscript{14,21}. The extreme stability of this HIV-1 reservoir precludes eradication with HAART alone and suggests that, without disruption of this reservoir, infected individuals must remain on HAART for the remainder of their lives\textsuperscript{14,21}.

Recent studies have identified small molecules capable of reactivating HIV-1 gene expression\textsuperscript{22-28}. While resting CD4\textsuperscript{+} T cells harboring a latent HIV-1 provirus are not susceptible to viral cytopathic effects or immune clearance, pharmacological reactivation of latent proviruses could lead to depletion of this latent reservoir.
Recent in vitro work suggests that reactivation paired with a T cell vaccination strategy could be used to eradicate the latent reservoir of HIV-1 in resting CD4\(^+\) T cells\(^{29}\). Several clinical trials are investigating the ability of small molecule reactivators to perturb the latent state of the provirus and reduce the size of the latent reservoir\(^{30,31}\).

Assessing strategies to perturb or eliminate the latent reservoir requires assays that can accurately quantitate the size of the latent reservoir and that can be scaled for use in large clinical trials. The reservoir was originally identified using a viral outgrowth assay carried out on highly purified resting CD4\(^+\) T cells isolated from patient peripheral blood mononuclear cells (PBMC)\(^{10,14,20,32}\). These resting cells do not actively produce virus without stimulation\(^9\). Limiting dilutions of the resting CD4\(^+\) T cells are activated with the mitogen phytohemagglutinin (PHA) in the presence of irradiated allogeneic PBMC. This activation reverses latency and reinitiates the production of infectious HIV-1 from the subset of resting CD4\(^+\) T cells harboring replication-competent proviruses. The viruses that are produced are expanded in PHA-stimulated CD4\(^+\) lymphoblasts from uninfected donors, which are added to the culture at two time points. After two weeks, viral outgrowth is assessed by an ELISA assay for HIV-1 p24 antigen in the culture supernatant. The frequency of latent infection, expressed as infectious units per million (IUPM) resting CD4\(^+\) T cells, is determined using Poisson statistics. Typically, patients on long term HAART exhibit IUPM values between 0.1 and 1\(^{10,14,20}\).

While this viral outgrowth assay is widely recognized as the definitive assay for determining the minimum frequency of CD4\(^+\) T cells harboring replication-
competent proviruses, it is time-consuming, labor-intensive, and expensive, requiring
two weeks of cell culture and PBMC from at least three uninfected blood donors per
assay. In its current form, this assay is not suitable for use in large clinical trials
evaluating the efficacy of eradication strategies, and the identification of simpler
assays for the latent reservoir has been identified as an AIDS research priority\textsuperscript{33}. A
recent study compared 11 different approaches for measuring persistent HIV-1 in
patients on HAART\textsuperscript{34}. Infected cell frequencies measured by PCR-based methods
were generally at least two orders of magnitude higher than and poorly correlated
with IUPM values. This likely reflects the presence of large numbers of defective
proviruses that are detected by PCR-based assays. The results raise doubts about
whether PCR-based assays can be used to assess the frequency of cells harboring
replication-competent proviruses. Therefore we developed a rapid and simple viral
outgrowth assay that can nevertheless detect and provide at least a minimal
estimate of the frequency of cells that must be eliminated to cure HIV-1 infection.
Methods

Patient Cohort

Twenty HIV-1 infected patients were enrolled in this study; 19 were recruited from the Moore Clinic at The Johns Hopkins Hospital and 1 patient was recruited from the SCOPE cohort at the University of California San Francisco. All study participants provided written informed consent for participation. This study was approved by the Johns Hopkins Institutional Review Board. Seventeen of the 20 patients were recruited on the basis of prolonged continuous suppression of plasma HIV-1 viremia on HAART to below the limit of detection of standard clinical assays (<50 copies HIV-1 RNA/mL). Three of the 20 patients were recruited on the basis of detectable plasma HIV-1 viremia; two patients were reported non-adherent to their HAART regimens and one patient had not yet initiated therapy.

Isolation of resting CD4⁺ T lymphocytes

Peripheral blood mononuclear cells (PBMC) were isolated using density gradient centrifugation. CD4⁺ T lymphocytes were enriched by negative depletion (CD4⁺ T cell Isolation Kit, Miltenyi Biotec). Resting CD4⁺ T lymphocytes were further enriched through negative depletion of cells expressing CD69, CD25, or HLA-DR (CD69 MicroBead Kit II, Miltenyi Biotec; CD25 MicroBeads, Miltenyi Biotec; Anti-HLA-DR MicroBeads, Miltenyi Biotec). Resting CD4⁺ T cells purified using the above described two-step bead depletion procedure were stained with CD4-PE and HLA-DR-APC (BD Biosciences). The purity of these cells was analyzed by flow cytometry using a FACS Canto II (BD Biosciences) and FlowJo software (Treestar).

Limiting dilution viral outgrowth assay
For each patient, both a standard viral outgrowth assay and a MOLT-4/CCR5 viral outgrowth assay were performed using freshly purified resting CD4+ T lymphocytes obtained from a single blood draw as described above. Briefly, five-fold serial dilutions of resting CD4+ T lymphocytes from HIV-1 infected patients were stimulated by co-culture with a 10-fold excess of γ-irradiated allogeneic PBMC from uninfected donors and the mitogen PHA (Remel) in RPMI containing 10% fetal bovine serum, 100 U/mL IL-2 (Novartis) and 1% T-cell growth factor (produced in house, as described previously32). These conditions are sufficient to activate 100% of the resting CD4+ T lymphocytes, as previously demonstrated by CFSE dilution and expression of cell surface activation markers10,35. T cell activation reverses HIV-1 latency in at least a fraction of the latently infected cells. After one day of stimulation, the mitogen containing media is removed and either MOLT-4/CCR5 cells or health donor CD4+ lymphoblasts are added in fresh media to propagate replication-competent HIV-1 in the culture wells. The standard viral outgrowth assay utilizes two additions of CD4+ lymphoblasts from uninfected donors as target cells for HIV-1 outgrowth on days 2 and 9. The MOLT-4/CCR5 viral outgrowth assay utilizes a single addition of MOLT-4/CCR5 cells on day 2. The ratio of target cells added is the same for both assays32, with 1×10^7 target cells added to wells containing 1×10^6 patient resting CD4+ T cells and 2.5×10^6 target cells added to all other wells. Five days after initial mitogen stimulation of input resting CD4+ T lymphocytes, the culture media was changed and the cells in each well were split. Supernatants from each well were tested for HIV-1 RNA and/or HIV-1 p24 protein at various time points by RT-PCR and ELISA (Alliance HIV-1 p24 antigen ELISA Kit, Perkin Elmer),
respectively. The frequency of latently infected cells among the input resting CD4+ T lymphocytes was calculated by a maximum likelihood method, as described previously and is expressed as infectious units per million cells (IUPM)\textsuperscript{32}. The 95% confidence intervals for individual IUPM determinations are ±0.7 log IUPM, or 5 fold\textsuperscript{32}. With a sample size of 25, this assay can detect with 80% power a 0.2 log reduction in the reservoir assuming a type 1 error of 0.05.

**HIV-1 RNA detection by RT-PCR**

After reversal of latency and subsequent release of HIV-1, virion-associated HIV-1 RNA was isolated from 60µL of culture supernatant using the ZR-96 Viral RNA Kit (Zymo Research), a 96-well column based RNA isolation kit. cDNA was synthesized from the isolated HIV-1 RNA using the SuperScript III First-Strand Synthesis System (Invitrogen) with oligo-dT primers. Isolated cDNA was assayed for HIV-1 by RT-PCR as described previously\textsuperscript{36} using an Applied Biosystems 7300 Real Time PCR System (Applied Biosystems) and TaqMan Universal PCR Mastermix (Applied Biosystems). RNA copy number was determined using a standard curve generated with the DNA plasmid pVQA, described previously\textsuperscript{37}. RNA copy numbers below 10 copies or above 10\textsuperscript{6} copies were extrapolated based on the standard curve generated.

**Statistics**

Infected cells frequencies in limiting dilution assays were calculated as described by Myers et al.\textsuperscript{38}. Where appropriate, results were expressed as mean ± standard deviation. A Wilcoxon rank sum test (paired comparison) was used for
statistical analysis. All statistical analyses were performed with MedCalc software, v12.4.0.0. A p value of <0.05 was considered significant.
Results

Patient characteristics

A rapid and simple viral outgrowth assay was developed and evaluated using samples from 20 patients with HIV-1 infection. The baseline characteristics of the patient cohort enrolled in this study are summarized in Table 1. Seventeen patients were recruited on the basis of prolonged suppression of viremia to <50 copies of HIV-1 RNA/mL on HAART, with a duration of suppression from 12 to 156 months. We intentionally enrolled three additional patients who were viremic at the time of blood draw with viral loads of 5,392, 452,059, and 31,238 copies/mL. Of the three viremic patients, one patient had not yet started HAART and two were known to be non-adherent. The average age of the cohort (± SD) was 50.8 ± 10.8 years, and the cohort was overwhelmingly comprised of black males. The CD4 nadir of the cohort ranged from 6 to 755 cells/µL, and 8 patients had a history of an AIDS diagnosis.

Highly purified resting CD4+ T cells can be obtained using a two-step bead depletion procedure

The original viral outgrowth assay used to define the latent reservoir was performed on resting CD4+ T cells purified from PBMC in a multistep process that included fluorescence activated cell sorting (FACS) and required a BSL-3 sorting facility. Resting CD4+ T cells were differentiated from activated CD4+ T cells by the absence of cell surface markers CD69, CD25, and HLA-DR. To simplify the purification of resting CD4+ T cells, we devised a two-step bead depletion purification procedure. Initially, CD4+ T cells were purified from PBMC by negative selection as
described in Methods. Subsequent bead depletion of cells expressing CD69, CD25, or HLA-DR yielded a highly purified, unmanipulated population of resting CD4⁺ T cells. The purity of these cells was routinely assessed by staining with antibodies to CD4 and HLA-DR.

Representative examples are shown in Figure 1.1 and Figure 1.2. Typical purities were 96-97% with less than 0.1% contamination with activated (HLA-DR⁺) CD4⁺ T cells. As is discussed below, IUPM values obtained with these purified resting CD4⁺ T cells were in the same range as observed with sorted cells.

The use of MOLT-4/CCR5 cells for virus expansion gives statistically comparable results at 14 days

The standard viral outgrowth assay requires a minimum of 3 separate blood samples from healthy donors in addition to a blood sample from the test patient (Figure 1.3). CD4⁺ T lymphoblasts from two of these samples are added to cultures at days 2 and 7 to expand virus released from patient cells in which latency has been reversed by T cell activation. These donor lymphoblasts are prepared by PHA stimulation of donor PBMC for 2 days followed by depletion of CD8⁺ T cells. Eliminating the need for donor lymphoblasts in virus expansion would significantly simplify the assay. We hypothesized that a single addition of a cell line expressing high levels of CD4 and the co-receptors CCR5 and CXCR4 would allow for efficient expansion of viruses released from latently infected cells. We chose the MOLT-4/CCR5 cell line.³⁹ This cell line was derived from MOLT-4 cells, which express high levels of CD4 and CXCR4, and has been engineered to stably express CCR5. It is thus capable of supporting replication of both X4-tropic and R5-tropic variants of
HIV-1. To verify that the MOLT-4/CCR5 cells are an acceptable alternative for donor-derived CD4+ T lymphoblasts, we compared IUPM values obtained using the standard viral outgrowth assay with donor derived lymphoblasts to IUPM values obtained using a simplified assay in which a single addition of MOLT-4/CCR5 cells was used in place of donor lymphoblasts. All 3 viremic patients (V1, V2, V3) and 14 of the 17 patients on suppressive HAART (S1-S14) were included in this comparison. Resting CD4+ T cells obtained from each patient using the bead depletion method described above were split and tested using the standard assay and the simplified MOLT-4/CCR5 assay as outlined in Figure 1.3. The p24 ELISA at day 14 was used as an assay endpoint. Replication-competent HIV-1 was isolated from purified resting CD4+ T lymphocytes in all 3 of the viremic patients and 10 of the 14 patients on HAART by both assays (Figure 1.4A, Table 1.2). In 3 of the 14 patients on HAART, replication-competent HIV-1 was isolated in only one of the two viral outgrowth assays (Figure 1.4A, Table 1.2, Patients S4, S7, and S14). No replication-competent HIV-1 was recovered from purified resting CD4+ T lymphocytes by either viral outgrowth assay in 2 of the 14 patients on HAART (Figure 1.4A, Table 1.2, Patients S8 and S13). These results are expected because splitting the sample reduces the input number of resting CD4+ T cells. The frequency of latently infected resting CD4+ T lymphocytes was markedly higher in the viremic patients compared to patients on HAART (Figure 1.4A, Table 1.2), consistent with our previous results21.

No significant difference was observed between the frequency of latently infected resting CD4+ T lymphocytes measured in the standard viral outgrowth assay
versus the MOLT-4/CCR5 viral outgrowth assay (Figure 1.4B, Wilcoxon rank sum test, p=0.9032). Furthermore, the frequency of latently infected cells as measured by the MOLT-4/CCR5 viral outgrowth assay correlates highly with that of the standard viral outgrowth assay (Figure 1.4C, Pearson’s correlation coefficient, r=0.9381, p<0.0001). When only patients on suppressive HAART were considered, the correlation remained highly significant (r=0.7602, p=0.0016).

**HIV-1 specific RT-PCR assay detects HIV-1 outgrowth from the latent reservoir earlier than p24 antigen ELISA**

To shorten the time required to measure latently infected cells by the viral outgrowth assay, we explored the use of RT-PCR as an alternative to the p24 ELISA to detect virus production. Twenty-nine replicate wells were set up with 200,000 patient resting CD4+ T cells/well from a patient on suppressive HAART (S15). The cells were activated with PHA and irradiated feeders and then cultured with MOLT-4/CCR5 cells over 14 days using the protocol developed for the viral outgrowth assay. Culture supernatants were assayed for released virus at multiple time points during the 14-day culture using both the p24 antigen ELISA and a novel RT-PCR assay. This assay detects polyadenylated HIV RNAs without interference from proviral or plasmid DNA, and when applied to virion-containing supernatants, detects mainly genomic viral RNA, allowing accurate quantitation of virus release36. For both assays, positive wells showed an exponential increase in the amount of virus in the supernatant (Figures 1.5A and 1.5B). Among the wells positive for outgrowth, there was complete concordance between HIV-1 p24 antigen ELISA and HIV-1 specific RT-PCR at 14 days. All of the wells that were positive for outgrowth by RT-PCR
eventually tested positive by p24 ELISA (Figure 1.5C). Under conditions where a majority of the positive wells are predicted to contain a single latently infected cell, HIV-1 specific RT-PCR detected viral outgrowth significantly earlier than HIV-1 p24 antigen ELISA (Figure 1.5D, Wilcoxon rank sum test, p=0.0020), with the average days (± S.D.) of detection being 6.1 ± 2.1 and 9.6 ± 2.7 days, respectively. These results indicate that HIV-1 specific RT-PCR accurately detects viral outgrowth in a shorter time frame.

**Accurate measurement of the frequency of latently infected cells can be obtained in 7 days using RT-PCR to detect outgrowth in the MOLT-4/CCR5 viral outgrowth assay.**

Given that HIV-1 specific RT-PCR accurately detected HIV-1 outgrowth from the latent reservoir significantly earlier than the HIV-1 p24 antigen ELISA, we sought to determine whether HIV-1 specific RT-PCR could be used to detect positive wells more rapidly in the MOLT-4/CCR5 viral outgrowth assay. As shown in Figure 1.5B, nearly all wells that eventually became positive by p24 ELISA were positive by RT-PCR on day 7. Therefore, supernatants from viral outgrowth cultures from patients S8-S14 and V1-V3 were tested at day 7 using the HIV-1 specific RT-PCR assay. For comparison, an HIV-1 p24 antigen ELISA was also performed on culture supernatants from these viral outgrowth cultures on day 7. The frequencies of latently infected cells obtained using both assays on day 7 of the viral outgrowth assay were compared to the frequencies determined on day 14 using the HIV-1 p24 antigen ELISA (Figure 1.6A). The frequencies determined with HIV-1 p24 antigen ELISA on day 7 were significantly lower than the frequencies determined with the
same assay on day 14 (Figure 1.6A, Wilcoxon rank sum test, p=0.0010). However, the frequencies determined with HIV-1 specific RT-PCR on day 7 and with HIV-1 p24 antigen ELISA on day 14 were not significantly different (Figure 1.6A, Wilcoxon rank sum test, p=0.9219). These results suggest that the use of a sensitive assay for free virus on day 7 on the culture may effectively substitute for an ELISA assay on day 14.

We next sought to evaluate the agreement between the day 7 and day 14 endpoint assays. A total of 74 wells from patients S8-S14 and V1-V3 were positive for HIV-1 outgrowth by either HIV-1 specific RT-PCR or HIV-1 p24 antigen ELISA. Outgrowth was detectable on day 7 using HIV-1 p24 antigen ELISA in only 60% of the positive wells. However, outgrowth was detectable in 81% of the positive wells on day 7 using HIV-1 specific RT-PCR. The correlation of the frequency of latently infected resting CD4⁺ T cells calculated at day 7 and day 14 was evaluated using Pearson’s correlation coefficient. The correlation between the frequency of latent infection calculated at day 14 using HIV-1 p24 antigen ELISA and the frequency determined on day 7 was markedly higher when the HIV-1 specific RT-PCR assay was used rather than the HIV-1 p24 antigen ELISA (Figure 1.6C versus Figure 1.6B, r=0.9698 versus r=0.9133). When only patients on suppressive HAART were considered, the frequency determined at day 7 using the RT-PCR assay was positively correlated with the frequency determined at day 14 by HIV-1 p24 antigen ELISA (r=0.8516, p=0.0001) while the frequency determined at day 7 using the HIV-1 p24 antigen ELISA no longer correlated with the frequency determined at day 14 by ELISA (r=0.4448, p=0.1110). Two additional patients on suppressive HAART
(S16, S17) were included in a final comparison of the rapid MOLT-4/CCR5 viral outgrowth assay and the standard viral outgrowth assay. A statistically significant positive correlation was seen when the frequency of latently infected resting CD4$^+$ T cells determined using the rapid MOLT-4/CCR5 viral outgrowth assay was compared to the frequency determined using the standard viral outgrowth assay (Figure 1.6D, $r=0.9591$, $p<0.0001$). When only patients on suppressive HAART were considered, a statistically significant positive correlation was still observed ($r=0.7522$, $p=0.0194$).
Discussion

Latent HIV-1 infection of resting CD4\(^+\) T cells remains the major barrier to HIV-1 eradication. A number of small molecules have been identified that are capable of reactivating transcription of otherwise silent HIV-1 proviruses\(^{22-27}\). Some of these compounds have already entered clinical trials\(^{30}\), and drug discovery efforts to find additional compounds that can perturb or eliminate latent HIV-1 continue. Concurrently, immunological approaches are being investigated and have shown promise\(^{29}\). However, a key hurdle facing HIV-1 eradication efforts has, until recently, been largely ignored: the development of a reliable and simple assay to measure the size of the HIV-1 latent reservoir. Such an assay is absolutely required for evaluating the effectiveness of an eradication strategy. PCR based assays are being used to quantify proviruses in T cell subsets and the level of residual viremia in HIV-1 infected patients\(^{30,41-45}\). A recent study has compared results of various PCR based assays with those obtained with the viral outgrowth assay using a set of samples from two well characterized cohorts of patients on HAART\(^{34}\). Because current PCR assays detect both replication-competent and defective proviruses, the correlation between infected cell frequencies measured by PCR and viral outgrowth was not strong, with the exception of an assay measuring integrated HIV-1 DNA in PBMC\(^{34}\). The measurement of integrated HIV-1 DNA by Alu PCR\(^{44}\) is of particular interest because the stable reservoir for HIV-1 consists of resting CD4\(^+\) T cells harboring integrated HIV-1 DNA\(^{8,9}\). It is likely that this and other PCR based assays will play an important complementary role to viral outgrowth assays. Prior to the present study, the standard viral outgrowth assay was the only assay available to
directly quantify the frequency of resting CD4+ T cells harboring latent but replication-competent viral genomes.

The development of a rapid isolation procedure to obtain unperturbed resting CD4+ T cells was an essential first step towards creating a viral outgrowth assay suitable for widespread use in eradication studies and clinical trials. The latent reservoir was originally defined using viral outgrowth assays performed on highly purified populations of resting CD4+ T cells obtained through a combination of magnetic bead depletion and cell sorting. Thus, the assay required a BSL-3 cell sorting facility. While feasible for small-scale studies, this approach cannot be utilized for large-scale studies in which numerous measurements of the size of the latent reservoir must be taken across many patients. As we have demonstrated here, our isolation procedure yields a highly purified resting CD4+ T cell population. Moreover, the frequencies of latently infected cells measured in these populations are very similar to those obtained with the sorting method8-10.

The standard viral outgrowth assay relies on a minimum of 3 separate blood samples from healthy donors. PBMC from two of the subsequent donations are added to the cultures to propagate the HIV-1 released following the reversal of latency. These healthy donor cells may also provide subsequent allogeneic stimulation to the patient cells in culture. We sought to replace these healthy donor cells with a cell line that supports infection by both X4-tropic and R5-tropic HIV-1. Furthermore, we believed that a cell line would provide greater uniformity to the viral outgrowth assay, since every viral isolate growing out of a patient’s latent reservoir would propagate in identical culture conditions. As we have clearly demonstrated,
the MOLT-4/CCR5 cell line performs robustly in place of mitogen stimulated CD8-depleted healthy donor PBMC in the viral outgrowth assay. This modification significantly simplifies the assay and allows the assay to be more easily scaled for large studies or clinical trials. Other cell lines that support replication of both X4 and R5 isolates could potentially be used as target cells in this assay. Of note, our rapid assay in its current form still requires a single blood donation from a healthy donor for the generation of irradiated PBMC used in the initial mitogen stimulation. It is possible that the replacement of mitogen stimulation with co-stimulation via anti-CD3 and anti-CD28 monoclonal antibodies could alleviate the need for any healthy blood donors.

Interestingly, the success of MOLT-4/CCR5 cells in propagating reactivated HIV-1 suggests that the allogeneic stimulation provided by the healthy donor CD4+ T lymphoblasts was not required. As MOLT-4/CCR5 cells do not express MHC class II46,47, no allogeneic stimulation of patient CD4+ T cells should occur. The lack of allogeneic stimulation was noted in the early characterization of the MOLT-4 cell line48-50. Thus, we can infer from our study that the initial mitogen stimulation alone is generally sufficient for reactivation of latent HIV-1 and viral outgrowth. However, we cannot conclude that this initial mitogen stimulation is sufficient for reactivation of all latent proviruses. Studies are ongoing to determine whether any replication-competent proviruses remain non-induced after a single round of mitogen stimulation. If this is the case, any measurement of the size of the latent reservoir that relies upon reactivation of latent proviruses through a single round of T cell stimulation may in fact be underestimating the size of the reservoir. The strategic
use of both culture and PCR based assays may allow us to bracket the true size of the latent reservoir.

The standard viral outgrowth assay requires 14 days to complete. This 14 day period includes two additions of CD4+ T lymphoblasts from healthy donor PBMC as well as multiple media changes. The length of time required for the standard viral outgrowth assay is a function of the endpoint assay used to measure viral outgrowth: the HIV-1 p24 antigen ELISA. With the goal of reducing the length time required for detecting viral outgrowth, we adapted a recently developed HIV-1 specific RT-PCR assay for use as an endpoint assay. On average, viral outgrowth from the latent reservoir under conditions resembling the MOLT-4/CCR5 viral outgrowth assay was detectable by the HIV-1 specific RT-PCR assay after 6.1 ± 2.1 days (± S.D) versus 9.6 ± 2.7 days (± S.D) for HIV-1 p24 antigen ELISA. This result indicated that utilizing a more sensitive endpoint assay for viral outgrowth could indeed reduce the length of time required to complete the MOLT-4/CCR5 viral outgrowth assay. The data presented here demonstrate that no significant difference exists between the frequency of latent infection of resting CD4+ T cells as measured at day 7 using HIV-1 specific RT-PCR and at day 14 as measured by HIV-1 p24 antigen ELISA.

The utilization of an RT-PCR based measurement for HIV-1 outgrowth not only allows more sensitive detection of viral replication, but it will allow for more high-throughput measurement of HIV-1 replication. Of note, extremely high sequence conservation has been observed at the primer and probe binding sites, especially amongst subtype B isolates\textsuperscript{36}. The degree of conservation is actually higher than is observed in the regions of \textit{gag} that are amplified in many other PCR assays\textsuperscript{36}. It
remains possible that in rare patients, sequence variation in these conserved regions could interfere with PCR detection. In these rare cases, negative results in the PCR assay may be due to primer mismatch rather than low frequency of latent infection.

Given the greater sensitivity of the HIV-1 specific RT-PCR assay, it is possible that small amounts of replication-defective virus released after mitogen stimulation of resting CD4+ T cells might be detected, resulting in a false positive readout of viral outgrowth. Our data suggest that the release of replication-defective virus is not widely detected by the RT-PCR assay at day 7. In rare cases (patient S13), we observed weak positive signals by RT-PCR at day 7 in wells that remained negative by p24 ELISA at day 14. These data could represent viruses that have low fitness and a slower replication rate than needed to expand to beyond the limit of detection for HIV-1 p24 antigen ELISA on day 14. It is possible that such viruses could be detected by HIV-1 p24 antigen ELISA with a longer culture period. As shown in Figure 4C, only 11 out of 29 wells containing an input of 200,000 resting CD4+ T cells from patient S15 were positive for viral outgrowth. The frequency of latent HIV-1 infection measured in the resting CD4+ T cells of patient S15 (3.25 IUPM) suggests that each of the 29 replicate wells likely contained dozens of integrated, defective proviruses. However, only 11 wells were positive for when tested by RT-PCR. Furthermore, of the wells that were positive for viral outgrowth, detection by HIV-1 specific RT-PCR was not possible until day 6.1 ± 2.1 days (± S.D), and these wells remained positive for the duration of the outgrowth time course, with an exponentially increasing amount of virus. These data suggest that
our HIV-1 specific RT-PCR requires a level of viral replication achieved after a nearly one week and is not sensitive enough to detect replication defective viruses that do not expand further.

The MOLT4/CCR5 viral outgrowth assay does require a single large blood sample of 150-200mL. This is due to the low frequency of replication-competent proviruses harbored within resting CD4\(^+\) T cells. Given this fundamental aspect of the biology of HIV-1 latency, it is unlikely that any version of a viral outgrowth assay can be performed without a large input of resting CD4\(^+\) T cells. However, because no alternative assay exists that specifically measures latent, replication-competent proviruses in resting CD4\(^+\) T cells, viral outgrowth assays will likely continue to play an important role in evaluating HIV-1 eradication strategies.

The MOLT-4/CCR5 utilizing viral outgrowth assay presented here is the most rapid and scalable assay available for measuring the size of the HIV-1 latent reservoir. As such, we believe that this assay will be an indispensable tool in evaluating the success of strategies to perturb or eradicate the HIV-1 latent reservoir.
Table 1.1 Characteristics of study patients.

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<th>Viral Load at Enrollment</th>
<th>ART§</th>
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† Race abbreviations:  W, white, non-Hispanic; H, Hispanic; B, black
§ Time of documented continuous suppression of plasma viremia to <50 copies/mL on HAART
‡ Drug abbreviations:  DRV/r, darunavir boosted with ritonavir; FTC, emtricitabine; TDF, tenofovir disoproxil fumarate; EFV, efavirenz; RAL, raltegravir; RAL/r, raltegravir boosted with ritonavir; ATV/r, atazanavir boosted with ritonavir; 3TC, lamivudine; MVC, maraviroc; FPV/r, fosamprenavir boosted with ritonavir; NVP, nevirapine
Table 1.2  Summary of infectious units per million (IUPM) measurements.

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Figure 1.1 Two-step bead depletion procedure yields highly purified resting CD4⁺ T cells from HIV-1 infected patients. A two-step negative selection strategy to purify resting CD4⁺ T cells from patient PBMC. (A) Representative FSC/SSC plot indicating live cell population after the two-step bead depletion procedure. (B) Representative dot plot indicating purity of resting CD4⁺ T cells. Purified cells were stained with antibodies to CD4 and HLA-DR.
Figure 1.2 Purity staining of resting CD4\(^+\) T cells obtained from an HIV-1 infected patient via two-step bead depletion procedure. Resting CD4\(^+\) T cells obtained from an HIV-1 infected patient on suppressive HAART. (A) Representative dot plot indicating CD4 and CD25 expression. (B) Representative dot plot indicating CD69 and HLA-DR expression.
Figure 1.3 The standard and the MOLT-4/CCR5 viral outgrowth assays. The frequency of HIV-1 latent infection of resting CD4⁺ T cells can be measured using a viral outgrowth assay. PBMC are collected from HIV-1 infected individuals and resting CD4⁺ T cells (CD25⁻, CD69⁻, HLA-DR⁻) are purified. Resting T cells are plated in 5-fold serial dilutions in duplicate, such that the input number of patient cells ranges from 1,000,000 to 320 cells per well. To reverse latency in the cells that harbor a replication-competent HIV-1 provirus, patient cells are activated with PHA and a 10-fold excess of irradiated PBMC from healthy donors. The next day, target cells for HIV-1 infection are added to allow outgrowth of replication-competent HIV-1 released from infected cells in which latency has been reversed. In the standard viral outgrowth assay, CD4⁺ lymphoblasts from healthy donors are added on days 2 and 7 of the assay. In the MOLT-4/CCR5 viral outgrowth assay, MOLT-4/CCR5 cells are added on day 2 only. For the standard assay, HIV-1 p24 antigen ELISA is used to identify wells positive for HIV-1 outgrowth at 14 days. For the MOLT-4/CCR5 assay, RT-PCR is used to identify wells positive for outgrowth at 7 days. The frequency of latently infected cells can be determined using limiting dilution statistics based on the input number of patient cells in the wells positive for outgrowth. This frequency is reported in infectious units per million (IUPM).
Figure 1.4 The standard and the MOLT-4/CCR5 viral outgrowth assays yield comparable frequencies of latent infection. (A) The frequency of latently infected resting CD4⁺ T cells was measured in 3 viremic patients and 14 HAART-suppressed patients using both the standard and the MOLT-4/CCR5 viral outgrowth assays, with HIV-1 p24 antigen ELISA used as the endpoint assay of viral outgrowth at day 14. (B) Statistical comparison of the IUPM values measured using the standard viral outgrowth assay and the MOLT-4/CCR5 viral outgrowth assay by Wilcoxon rank sum test. (C) The correlation of IUPM values measured using the standard viral outgrowth assay and the MOLT-4/CCR5 viral outgrowth assay (Pearson’s correlation coefficient, r).
Figure 1.5 Kinetics of HIV-1 outgrowth from latently infected CD4⁺ T cells measured by HIV-1 p24 antigen ELISA and HIV-1 specific RT-PCR. Resting CD4⁺ T cells were isolated from HAART patient S15, whose latent reservoir was previously measured to be 3.25 IUPM. Twenty-nine replicate wells were plated in which 200,000 resting cells were activated with PHA and irradiated PBMC from a healthy donor and subsequently cultured with MOLT-4/CCR5 cells. Outgrowth of reactivated HIV-1 was measured in positive wells over 14 days using both (A) HIV-1 p24 antigen ELISA and (B) HIV-1 specific RT-PCR. (C) The difference between the day on which a particular well becomes positive by RT-PCR versus p24 ELISA. (D) The day of detection of HIV-1 outgrowth from the latent reservoir is shown for HIV-1 p24 antigen ELISA and HIV-1 specific RT-PCR.
Figure 1.6 Accurate measurement of IUPM at day 7 using HIV-1 specific RT-PCR. (A) Using the rapid MOLT-4/CCR5 viral outgrowth assay, the frequency of latently infected cells was measured for HAART patients S1-S14 and viremic patients V1-V3 at day 7 with the HIV-1 specific RT-PCR assay and at both days 7 and 14 with HIV-1 p24 antigen ELISA. Statistical significance of the differences in IUPM values was assessed by Wilcoxon rank sum test. (B) Correlation of the IUPM measured at day 7 using HIV-1 p24 antigen ELISA with the IUPM measured at day 14 using HIV-1 p24 antigen ELISA (Pearson’s correlation coefficient, r). (C) Correlation of the IUPM measured at day 7 using HIV-1 specific RT-PCR with the IUPM measured at day 14 using HIV-1 p24 antigen ELISA (Pearson’s correlation coefficient, r). (D) Correlation of the IUPM measured at day 7 using the rapid MOLT-4/CCR5 outgrowth assay with the IUPM measured at day 14 using the standard outgrowth assay (Pearson’s correlation coefficient, r).
Chapter 2: A novel cell-based high throughput screen for inhibitors of HIV-1
gene expression and budding identifies the cardiac glycosides.

The work presented in this chapter was published in *Journal of Antimicrobial Chemotherapy* in April, 2014, volume 69(4):988-94.

**Introduction**

Current antiretroviral therapy effectively reduces the plasma level of human immunodeficiency virus type 1 (HIV-1) in most infected individuals to below limit of detection of clinical assays\(^{5-7}\). However, the ongoing problems of undesirable off-target effects, high cost, and drug resistant viral strains indicate a need for additional classes of antiretroviral drugs\(^{51,52}\). Viral assembly and budding have both been previously suggested as potential targets for such new drug classes. Late pre-assembly stages of the replication cycle include viral transcription, translation, and transport of viral proteins and RNA to the plasma membrane. Although these stages are primarily mediated by host proteins, they could be targeted by novel antiretroviral drugs. With regard to virus assembly, previous studies have identified potentially targetable interactions mediated by both the C-terminal and N-terminal domains of capsid, as well as interactions between zinc and the zinc finger of the nucleocapsid protein\(^{53}\). In the case of budding, the interaction between the cellular protein Tsg101, a member of the ESCRT-1 family, and the PTAP sequence in the p6 domain of the viral protein Gag, has been identified as a target\(^{54,55}\). Despite several potential drug targets, there are currently no clinically approved drugs that inhibit stages of the HIV-
1 replication cycle following integration and preceding maturation, leaving a significant portion of the HIV-1 replication cycle unopposed by antiretrovirals.

The aim of this study was to develop a high throughput method to screen for compounds with late-stage inhibitory potential against HIV-1. To do so, we transfected 293T cells with plasmids containing HIV-1 proviral DNA and then measured virus production using a qPCR assay of nucleic acid extracted from the cellular supernatant. The reverse transcription step of this assay made use of an oligo-dT primer to prevent the detection of both HIV-1 DNA and unprocessed RNA transcripts. Additionally, the qPCR step used a primer specific for polyadenylated HIV-1 RNA, again preventing the detection of unprocessed HIV-1 RNA transcripts and HIV-1 DNA, including the plasmid DNA used in transfection. To test this approach, we screened the Spectrum 2000 drug library.

Of the compounds that inhibited late stages of the HIV-1 replication cycle, there was a striking overrepresentation of drugs from the cardiac glycoside class. We identified compounds belonging to both subclasses of cardiac glycosides: the cardenolides and the bufadienolides. Although both are C(23) steroids, they differ in that cardenolides contain a butenolide five-membered lactone ring at C-17, whereas bufadienolides contain a six-membered lactone ring. Members of both classes of cardiac glycosides inhibited late stages of HIV-1 production, and changes in structure resulted in changes in inhibition.
Methods

293T cell screen for inhibitors of post-integration stages of the HIV-1 replication cycle

Lipofectamine 2000 (Invitrogen, USA) was used to co-transfect 293T cells with a plasmid expressing a CXCR4-tropic HIV-1 envelope protein (pX4) as well as a plasmid containing a variant of the reference HIV-1 isolate NL4-3 in which the env gene has been replaced with green fluorescent protein (GFP) (pNL4-3ΔEnvGFP). After 6 hours, the cells were treated with 0.05% Trypsin-EDTA (Gibco, USA), pelleted, and plated in flat-bottom 96 well plates at 180,000 cells/well in Dulbecco’s Modified Eagle Medium + 10% fetal bovine serum. Each test compound was added at a final concentration of 10 µM. Compound library (Microsource, USA). The compounds were dissolved in DMSO, and an equivalent concentration of DMSO (0.1% v/v) was present in each well, including the control wells. The proteasome inhibitor epoxomicin (Sigma-Aldrich) was added at a concentration of 1.8 µM as a positive control. The cells were incubated for 18 hours at 37°C in 5% CO₂. The supernatant was then removed, and the released HIV-1 RNA was extracted and quantitated.

HIV-1 RNA extraction and RT-qPCR

HIV-1 RNA was extracted from 60 µL of treated the ZR-96 Viral RNA kit (Zymo Research, USA). Eluted RNA was reverse transcribed to cDNA using the Superscript III First-Strand Synthesis System (Invitrogen, USA). The reverse transcription reaction was primed with oligo-dT, as described in the manufacturer’s protocol. The HIV-1 cDNA was detected using
primers and probes specific for sequences corresponding to polyadenylated HIV-1 RNA. Inhibition was calculated relative to the untreated control.

**Flow cytometry**

Flow cytometry was performed using a BD FACS Canto II (BD Biosciences, USA). Analysis was performed using FlowJo software (Tree Star, USA). Evaluation of the toxicity of the compounds screened was performed by fixing the cells with 2.5% paraformaldehyde and examining the resulting forward/side scatter plots. Any compounds that caused significant toxicity were not included in the list of hits and were not used in subsequent experiments.

**Measurement of intracellular Ca^{2+} levels with Fura Red AM dye**

Increases in intracellular Ca^{2+} were visualized using Fura Red AM dye (Invitrogen, USA). After treatment, cells were incubated with Fura Red AM dye (10 μM) for 30 minutes and then washed with PBS. Cells were analyzed for Fura Red AM by flow cytometry using the AmCyan channel. Decreases in the AmCyan signal corresponded to quenching of the Fura Red AM fluorescence as a result of increases in intracellular Ca^{2+}. The inhibitor KB-R7943 was obtained from Sigma-Aldrich (USA).

**Plasmids for the expression of the Na^{+}/K+-ATPase**

The plasmid containing the murine Na^{+}/K+-ATPase was obtained from Open Biosystems (plasmid MMM1013-7512721). The plasmid containing the human Na^{+}/K+-ATPase was obtained from Open Biosystems (plasmid MHS1010-9205172).
Results

Development of a cell-based screen for post-integration inhibitors of the HIV-1 replication cycle

We designed a cell-based system to screen for compounds that inhibit the release of packaged HIV-1 genomic RNA. Briefly, 293T cells were co-transfected with plasmids containing a CXCR4-tropic HIV-1 envelope (pX4) and an NL4-3 genome in which the env gene was deleted and replaced with GFP (pNL4-3ΔEnvGFP). The transfected 293T cells were transferred to 96-well plates, and assay compounds were added at the screening concentration of 10 μM. After 18 hours, cell-free supernatants were collected and viral RNA isolated. The eluted viral RNA was reverse transcribed to cDNA and then analyzed by quantitative PCR using a probe and primers specific to the DNA sequence corresponding to the polyadenylated HIV-1 RNA36.

To verify that this screen was capable of detecting compounds that inhibited the late stages of the HIV-1 life cycle, we utilized epoxomicin, a potent inhibitor of the 26S proteasome, as a positive control. Treatment of HIV-1-producing cells with epoxomicin has been shown to reduce the release of virus particles, presumably through the reduction in the levels of free ubiquitin required for HIV-1 gag processing57. Treatment with epoxomicin reduced the amount of HIV-1 genomic RNA released into the supernatant (Figure 2.1A), indicating that this system can detect known post-integration inhibitors of HIV-1 replication.

Identification of the cardiac glycosides as potent inhibitors of late stages of the HIV-1 replication cycle
With the goal of identifying novel late-stage inhibitors of the HIV-1 replication cycle, we utilized the 293T cell-based system described above to screen the Spectrum 2000 compound library (Microsource Discovery), a 2000-compound collection of approved drugs, natural products, and other bioactive molecules. Our screen yielded a number of compounds that inhibited HIV-1 genomic RNA release to varying degrees, with some compounds causing greater than five logs of inhibition relative to the negative controls (data not shown).

Among the compounds identified in our screen were the transcription inhibitor actinomycin D and the translation inhibitor cycloheximide. Actinomycin D has been shown to inhibit the replication of several other viruses\textsuperscript{58-60}. The identification of these general inhibitors served to validate the screening approach. Furthermore, other previously described inhibitors of HIV-1 were identified, including celastrol and digoxin\textsuperscript{61,62}. Recently, digoxin has been shown to strongly inhibit HIV-1 structural protein synthesis via alteration of RNA processing\textsuperscript{62}. Whether other cardiac glycosides also inhibit HIV-1 was previously unknown. Here, we observed that numerous other cardiac glycosides inhibited HIV-1 production (Table 2.1). Of these, the majority of the screened cardiac glycosides were cardenolides; the single bufadienolide that was screened also inhibited HIV-1 production (Table 2.1).

Because of the striking overrepresentation of cardiac glycosides among the inhibitors identified in the screen, we chose to focus specifically on these compounds. To determine whether the reduction in supernatant HIV-1 RNA by the cardiac glycosides resulted in a functional reduction in the infection of primary CD4\textsuperscript{+} T cells by those virus-containing supernatants, we spinoculated primary CD4\textsuperscript{+}
lymphoblasts from healthy donors with the supernatant from 293T cells that had been transfected with pX4 and pNL4-3ΔEnvGFP and then treated with cardiac glycosides. Because we observed that the cardiac glycosides inhibited HIV-1 production in our 293T cell screen, we expected that the supernatant from treated transfected cells would contain fewer viruses than supernatant from transfected cells that were not treated with cardiac glycosides. The resulting level of infection in CD4+ lymphoblasts therefore reflects how much virus was produced in the presence of the cardiac glycosides. Infection was measured 3 days after spinoculation using flow cytometry to detect GFP expression. The percentage inhibition was calculated relative to the maximum infection observed from infection with the supernatants from untreated, transfected 293T cells, and IC₅₀ values were calculated. We observed dose-dependent reductions in the infection of CD4+ lymphoblasts by supernatants from cardiac glycoside-treated cells, with IC₅₀ values ranging from 349.5 nM down to 4.3 nM for the compounds tested (Table 2.2). This confirms that the reductions in supernatant HIV-1 RNA caused by cardiac glycoside treatment resulted in reductions of infectious virus in the supernatant.

We sought to further verify that the cardiac glycosides inhibit production of HIV-1 structural proteins. Therefore, we directly examined the effects of cardiac glycosides on the expression of GFP from a proviral construct with GFP in the env ORF. We observed dose-dependent reductions in HIV-1 gene expression by the cardiac glycosides in 293T cells transfected with pNL4-3ΔEnvGFP and pX4, as measured by flow cytometry analysis of gene expression (data not shown). Expression of GFP is expected to require a single splicing reaction of the type used
to generate env mRNAs. Our finding is consistent with a previous report indicating that digoxin interferes with the Rev-dependent export of singly spliced HIV-1 RNA\(^62\). Our results extend these findings by demonstrating that the cardiac glycosides as a class inhibit HIV-1 gene expression.

**Cardiac glycoside inhibition of HIV-1 gene expression is dependent upon the Na\(^+\)/K\(^+\)-ATPase**

Cardiac glycosides inhibit the Na\(^+\)/K\(^+\)-ATPase by binding to the \(\beta1\) subunit, blocking Na\(^+\) ion extrusion from treated cells\(^63,64\). However, the murine \(\alpha\)-1 subunit is resistant to inhibition by cardiac glycosides\(^65\). To determine whether the inhibition of HIV-1 gene expression by the cardiac glycosides was dependent upon the inhibition of the human Na\(^+\)/K\(^+\)-ATPase, 293T cells were transiently co-transfected with the murine or human \(\beta1\) subunit in addition to pNL4-3ΔEnvGFP and pX4. Overexpression of the murine \(\beta1\) subunit inhibition by digoxin, as measured by GFP expression (Figure 2.2A versus 2.2B). Thus, expression of the digoxin-resistant murine \(\beta1\) subunit of the Na\(^+\)/K\(^+\)-ATPase is sufficient to overcome digoxin inhibition. When the human \(\beta1\) subunit overexpressed in 293T cells, HIV-1 inhibition by digoxin was still observed (Figure 2.2C versus 2.2D), indicating that overexpression of a \(\beta1\) subunit of the Na\(^+\)/K\(^+\)-ATPase is not sufficient for overcoming digoxin inhibition. Taken together, these data suggest that digoxin inhibits HIV-1 gene expression via inhibition of the \(\beta1\) subunit of the Na\(^+\)/K\(^+\)-ATPase.

**Cardiac glycoside inhibition of HIV-1 gene expression is independent of increases in intracellular Ca\(^{2+}\) concentration**
Cardiac glycoside inhibition of the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase has been shown to induce increases in intracellular Ca\textsuperscript{2+} concentrations\textsuperscript{63,64}. Therefore, cardiac glycoside inhibition of HIV-1 via interaction with the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase may also depend upon increases in intracellular Ca\textsuperscript{2+}. Previously, the small molecule KB-R7943 was shown to inhibit digoxin-induced increases in intracellular Ca\textsuperscript{2+} via inhibition of a sodium-calcium exchanger\textsuperscript{66,67}. We first verified that co-administration of digoxin and KB-R7943 prevents digoxin-induced increases in intracellular Ca\textsuperscript{2+} (Figure 2.3A). Next, we tested whether HIV-1 inhibition by digoxin depended on digoxin-induced increases in intracellular Ca\textsuperscript{2+}. Thus, we co-administered digoxin and KB-R7943 to 293T cells transfected with pNL4-3\textDeltaEnvGFP and pX4. Co-administration of digoxin with KB-R7943 did not relieve the inhibitory effect of digoxin on HIV-1, as measured by GFP expression using flow cytometry (Figure 2.3B). This result demonstrates that HIV-1 inhibition by cardiac glycosides is independent of increases in intracellular Ca\textsuperscript{2+}. 
Discussion

The principal components of current highly active antiretroviral therapy (HAART) regimens target only select stages of the HIV-1 life cycle: entry, reverse transcription, integration, and maturation. While there is no doubt that HAART has successfully reduced the morbidity and mortality associated with HIV-1 infection, the problems of drug resistance, associated toxicities, and cost remain. As a result, the development of novel classes of inhibitors remains a priority. Few inhibitors of HIV-1 gene expression and budding have been identified to date. Therefore, we sought to identify compounds that inhibit HIV-1 at these stages, with a preference for compounds that have been tested or approved for use in humans. To accomplish this, we developed a cell-based screen that could be scaled for rapid testing of large compound libraries.

Our screening uncovered a number of compounds that inhibit HIV-1 virion production by cells transfected with proviral DNA constructs, including numerous compounds from the cardiac glycoside family. The cardiac glycosides were initially described as inhibitors of the Na\(^+\)/K\(^+\)-ATPase and have enjoyed wide use in the treatment of heart failure and arrhythmia. Recent studies have suggested that this family of compounds may also have potent anti-cancer, immunomodulatory, and anti-hypertensive properties, as well as activity against HIV-1 replication.

With respect to the antiretroviral effects of the cardiac glycosides, a recent study has shown that a single drug in the cardiac glycoside class, digoxin, inhibits HIV-1 replication by altering viral RNA splice site use, resulting in the decreased production of Rev protein. This effect is mediated through the activity of a subset of
the SR family of cellular splicing factors. However, overexpression of the Rev protein alone did not counter the inhibitory effect of digoxin, suggesting that other mechanisms of inhibition may be involved. Wong et al. indicate that digitoxin, a compound that is closely related to digoxin, has a similar effect on certain SR proteins and suggest that other cardiac glycosides may also be effective at preventing HIV-1 replication. Here, we demonstrate that this is indeed the case.

Our studies demonstrate that not only digoxin but also the entire cardiac glycoside family of compounds exhibits antiretroviral effects. Cardiac glycosides are known Na⁺/K⁺-ATPase inhibitors; this inhibition results in the inhibition and then reversal of the sodium-calcium exchanger (NCX), leading to the buildup of calcium in the cell. Although Wong et al. demonstrated that the inhibition of HIV-1 replication was related to certain SR splicing proteins, we asked whether the action of the cardiac glycosides depended on both the Na⁺/K⁺-ATPase and the cardiac glycoside-induced increase in intracellular Ca²⁺. Our study demonstrates that although HIV-1 inhibition is dependent on the Na⁺/K⁺-ATPase, it was independent of the induced increase in intracellular Ca²⁺. Previous studies have shown that the Na⁺/K⁺-ATPase plays a role in several signal transduction pathways. Additionally, some previous studies have also shown that the induction of signaling is independent of the intracellular ion concentrations. It is possible that the inhibition of this ATPase triggers a signaling mechanism leading to the modulation of the action of the SR splicing proteins, another question that warrants further investigation. A detailed analysis of the mechanism of inhibition by each member of the cardiac glycoside family along with structure-activity relationship studies are
extremely important to the potential development of the cardiac glycosides as antiretrovirals. Of note, the dose-limiting toxicities observed with cardiac glycosides in humans are related to toxic increases in cardiac contractility, driven by increases in intracellular Ca\(^{2+}\). As the mechanism of cardiac glycoside inhibition of HIV-1 appears to be independent of such Ca\(^{2+}\) increases, it is possible that structural modification of the cardiac glycosides or development of alternative inhibitors could avoid the cardiac toxicity while maintaining HIV-1 inhibition.

A preponderance of evidence in the literature suggests that current HAART regimens are capable of fully suppressing ongoing HIV-1 replication in treated individuals. However, treatment intensification with raltegravir has been shown to lead to a rapid increase in episomal 2-LTR circles in a proportion of treated individuals even though no effect on residual low-level viremia in these individuals was observed\(^8\). Such reductions in low-level viremia upon intensification would further suggest that ongoing replication is occurring. As the cardiac glycosides inhibit HIV-1 at a post-integration stage of the viral life cycle, treatment intensification studies employing the cardiac glycosides may offer some new insights into the source of low-level viremia. Our present study demonstrates that the cardiac glycosides prevent the production and release of HIV-1 RNA-containing virions. Therefore, we predict that intensification with cardiac glycosides would lead to a decline in low-level plasma HIV-1 RNA. The kinetics of this decline could provide evidence for the source of this low-level plasma HIV-1 RNA, be it ongoing replication in activated CD4+ T cells, release of virus from cognate antigen-reactivated cells of the latent reservoir, or another source entirely.
<table>
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<th>Structural class</th>
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Table 2.2 IC$_{50}$ values for inhibition of infection of primary CD4$^+$ lymphoblasts with supernatant from transfected 293T cells treated with cardiac glycosides

<table>
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<th>FDA approved?</th>
<th>Structural class</th>
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Figure 2.1 Screening for compounds that inhibit late stages of HIV-1 replication. 293T cells were transiently transfected with pNL4-3ΔEnvGFP and pX4, and then some wells were treated with epoxomicin (1.8 µM). Eighteen hours after treatment, RNA was extracted from culture supernatant, cDNA was synthesized, and HIV-1 RNA-specific qPCR was performed. Supernatant from untreated cells was used as a control. No-template controls and no-reverse-transcription (RT) controls were also included.
Figure 2.2 Overexpression of the murine $\mu_1$ subunit of the Na$^+$/K$^+$-ATPase abrogates HIV-1 inhibition by digoxin. (A) Flow cytometric analysis of GFP expression in 293T cells transfected with murine $\mu_1$ subunit of the Na$^+$/K$^+$-ATPase, pNL4-3ΔEnv, and pX4. (B) Flow cytometric analysis of GFP expression in 293T cells transfected with murine $\mu_1$ subunit of the Na$^+$/K$^+$-ATPase, pNL4-3dEnv, and pX4 and then treated with digoxin (10 $\mu$M) for 18 hours. (C) Flow cytometric analysis of GFP expression in 293T cells transfected with human $\mu_1$ subunit of the Na$^+$/K$^+$-ATPase, pNL4-3dEnv, and pX4. (D) Flow cytometric analysis of GFP expression in 293T cells transfected with human $\mu_1$ subunit of the Na$^+$/K$^+$-ATPase, pNL4-3dEnv, and pX4 and then treated with digoxin (10 $\mu$M) for 18 hours.
Figure 2.3 Digoxin inhibition of HIV-1 is independent of digoxin-induced increases in intracellular Ca$^{2+}$. (A) Flow cytometric analysis of 293T cells treated with DMSO alone, digoxin (1 µM), or digoxin (1 µM) plus KB-R7943 (10 µM) for 18 hours, then incubated with Fura Red AM dye (10 µM) for 30 minutes. Reduced AmCyan fluorescence indicates increased intracellular Ca$^{2+}$. (B) Flow cytometric analysis of GFP expression in 293T cells transiently transfected with pNL4-3ΔEnvGFP and pX4, and then treated with the indicated concentrations of KB-R7943 alone or in combination with digoxin (1 µM) for 18 hours. The percent of cells expressing GFP is normalized to GFP expression in transfected DMSO only control 293T cells.
Chapter 3: Novel ex vivo approaches distinguish effective and ineffective single agents for reversing HIV-1 latency in vivo


**Introduction**

HIV-1 persists in a latent reservoir (LR) despite antiretroviral therapy (ART)\textsuperscript{10-12,14,81}. This reservoir is the major barrier to HIV-1 eradication\textsuperscript{31,33}. Current approaches to purging the LR involve pharmacologic induction of HIV-1 transcription and subsequent killing of infected cells by cytolytic T lymphocytes (CTL) or viral cytopathic effects\textsuperscript{82-84}. 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 novel *ex vivo* assay, we demonstrate that none of the latency reversing agents (LRAs) tested induced outgrowth of HIV-1 from the LR of patients on ART. Using a novel RT-qPCR assay specific for all HIV-1 mRNAs, we demonstrate that LRAs that do not cause T cell activation do not induce significant increases in intracellular HIV-1 mRNA in patient cells; only the PKC agonist bryostatin-1 caused substantial 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 LR *in vivo* when administered individually.
HIV-1 cure is hindered by viral persistence in a small fraction (~1/10^6) of resting CD4^+ T cells (rCD4s) that harbor latent but replication-competent proviruses^{10-12}. 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 latency-reversing agents (LRAs) that do not activate T cells. Due to the low frequency of latently infected rCD4s in vivo, cell models have been used to identify a number of mechanistically distinct LRAs. These include: (1) histone deacetylase (HDAC) inhibitors, thought to function through epigenetic and other mechanisms^{22,23,85,86}; (2) disulfiram, postulated to involve nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)^{26,87}; and (3) the bromodomain-containing protein 4 (BRD4) inhibitor JQ1, which elicits effects through positive transcription elongation factor (P-TEFb)^{88-91}. Acting through signaling pathways associated with T cell activation, protein kinase C (PKC) agonists such as phorbol esters, prostratin^{24,92,93} and bryostatin-1^{85,94-96} also reverse latency in cell models.

Evidence that putative LRAs reverse latency ex vivo in primary rCD4s from HIV-1-infected individuals is limited; disulfiram and the HDAC inhibitor vorinostat have been tested in patient cells with inconsistent results. Clinical trials in patients on ART are ongoing with disulfiram and the HDAC inhibitors vorinostat, romidepsin, and panobinostat. A recent trial of disulfiram showed no consistent evidence of latency reversal. In another clinical trial, a single dose of vorinostat modestly increased intracellular RNAs containing HIV-1 *gag* sequences in rCD4s of patients on ART^{30}. Ex vivo treatment of patient cells with vorinostat induced outgrowth in
some studies\textsuperscript{22,23} but no virion production in another study\textsuperscript{28}. Importantly, no LRA has been shown to reduce the size of the LR.
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. Infected individuals were enrolled under the criteria of suppression of viremia to undetectable levels (<50 copies mL\(^{-1}\)) on ART for at least 6 months. PBMC 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 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 \(\mu\)M T20 to prevent new infection events.

Treatment of rCD4s with LRAs

rCD4s were treated with the following concentrations: 335 nM vorinostat, 40 nM romidepsin, 30 nM panobinostat, 500 nM disulfiram, 1 \(\mu\)M JQ1, 10 nM bryostatin-1, or 50 ng mL\(^{-1}\) PMA plus 1 \(\mu\)M ionomycin.

MOLT-4/CCR5 outgrowth assay

Five million purified rCD4s 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. rCD4s were then cultured with MOLT-4/CCR5 cells in 8 mL RPMI + 10% FBS in individual wells in 6 well plates. After 4 days of culture, cells were resuspended and split into two wells of a 6 well plate with the media volume adjusted to 8 mL per well. After 7 days of culture, wells were resuspended and split 1:2 with the media volume adjusted to 8 mL per well. Viral outgrowth was assessed at 14 days using the Alliance HIV-1 p24 antigen ELISA kit (Perkin Elmer).

**Cell lines**

MOLT-4/CCR5 cells from Dr. Masanori Baba, Dr. Hiroshi Miyake, and Dr. Yuji Iizawa were obtained from the NIH AIDS Reagent Program, NIAID, NIH.

**Generation of latently HIV-1 infected BCL-2 transduced cells**

Briefly, primary CD4+ lymphoblasts were transduced with BCL-2 and allowed to return to a resting state in the absence of exogenous cytokines. BCL-2 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 x 10^6 cells for experiments measuring only HIV mRNA and 10 x 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
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 re-extracted using Acid-Phenol:Chloroform, pH 4.5 (Ambion) per 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 one 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 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 rCD4 equivalents. The limit of quantification was set as the dilution point at which the Ct of the plasmid molecular standard replicates had a standard deviation > 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 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 0.2mL of supernatant from five million cultured rCD4s 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 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:

Forward (5’→3’) CAGATGCTGCATATAAGCAGCTG (9501-9523)
Reverse (5’→3’) TTTTTTTTTTTTTTTTTTTTTTTTTGAAGAC (9629-poly A)
Transcripts containing HIV-1 gag sequence were detected using the following primers and probe:

Forward (5′→3′) ACATCAAGCAGCCATGCAAAT (1368-1388)
Reverse (5′→3′) TCTGGCCTGGTGCAATAGG (1453-1471)
Probe (5′→3′) VIC-CTATCCCATTCTGCAGCTTCTCATTGATG-TAMRA (1401-1430)

Chimeric host/HIV-1 read-through 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)
Probe (5′→3′) FAM-CCTGTACTGGGTCTCTCTGG-MGB (446-465, 9531-9550)

cDNA synthesis reaction with gag primer sequence:
Reverse (5′→3′) GTCACTTCCCCTTGG (1480-1494)
Results and Discussion

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 utilized three independent assays to evaluate the efficacy of LRAs in cells from HIV-1 infected individuals on suppressive ART (participant characteristics in Table 3.1).

We first tested LRAs in a modified viral outgrowth assay\(^1\). In the original assay, patient-derived rCD4s were activated and co-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, T cell activation was replaced with LRA treatment. The subsequent co-culture of patient rCD4s with healthy donor lymphoblasts constitutes a mixed lymphocyte reaction, which induces background reactivation of latent HIV-1\(^9\) and complicates LRA evaluation. Therefore, we treated rCD4s with LRAs and then cultured the cells with a transformed CD4\(^+\) T cell line (MOLT-4/CCR5) (Figure 3.1A) that supports robust HIV-1 replication but does not induce allogeneic stimulation of rCD4s (Figure 3.2A–C). We treated five million purified rCD4s from infected individuals on ART with single LRAs for 18 h and then co-cultured the cells with MOLT-4/CCR5 cells for 14 days to permit viral outgrowth. T cell activation with phorbol 12-myristate 13-acetate + ionomycin (PMA/I) served as a positive control. We concurrently measured the frequency of latently infected cells\(^9\). 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 that were not
toxic to rCD4s. No drug treatment induced cell death as shown by the lack of 7-AAD staining (Figure 3.1B). Surprisingly, none of the LRAs induced viral outgrowth from cells from any individual tested while PMA/I-treated cultures were positive for every patient with a detectable LR (Figure 3.1C).

We next asked whether LRA treatment induced rapid virus release. We collected culture supernatants from rCD4s from five infected individuals (S26–S30) after 18 h of LRA treatment and prior to 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) (Figure 3.1D). Bryostatin-1 treatment induced detectable supernatant HIV-1 mRNA from one infected individual (S27), whereas no other LRA had a measurable effect (Figure 3.1D). 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 (Figure 3.1C).

The most widely used method to detect induction of HIV-1 transcription in cells from infected individuals involves the measurement of RNAs containing HIV-1 gag sequences. Because this method lacks a stringent selection for poly-adenylated 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 (Figure 3.3). We detected baseline intracellular HIV-1 mRNA in rCD4s from ten out of 11 infected individuals. Stimulation with PMA/I for 18 h dramatically increased intracellular HIV-1 mRNA (mean increase = 115.5-fold,
Figure 3.3B). However, at clinically relevant concentrations that reverse latency in a primary cell model (Figure 3.3B and 3.3C), vorinostat, romidepsin, panobinostat, disulfiram, and JQ1 failed to increase intracellular HIV-1 mRNA in rCD4s from infected individuals when used as single agents (Figure 3.3B and 3.3C). Bryostatin-1 caused significant increases in some infected individuals (Figure 3.3C). We observed similar results after 6 h of LRA treatment (Figure 3.4).

While no effect was seen in latently infected cells from infected individuals, LRA treatment increased intracellular HIV-1 mRNA in a B-cell lymphoma 2 (BCL-2) transduced primary rCD4 model of latency (Figure 3.5). LRA-induced increases in HIV-1 mRNA were consistent with measurements of the fraction of cells that up-regulate HIV-1 gene expression, as assessed by GFP reporter (Figure 3.5B). The frequency of latent infection in this model is substantially higher than that observed in vivo\textsuperscript{14}. To confirm that our assay effectively detects intracellular HIV-1 mRNA increases at frequencies of latent infection seen in vivo, we treated model cells with a known percentage of latent infection and then serially diluted these cells into rCD4s from uninfected individuals immediately prior to RNA isolation. We detected proportionate increases in intracellular HIV-1 mRNA in vorinostat-treated cells down to a frequency of 1/10\textsuperscript{6} cells (Figure 3.3D and 3.3E). Therefore, 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.

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 rCD4s\(^{99,100}\), 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 LTR-initiated transcripts by conventional *gag* RT-qPCR assays (**Figure 3.6**). 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 read-through of the 5′ LTR or 3′ LTR, independent of proviral orientation (**Figure 3.6A**). We treated ten million rCD4s from infected individuals on ART with vorinostat or PMA/I for 6 h and compared the levels of HIV-1 mRNA, read-through transcripts, and transcripts containing *gag* sequence (**Figure 3.6A and 3.6B**). We detected a small increase (~2-fold) in transcripts containing *gag* sequence in vorinostat-treated rCD4s from four out of five infected individuals, consistent with previous reports\(^3^0\) (**Figure 3.6B**). Vorinostat treatment also induced increases in read-through transcripts (**Figure 3.6B**) comparable to the increases in transcripts containing *gag* sequence but had no effect on levels of HIV-1 mRNA (**Figure 3.6B**).

To prove that the read-through signal is amplified from a transcript that initiated upstream of the 5′ LTR and contains *gag* sequence, we primed cDNA synthesis with a *gag* primer (**Figure 3.6C**). We detected comparable, statistically significant inductions of read-through and *gag* transcripts after 6 h of vorinostat treatment (**Figure 3.6C**) \((P = 0.027, P = 0.011, \text{respectively} ; \text{ratio paired t-test of transcript copies})\), indicative of read-through transcription. PMA/I induction of *gag*
transcripts greatly exceeded that of read-through transcripts, indicative of LTR-initiated transcription (Figure 3.7). While not every potential LRA will induce read-through 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 novel assays presented herein facilitated 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 LR ex vivo. The striking discordance between the effects of non-stimulating LRAs in in vitro models of HIV-1 latency and the ex vivo effects in rCD4s 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 LR in vivo when administered individually. The only active single agent was the PKC agonist bryostatin-1, which is likely 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 remains to be seen, and further progress may depend on finding safe and active combinations of LRAs.
Table 3.1 Characteristics of HIV-1 infected study participants.

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<th>Sex</th>
<th>Race</th>
<th>Duration of infection (months)</th>
<th>ART regimen</th>
<th>Time on ART (months)</th>
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Abbreviations: abacavir (ABC), emtricitabine (FTC), lamivudine (3TC), tenofovir (TDF), efavirenz (EFV), etravirine (ETR), nevirapine (NVP), atazanavir boosted with ritonavir (ATV/r), darunavir boosted with ritonavir (DRV/r), fosamprenavir boosted with ritonavir (FPV/r), lopinavir boosted with ritonavir (LPV/r), elvitegravir boosted with cobicistat (EVG/c), raltegravir (RAL), maraviroc (MVC)
Figure 3.1 LRAs do not induce outgrowth of latent HIV-1. (A) Schematic of LRA outgrowth assay. (B) LRA-treated rCD4s were stained with Annexin-V and 7-AAD. Toxicity was defined as percent positivity by flow cytometry. (C) Viral outgrowth from LRA-treated rCD4s from infected individuals. Wells positive by ELISA for HIV-1 p24 antigen at 14 days are depicted with a positive sign. Negative wells are depicted with a negative sign. (D) Culture supernatant HIV-1 mRNA (copies mL$^{-1}$) from LRA-treated rCD4s obtained from five infected individuals (S26–S30). Dotted line indicates limit of detection (208.3 copies mL$^{-1}$). Error bars indicate mean ± s.e.m.
Figure 3.2 MOLT-4/CCR5 cells do not induce allogeneic activation of rCD4s. rCD4s were isolated from an uninfected individual and stained with CFSE. Cells were then cultured alone, co-cultured with MOLT-4/CCR5 cells, or stimulated with anti-CD3 and anti-CD28 antibodies for 7 days. Cells were analyzed by flow cytometry for (A) CD25 expression, (B) morphology defined by forward scatter and side scatter, and (C) CFSE dilution indicative of activation-induced cell division.
Figure 3.3 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. Intracellular HIV-1 mRNA from LRA-treated rCD4s obtained from infected individuals presented as (B) fold change relative to DMSO control (mean ± s.e.m.) and (C) copies of HIV-1 mRNA per million rCD4 equivalents. Data points represent mean effect of the LRA for each individual. Statistical significance was determined using a paired t-test. RT(-) controls were negative for all samples. Lines connect data points from each infected individuals. Dotted line indicates 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.
Figure 3.4 Short treatment with LRAs does not consistently induce HIV-1 mRNA production in cells from HIV-1 infected individuals on ART. Five million rCD4s from infected individuals on ART were cultured in triplicate with LRAs for 6 h. Intracellular HIV-1 mRNA from each replicate was measured using RT-qPCR. Mean effect of the LRA in each patient, depicted by individual dots, is presented as (A) fold change relative to DMSO control (mean ± s.e.m.) and (B) copies of HIV-1 mRNA per million rCD4 equivalents. Statistical significance was determined using a paired t-test. Lines connect data points from individual subjects.
Figure 3.5 A primary CD4+ T cell model of HIV-1 latency is responsive to LRAs. (A) Intracellular HIV-1 mRNA from LRA-treated BCL-2-transduced primary CD4+ T latency model cells. Changes are presented as fold induction relative to DMSO control (mean ± s.d.). (B) LRA-induced reactivation in latency models cells, defined as the percent GFP+ cells normalized to the effect of PMA/I treatment (mean ± s.d.) as measured by flow cytometry. Intracellular HIV-1 mRNA in serially diluted latency models cells, presented as (C) copies of HIV-1 mRNA per million rCD4 equivalents (mean ± s.d.) and (D) fold change relative to DMSO control (mean ± s.d.). An x indicates sample was below the limit of detection. RT(–) controls were negative for all samples. For A and B, statistical significance was determined using unpaired t-test. Asterisk indicates $P$ value >0.05 (** indicates $10^{-2}$, *** indicates $10^{-3}$, **** indicates $10^{-4}$).
**Figure 3.6** 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 read-through 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 read-through, *gag*-containing, and mRNA transcripts in rCD4s from five infected individuals, presented as fold change relative to DMSO control. (C) Schematic of Gag specific cDNA synthesis and qPCR detection of read-through transcripts. (D) Effect of vorinostat on read-through transcripts containing *gag* in from five infected individuals, presented as fold change relative to DMSO control. RT(-) controls were negative for all samples.
Figure 3.7 PMA/I induces *bona fide* HIV-1 *gag* transcripts. Ten million rCD4s from five infected individuals on ART were cultured with DMSO alone or PMA/I for 6 h. DNase-treated intracellular RNA was measured by RT-qPCR. RT(-) controls were negative for all samples. Fold change relative to DMSO control is presented.
Chapter 4: Identifying effective HIV-1 latency-reversing drug combinations through ex vivo analysis

The work presented in this chapter was published in *Journal of Clinical Investigation* in March, 2015.

Introduction

HIV-1 persists in a latent reservoir despite suppressive antiretroviral therapy (ART). Resting CD4+ T cells (rCD4s) that harbor latent proviruses allow little to no HIV-1 gene expression, 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. This small but stable latent reservoir necessitates life-long ART 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. While global T cell activation effectively reverses latency, toxicity due to cytokine release precludes its clinical use. This has fueled the search for small molecule latency-reversing agents (LRAs) that do not induce T cell activation and cytokine release (reviewed previously).

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 previously). Many LRAs have been identified using these
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\textsuperscript{30,109}, and similar results have recently been reported by Rasmussen and colleagues with another HDAC inhibitor, panobinostat\textsuperscript{110}. 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\textsuperscript{37,111-113}. These results emphasize that LRAs should be validated by studies using rCD4 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\textsuperscript{10,98,112}.

We recently demonstrated that candidate LRAs including (i) HDAC inhibitors (vorinostat, panobinostat, romidepsin), (ii) disulfiram, which is believed to activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and (iii) JQ1, which is a bromo and extra terminal (BET) bromodomain inhibitor, were only minimally active at reversing latency in rCD4s from infected individuals\textsuperscript{111}. The protein kinase C (PKC) agonist bryostatin-1 was the only single LRA to significantly induce intracellular HIV-1 mRNA production ex vivo\textsuperscript{111}. This effect, however, was a mere four percent 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 was identified, provides an upper bound for latency reversal. LRA regimens that substantially reverse latency ex vivo compared to the benchmark of maximal T cell activation (typically ~100 fold induction) 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. While some combinations have previously been tested in CD4+ T cells from infected individuals, no comparative ex vivo study has been performed to assess the efficacy of multiple two-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 that 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. 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\textsuperscript{121}. 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.
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 RNAs level (<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\(^{111}\). Cells were cultured in RPMI medium supplemented with 10% fetal bovine serum at a concentration of 5x 10⁶ cells per mL for all experiments.

Latency reversing agent treatment conditions

Resting CD4+ T cells 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 µM JQ1, 30 nM panobinostat, 40 nM romidepsin, 335 nM vorinostat, 50 ng mL⁻¹ PMA plus 1 µM 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\(^{30,103,109,111-113}\) with the aim of selecting clinically relevant concentrations.
Measurement of intracellular HIV-1 mRNA

Five million resting CD4$^+$ T cells 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 h in a volume of 1 mL RPMI + 10% FBS. Total RNA was isolated, and cDNA synthesis and real-time quantitative PCR were performed as described$^{111}$. Briefly, each PCR reaction contained template from approximately one 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. No-RT control reactions were preformed on every treatment sample from only one 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$^{36}$.

Results from the triplicate samples for each drug treatment were averaged and presented as copies of HIV mRNA per million resting CD4$^+$ T cell equivalents, fold change relative to DMSO control, and normalized percentage of the effect of PMA plus ionomycin $[$(copies_{LRA\ x} - \text{copies}_{DMSO \ control})/(\text{copies}_{PMA+I} - \text{copies}_{DMSO \ control})]$. The limit of quantification was 10 copies as described$^{111}$. Some samples from one 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 plus ionomycin, 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 = $2^{-\Delta Ct}$, $\Delta Ct = Ct_{LRAx} - Ct_{DMSO\ control}$). Particular LRA treatments consistently changed expression of Pol2 and/or G6PD. Samples treated with the same LRA regiment had near 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 was performed as described. Results were presented as copies of HIV mRNA per mL supernatant and normalized percentage of the effect of PMA plus ionomycin $\{(\text{copies}_{LRAx} - \text{copies}_{DMSO\ control})/(\text{copies}_{PMA+I} - \text{copies}_{DMSO\ control})\}$. The limit of detection for each qPCR was 10 copies per reaction, which scaled to a limit of detection of 150 copies per 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 to predict 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:

$$f_{axy, P} = f_{ax} + f_{ay} - (f_{ax})(f_{ay})$$

where $f_{axy, P}$ is the predicted fraction affected by a combination of drug X and drug Y given the experimentally observed fraction affected for drug X ($f_{ax}$) and drug Y ($f_{ay}$) individually. The experimentally observed fraction affected by a combination of drug X and drug Y ($f_{axy, O}$) can be compared to the predicted fraction affected computed using the Bliss model ($f_{axy, O}$) as follows:

$$\Delta f_{axy} = f_{axy, O} - f_{axy, P}$$

If $\Delta f_{axy} < 0$ with statistical significance, then the combined effect of the two drugs exceeds that predicted by the Bliss model and the drug combination displays synergy. If $\Delta f_{axy} = 0$, then the drug combination follows the Bliss model for independent action. If $\Delta f_{axy} > 0$ with statistical significance, then the combined effect of the two 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:

$$f_{ax} = (\text{copies drug X} - \text{copies DMSO control}) / (\text{copies PMA/I} - \text{copies DMSO control})$$

**Flow cytometry**

Resting CD4$^+$ T cells isolated from three 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 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 Pharmingen). Samples were analyzed using a FACSCalibur flow cytometer and Cell Quest software (Becton Dickinson). 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 manufacturer's protocol (BD Biosciences). Briefly, 50 L standards were mixed with 50 μL mixed capture beads-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 mRNA measurement as described:\textsuperscript{111}:

forward (5′→3′) CAGATGCTGCATATAAGCAGCTG (9501-9523),
reverse (5′→3′) TTTTTTTTTTTTTTTTTTTTTTTTTAAAGCAC (9629-poly A),
probe (5′→3′) FAM-CCTGTACTGGGTCCTCTGG-MGB (9531-9550).
**Statistics**

Ratio paired Student's t-test was used to determine statistical significance where indicated. We considered $P < 0.05$ to be statistically significant. Approximately a quarter of the experiments measuring intracellular and supernatant HIV 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 granted approval for this study. All research participants enrolled in this study provided written informed consent prior to inclusion in this study.
Results

Quantifying the combined effects of two or more LRAs requires first understanding the effect of each drug alone. Therefore, we treated five million purified rCD4s from infected individuals on suppressive ART (participant characteristics in Table 4.1) 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. 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 4.1A, Figure 4.2). In contrast, the T cell activation control of PMA + ionomycin (PMA/I) dramatically elevated levels of intracellular HIV-1 mRNA (mean increase of 148.8-fold, Figure 4.1A). Treatment of CD4 T cells with PMA/I causes a dramatic up-regulation 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 are normalized as a percent of the effect elicited by T cell activation with PMA/I, it is apparent that individual LRAs generally show limited efficacy ex vivo (Figure 4.1B).

To identify effective two-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. Ten of the 11 combinations tested caused a significant increase in intracellular HIV-1 mRNA relative to the DMSO
control (Figure 4.1A, Figure 4.2). To compare the efficacy of these combinations, we plotted increases in intracellular HIV-1 mRNA levels as a percent of the effect of the T cell activation control, PMA/I. Combinations of the PKC agonist bryostatin-1 with JQ1 or with each of three different HDAC inhibitors were significantly more effective than bryostatin-1 alone (Figure 4.1B), with some combinations approaching the magnitude of induction stimulated by T cell activation with PMA/I. For example, treatment with a combination of bryostatin-1 and panobinostat caused increases in intracellular HIV-1 mRNA that were on average 51.5% of that seen with the PMA/I control, with increases of 89.1% seen in some infected individuals. Similarly, treatment with a combination of bryostatin-1 and JQ1 caused increases in intracellular HIV-1 mRNA that were on average 32.6% of that 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 4.1B).

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\textsuperscript{123}. 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\textsuperscript{124} (Figure 4.3). 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 4.3). Disulfiram containing combinations did not exhibit synergy, but rather conformed to the predictions of the Bliss independence model (Figure 4.3).

To further explore the synergistic relationship between bryostatin-1 and the HDAC inhibitors, we tested a ten-fold lower concentration of bryostatin-1 alone and in combination with the HDAC inhibitor romidepsin. Treatment with 1 nM bryostatin-1 did not induce significant intracellular HIV-1 mRNA. However, when 1 nM bryostatin-1 was combined with romidepsin, we observed significant induction of intracellular HIV-1 mRNA (Figure 4.4A, mean 20.2 fold induction) and this combination was synergistic (Figure 4.4B).

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 an RT-qPCR assay previously shown to provide sensitive and accurate quantitation of HIV-1 virion production. We focused on LRAs showing synergistic effects, particularly JQ1, romidepsin, and the PKC agonists bryostatin-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 $2.6 \times 10^5$ HIV-1 mRNA copies/mL supernatant. Of the LRAs tested, only bryostatin-1 and prostratin induced significant virus release as single agents (Figure 4.5A and 4.5B). Combinations of bryostatin-1 or prostratin with JQ1 or romidepsin also caused significant virus release (Figure 4.5A and 4.5B), but the combined effects did not significantly exceed those of bryostatin-1 or prostratin alone (Figure 4.5B). Surprisingly, combination LRA treatment exceeded the effect seen with maximal T cell activation by PMA/I in some instances (Figure 4.5B). 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 4.5C).

Next, we examined the relationship between intracellular HIV-1 mRNA levels and HIV-1 virion production by LRA-treated rCD4s (Figure 4.6). Treatments including the PKC agonists bryostatin-1 or prostratin clustered with PMA (also a PKC agonist) + ionomycin while treatments lacking a PKC agonist showed much lower activity, especially with regards to virion production. Tobit regression analysis of only the treatments containing a PKC agonist yielded a significant correlation between increases in intracellular HIV-1 mRNA and virion release (Figure 4.6, $P = 0.008$ for Chi-squared 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 4.7A), consistent with previous studies \(^{24,94}\). 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 4.7A). Treatments containing a PKC agonist caused minimal decreases in rCD4 cell viability as assessed by annexin V and 7-AAD staining (Figure 4.7B). Importantly, combination LRA treatment did not cause cellular toxicity exceeding that caused by single LRA treatment (Figure 4.7B). 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 4.8A). Similarly, treatment of unfractionated PBMC with PKC agonists alone or in combination with other LRAs caused little or no cytokine production (Figure 4.8B).

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 4.9A; complete description of model in Appendix 1) 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 regimens and have baseline plasma HIV-1 RNA levels below the limit of detection prior to LRA administration. **Figure 4.9B** 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 is administered continuously with activating potential comparable to that in the ex vivo assay, and if the latent reservoir is 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 4.9B** 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 4.9B** 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 > 100 copies/mL are predicted for all treatments investigated, except for romidepsin (**Figure 4.9C**)
which give detectable plasma HIV-1 RNA levels only if LRA-activated cells are assumed to survive three times as long as functionally activated cells (Fig 4.9D). 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\textsuperscript{111-113}. These studies suggested that combination therapy comprised of mechanistically distinct LRAs may by required to robustly reverse latency. In this study, we employed two distinct measures of latency reversal to evaluate the efficacy of two-drug LRA combinations in rCD4s from infected individuals.

We report here a number of new two-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 4.1). 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 4.3), 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\textsuperscript{85}. PTEF-b, 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 is a result of the induction of PTEF-b 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 by when disulfiram was combined with an HDAC inhibitor (Figure 4.1). By the rigorous Bliss independence criterion, we did not observe synergy for the disulfiram combinations we tested (Figure 4.3), 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\textsuperscript{87} and the HDAC inhibitors\textsuperscript{85,125,126}. 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 (Figure 4.5 and Figure 4.6). 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. We therefore measured virion production after ex vivo treatment of rCD4s with LRAs, which serves as a proxy for viral protein production. 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\textsuperscript{115}. 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 (CTL) to kill HIV-1 infected cells, both ex vivo and in in vitro models\textsuperscript{127}. 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 bryostatin-1 efficacy in the treatment of a variety of cancers have been safely completed. Lower doses of bryostatin-1 were well tolerated, but dose limiting toxicities 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 4.8). 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 LC/MS/MS assay in patients receiving bryostatin-1 in combination with GM-CSF\textsuperscript{128}. Plasma steady state concentrations of bryostatin-1 ranging from roughly 0.2 nM to 1 nM could be achieved in patients receiving the approximated maximally tolerated dose of 16 μg/m2/day continuously infused for 14 or 21 days, and these concentrations could be maintained over the course of the infusion. As presented in Figure 4.4, we found that 1 nM bryostatin-1 induced significant 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 bryostatin-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. Bryostatin-1 is a natural product available only in small amounts. Several synthetic analogs of both bryostatin-1 and prostratin have recently been developed\textsuperscript{94,129}. 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 non-human primate studies and clinical trials. Some LRAs also increase HIV-1 RNA production in ex vivo assays using cells from patients on ART\textsuperscript{111-113}. However, in general, the activity of individual LRAs in these systems is weak compared to maximal T cell activation\textsuperscript{111}. In clinical trials, HDAC inhibitors have been shown to cause modest increases in cell associated HIV-1 RNA in some studies\textsuperscript{30,109,110,120,121}, but clear changes in plasma HIV-1 RNA have been seen in only one study to date\textsuperscript{121}, 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 rCD4 from patients on ART. Quantitating virus production allowed us to make predictions about how LRA therapy would affect a readily measureable 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 increase 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 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\textsuperscript{121}. Clinical trials of disulfiram\textsuperscript{122}, vorinostat\textsuperscript{30,109}, and panobinostat\textsuperscript{110} 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\textsuperscript{111} and consistent with the predictions of this mathematical model. As plasma HIV-1 RNA is predicted to change rapidly following LRA administration (\textbf{Figure 4.9C}), 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\textsuperscript{19}, 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\textsuperscript{28,103,130}. Further studies of the fraction of cells induced ex vivo, as well as the lifespan 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\textsuperscript{29}.

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 4.6 indicates 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 co-stimulation to produce intracellular HIV-1 RNA or virions. They found that roughly 7.5% of proviruses produced intracellular HIV-1 RNA while only 1.5% produce virions after co-stimulation. This is consistent with the data we present here (Figure 4.6), 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 to 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 two-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 hopefully 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 like 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.
Table 4.1 Characteristics of HIV-1 infected study participants.

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<th>Sex</th>
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<th>ART regimen</th>
<th>Time on ART (months)</th>
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</table>

Abbreviations: male (M), female (F), Caucasian/white (W), African American/Black (B), abacavir (ABC), emtricitabine (FTC), lamivudine (3TC), tenofovir (TDF), efavirenz (EFV), etravirine (ETR), nevirapine (NVP), atazanavir boosted with ritonavir (ATV/r), darunavir boosted with ritonavir (DRV/r), fosamprenavir boosted with ritonavir (FPV/r), lopinavir boosted with ritonavir (LPV/r), elvitegravir boosted with cobicistat (EVG/c), raltegravir (RAL), maraviroc (MVC)
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 two 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 percent of the effect of maximal reactivation with PMA + ionomycin. Data points represent the mean effect of 2 or 3 replicate LRA treatments of 5 million cells for each individual. For panels A and B, statistical significance was calculated from the HIV-1 mRNA copy number values using a ratio paired T test compared to (A) the DMSO control, (B) bryostatin-1 or prostratin alone, or disulfiram alone. *P < 0.05, **P < 0.005, ***P < 0.0005, ****P < 0.00005. Error bars represent SEM.
Figure 4.2 LRA combinations induce intracellular HIV-1 mRNA production in rCD4s from infected individuals on ART. Intracellular HIV-1 mRNA levels in rCD4s, obtained from infected individuals and treated ex vivo with a single LRA or a combination of two LRAs, presented as copies per million rCD4 equivalents. Numbers in parentheses in Fig. 1A indicate number of individuals used for each treatment.
Figure 4.3 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 + ionomycin (fraction affected, \( f_a \)) presented in Figure 1. See Methods for more detail. 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 \( f_a \) was calculated using ratio paired T test compared to the predicted \( f_a \) for each combination. *\( P < 0.05 \), **\( P < 0.005 \), ***\( P < 0.0005 \), ****\( P < 0.00005 \).
Figure 4.4 Lower dose 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 to the DMSO control. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$. (B) Calculation of synergy for bryostatin-1 (1nM) and romidepsin using the Bliss independence model. Data are presented as the difference between the observed and predicted fractional response relative to PMA + ionomycin (fraction affected, $f_{a,x}$). See Methods for more detail. Statistical significance for the experimental $f_a$ was calculated using paired T test compared to the predicted $f_a$ for each combination. *$P < 0.05$. rCD4s from four HIV-1 infected individuals were tested per condition.
**Figure 4.5 PKC agonists alone or in combination with another 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 two LRAs, presented as (A) HIV-1 mRNA copies/mL supernatant and (B) as a percent of the effect of maximal reactivation with PMA + ionomycin. 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 ± s.e.m. Statistical significance was calculated from the HIV-1 mRNA copy number values using a ratio paired T test compared to (A) DMSO control, or (B) bryostatin-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 + ionomycin (fraction affected, f_a). See Methods for more detail. Statistical significance for the experimental f_a was calculated using a ratio paired T test compared to the predicted f_a for each combination. *P < 0.05, **P < 0.005, ***P < 0.0005, ****P < 0.00005.
Figure 4.6 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 for Chi-squared test) (see Methods).
Figure 4.7 Effect of LRA treatment on T cell activation-associated surface markers and toxicity. Primary rCD4s treated with a single LRA or a combination of two LRAs were assayed for (A) surface expression of CD25 and CD69 and (C) positivity for annexin V and 7-AAD staining. Data are the mean effect of 2 or 3 independent experiments. Error bars represent SEM.
Figure 4.8 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 two LRAs were assayed for supernatant cytokine concentrations (pg/mL). Data are the mean effect of 2 or 3 independent experiments.
Figure 4.9 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 Appendix 1) 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. (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, D indicates duration of LRA activity. Parameters: $d_y = 1$/day; $d'_y = 1$ day$^{-1}$ (blue curves in B, D, all curves in C) or $1/3$ day$^{-1}$ (red curves in B, D); $a + d_z = 5.2 \times 10^{-4}$ day$^{-1}$ (reservoir half-life of 44 months), initial viral load = 2 copies/mL. Other parameters: See Appendix 1.
$z$ – size of latent reservoir (LR)
$y$ – naturally activated LR
$y'$ – LRA-activated LR
$v$ – virus produced by y
$d_y$ – rate of death for $y$
$a$ – rate of natural activation of LR
$k$ – rate of virus production by y
$d_y'$ – rate of death for $y$
$a'$ – rate of LRA-activation of LR
$k'$ – rate of virus production by y
$d_y'$ – rate of death for $y$
Appendix 1: Mathematical model of viral dynamics

A system of differential equations was used to describe *in vivo* viral dynamics during administration of LRA therapy, assuming that co-administered ART suppresses all viral replication. Let $z$ be the abundance of latently infected resting $CD_4^+$ T cells, let $y$ be the abundance of activated infected $CD_4^+$ T cells, and let $y'$ be the abundance of LRA-stimulated infected $CD_4^+$ T cells that are induced to produce virus, though they may not be functionally activated. Here, activation includes any LRA-independent transition to virus production, such as that caused by stochastic transcriptional changes or by antigenic stimulus. Let $v$ be the plasma viral load, in copies per mL ($c \text{ mL}^{-1}$). Since our conclusions will rely only on observed viral load, arbitrary units can be used for the cellular quantities. During fully suppressive ART, viral dynamics can be described by the system,

\begin{align*}
\dot{z} &= -(a + a' + d_z)z \\
\dot{y} &= az - d_yy \\
\dot{y}' &= a'z - d'_yy' \\
\dot{v} &= ky + k'y' - d_vv.
\end{align*}

(S1)

Here, $a$ and $a'$ are the rates of activation and LRA-driven induction, respectively. Latently infected cells die at rate $d_z$. To represent the baseline (untreated) rate of reservoir decay due to combined effects of activation and death, we will use the compound parameter $\delta = a + d_z$. Activated cells produce virus at rate $k$ and die at
rate $d_y$; LRA-induced cells produce virus at rate $k'$ and die at rate $d'_y$. Since induction is likely not as drastic as functional T cell activation, it is likely for $d'_y$ and $k'$ to be less than $d_y$ and $k$, respectively. Virus is cleared at rate $d_v$. The values of $a'$, $k'$, and $d'_y$ depend on the LRA treatment given. The binary "switch" between latency and (either form of) activity is an idealization; it is possible that transient viral production occurs in cells experiencing varying degrees of latency.

The effect of LRA can be detected by the transient increase in viral load that it causes. To estimate this increase, we rely on observations of the ex vivo system. Specifically, we assume that this system also follows the above viral dynamics, with abundance of extracellular mRNA taking the place of plasma viral load for variable $v$. We assume moreover that parameter values are the same in vivo as ex vivo, with the exception that $d_v$ is zero ex vivo. See Appendix Table 1 for discussion of these assumptions.

Generally, we assume $d_y \geq d'_y$ and that both of these cell death rates are much larger than, $a$, $a'$, and $d_z$. Below we state explicitly where these assumptions are used.

**Analysis of ex vivo dynamics**

Following the above discussion, we assume $d_v = 0$. The assay begins with only resting CD4$^+$ T cells, implying initial condition $v(0)=y(0)=y'(0)=0$ and $z(0) = z_0$, where $z_0$ is the number of latently infected cells collected from the cell donor (a small fraction of the 5 million cells). Since $d_v = 0$, the virus simply accumulates over time.
The DMSO control provides no inducing effect beyond the baseline rate $a$, and the solution of system (S2) for this case is

$$v_{DMSO}(t_a) = \frac{akz_0}{d_y\delta(d_y-\delta)} \left( d_y(1 - e^{-\delta t_a}) - \delta(1 - e^{-d_y t_a}) \right),$$

(S2)

where the subscript in $t_a$ indicates time in the assay, which will later be distinguished from time *in vivo*. Adding treatment applies a non-zero $a'$. The amount of extracellular mRNA is therefore increased by a factor:

$$\frac{v_{LRA}(t_a)}{v_{DMSO}(t_a)} \approx 1 + \frac{a'k'}{ak} \left( 1 + \frac{t_a}{3} \left( d_y - d_y' - a' - \frac{ak}{k'} \right) \right).$$

(S3)

This approximation holds for $\delta t_a \ll 1$ and $(t_a/3)(d_y - d_y' - a' - ak/k')$ near to or less than one; both are expected as $t_a \leq 1$ day in the assay, $\delta$ is the slow rate of reservoir decay (half-life of many months), and the other rate parameters are no more than $1$ day$^{-1}$. Let $\Box$ be the observed value of $v_{LRA}(t_a)/v_{DMSO}(t_a)$ at the end of the assay. From this observation, we can estimate the following parameter ratio:

$$\frac{k'}{k} \approx \left( \frac{a'}{a'} \right)^{\frac{3(\rho-1)+t_a a'}{3-t_a(a'+d_y-d_y')}}.$$  

(S4)

This parameter ratio estimate is used to predict viral load *in vivo*, below.
Analysis of *in vivo* dynamics

Since virus is subject to rapid decay *in vivo*, we can treat it using the commonly used quasi-steady state approximation: \( v(t) = \left( ky(t) + k'y'(t) \right)/d_v \).

Likewise, since death rate \( d_y \) greatly exceeds baseline activation rate \( a \), the initial number of actively infected cells can be approximated by activation-death equilibrium, \( y(0) = az_0/d_y \), implying a residual viral load of \( v(0) = akz_0/(d_yd_v) \). The fractional increase in viral load caused by administering the LRA for a period of time \( t \) follows from these assumptions and system (S5):

\[
\frac{v_{LRA}(t)}{v(0)} = \frac{dy e^{-(\delta+a')t} - (\delta + a') e^{-dy t}}{d_y - \delta - a'}
\]

Here, eq. (S5) has been used to eliminate both \( k \) and \( k' \) by introducing the *ex vivo*-observed parameter \( \rho \). The first line of (S5) represents viremia due to activated cells, while the second line represents viremia due to LRA-induced cells.

The *in vivo* viral load ratio in (S5) approximates a bi-exponential curve, initially rising linearly from 1 at rate \( \approx d_y(\rho - 1) \) and ultimately decaying exponentially at rate \( \delta + a' \). The maximum value cannot be expressed in a simple form, but the peak viral load ratio can be approximated by noting that the first line of () falls between 0 and 1, while the second line (for which the maximum can be expressed in closed form) has a peak much larger than 1 for typical parameter values \( (d_y \geq d'_y > a' > \delta) \).
none of these rates much larger than 1 day$^{-1}$, and $\rho \gg 1$). The peak viral load, relative to the baseline residual viral load, is therefore approximately

$$\frac{\max(v_{\text{LRA}}(t))}{v(0)} \approx 1 + \left(\frac{d_y}{d'_y}\right) (3(\rho - 1) + a't_a) \left(\frac{(\delta+a')(d'_y/3)\ln(d'_y/\delta+a')}{d'_y-a'}\right),$$

(S6)

and it occurs approximately at time

$$t_{\text{max}} \approx \frac{\ln(d'_y/\delta+a')}{d'_y-a'\delta}.$$

(S7)

The approximation in Eq. (S6) never overestimates the true peak ratio by more than 1. Note that the exponentiated expression decreases with the sum $(\delta+a')$, indicating the effect of a rapidly decaying reservoir on the peak viral load. If $(\delta+a')$ is very small relative to $d'_y$, then the peak viral load is simply

$$\frac{\max(v_{\text{LRA}}(t))}{v(0)} \lesssim 1 + (\rho - 1) \frac{d_y}{d'_y}.$$

(S8)

This approximation is used in Figure 4.8B. Note that this result does not depend on the LRA-driven induction rate $a'$ nor the viral production rate $k'$ of LRA-induced cells; the experimentally observed parameter $\rho$ depends on a combination of induction and production. Further experiments — involving measurement of the fraction of cells induced or the decay in viral production over time — would be needed to
resolve rate $a'$, which determines the rate at which LRA therapy would ultimately
deplete the latent reservoir.
In vivo dynamics for short treatment window

The previous section assumes that treatment is administered continuously, until the latent reservoir eventually decays completely, yet such a regimen may not be achievable. Suppose instead that the effect of treatment ceases at time \( t_{\text{Stop}} \), after which point \( a' \) is set to zero. For \( t > t_{\text{Stop}} \), the viral load ratio is:

\[
\frac{v_{\text{LRA}}(t)}{v(0)} = \frac{e^{-(d_y+\delta)t-(\delta+a')t_{\text{Stop}}}}{(d_y-\delta)(d_y-\delta-a')}
\times \left[ d_y^2 e^{d_y t+\delta t_{\text{Stop}}} - d_y \left( (\delta + a') \left( e^{d_y t+\delta t_{\text{Stop}}} + e^{\delta t+(\delta+a')t_{\text{Stop}}} \right) - a' e^{\delta t+d_y t_{\text{Stop}}} \right) 
+ \delta(\delta + a') e^{\delta t+(\delta+a')t_{\text{Stop}}} \right] 
+ e^{-d_y(t-t_{\text{Stop}})} \frac{d_y(3(\rho - 1) + a't_a)(e^{-(\delta+a')t_{\text{Stop}}} - e^{-d_y t_{\text{Stop}}})}{(d_y - \delta - a')(3 + t_a(d_y - a' - d_y))}.
\]

(S9)

As in Eq. (S9), the first term (spanning the first three lines) represents the portion due to activated cells, while the second term (on the final line) represents the portion due to LRA-induced cells. This expression is used to compute the curves in Figure 4.8C and 4.8D. Note that this dynamic treats the LRA as pharmacologically active at maximum concentration at the start of therapy; a more realistic model would include an absorption phase during which viral load may increase more gradually.
Parameters used in Figure 4.8

For each treatment described in Figure 4.8B, \( \rho \) was chosen to match the median value observed in the ex vivo assay, given in Table 2. To provide viral load estimates, pre-treatment residual viremia of 2 c ml\(^{-1} \) was used. Eq. (S8) was used to compute peak viral load, with \( d_y/d'_y \) of 1 or 3.

For Figure 4.8C and 4.8D, Eq. (S9) was used, and both \( \rho \) and pre-treatment residual viremia were as in Figure 4.8B. Baseline activation rate \( a=5.7\times10^{-5} \) day\(^{-1} \) and latent cell death rate \( d_z=4.66\times10^{-4} \) day\(^{-1} \) were chosen to be consistent with observed residual viremia and reservoir half-life of 44 months. Death rate \( d_y \) was set to 1 day\(^{-1} \), and \( d'_y \) was either 1 day\(^{-1} \) (blue curves) or 1/3 days\(^{-1} \) (red curves). For blue curves, \( a' \) for each treatment was chosen using the relationship (S4), assuming \( d_y/d'_y = k/k' = 1 \) (see Appendix Table 2). For red curves displaying romidepsin treatment, \( a' \) of 0.002 day\(^{-1} \) was chosen to be consistent with \( d_y/d'_y = k/k' = 3 \).
Appendix Table 1: Assumptions regarding comparison of *in vivo* and *ex vivo* parameters

<table>
<thead>
<tr>
<th>Parameter Description</th>
<th>Why assumed same <em>ex &amp; in vivo</em></th>
<th>Caveats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation rate</td>
<td>Stochastic factors governing viral transcription launch a program of viral production; these intracellular fluctuations may be similar in both settings.</td>
<td>Immune-activating effects (MHC class II presentation, cytokine signaling) not present in the assay may cause the <em>in vivo</em> value to exceed the <em>ex vivo</em> value.</td>
</tr>
<tr>
<td>LRA-driven induction rate</td>
<td>Assay treatment conditions replicate the <em>in vivo</em> drug environment. Mechanisms causing induction are believed to rely on the same cellular transcriptional machinery in both settings.</td>
<td>Immune-activating effects not present in the assay may interact with the LRA effect, causing the <em>in vivo</em> value to differ from the <em>ex vivo</em> value.</td>
</tr>
<tr>
<td>Rate of viral production by activated (LRA-induced) cells</td>
<td>Viral production occurs intracellularly, and primary CD4+ T cells studied in the assay are a close representation of intracellular activity <em>in vivo</em>.</td>
<td>Cytokine production by CD8+ T cells <em>in vivo</em> may suppress viral production compared to <em>ex vivo</em> rates.</td>
</tr>
<tr>
<td>Death rate of activated (LRA-induced) cells</td>
<td>Production of cytotoxic viral proteins is a major cause of cell death and may be similar in both settings (see parameters k, k' above).</td>
<td>CTL response, not present in the assay, may alter ( d_y (d'_y) ) <em>in vivo</em>, but evidence suggests that this generally is not the case; also evidence suggests that HIV-specific responses are generally weak in HIV-infected individuals.</td>
</tr>
<tr>
<td>Death rate of latently infected cells</td>
<td>Low levels of transcription and viral production in latently infected cells enable long cell lifespan <em>ex vivo as in vivo</em>.</td>
<td>Conditions in the assay may not be conducive to very long cellular lifespans. Even if this parameter differs between the two settings, decay over the short duration of the assay is not expected to have a large effect on observed viral production, as noted in discussion of Eq. ( ).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter Description</th>
<th>Why assumed zero <em>ex vivo</em></th>
<th>Caveats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral decay rate</td>
<td>Viral clearance occurs primarily in lymphoid and other organs</td>
<td>Some decay of viability of virus particles may also occur over the course of the day-long assay, at a rate slower than <em>in vivo</em>.</td>
</tr>
</tbody>
</table>
Appendix Table 2: Treatment-specific parameters used for blue curves in Figure 4.8C and 4.8D

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$\rho$</th>
<th>$\omega$ (day$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Romidepsin</td>
<td>15</td>
<td>$8 \times 10^{-4}$</td>
</tr>
<tr>
<td>Prostratin + romidepsin</td>
<td>104</td>
<td>0.0059</td>
</tr>
<tr>
<td>Bryostatin-1 + romidepsin</td>
<td>105</td>
<td>0.0059</td>
</tr>
<tr>
<td>Bryostatin-1</td>
<td>120</td>
<td>0.0068</td>
</tr>
<tr>
<td>Prostratin</td>
<td>209</td>
<td>0.012</td>
</tr>
<tr>
<td>Prostratin + JQ1</td>
<td>297</td>
<td>0.017</td>
</tr>
<tr>
<td>Bryostatin-1 + JQ1</td>
<td>401</td>
<td>0.023</td>
</tr>
<tr>
<td>PMA + ionomycin</td>
<td>554</td>
<td>0.032</td>
</tr>
</tbody>
</table>
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National Institute of Allergy and Infectious Diseases (NIAID)
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Gates Grand Challenges Exploration, Phase I 2011
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AWARDS
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AAAS/Science Program for Excellence in Science 2011–2013
Pollard Scholars in Cellular and Molecular Medicine 2012
Trinity College Dean’s List, Duke University 2006, 2007

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Ho YC*, **Laird GM***, Siliciano RF


*PMID: 24799684*

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“Novel ex vivo approaches distinguish effective and ineffective drugs for reversing latency in vivo”
Oral abstract presentation
Conference on Retroviruses and Opportunistic Infections (CROI), Boston, MA 2014

“The Road to Curing HIV”
Partnering Towards Discovery:
Conversations in Research and Medicine
Johns Hopkins University School of Medicine 2013

POSTER RESEARCH PRESENTATIONS
“Ex vivo identification of highly effective latency-reversing drug combinations”
Conference on Retroviruses and Opportunistic Infections (CROI), Seattle, WA 2015

“Novel viral release assay for HIV and SIV”
Keystone Symposium X7/X8, Whistler, BC, Canada 2012

“Discovery of novel non-cytidine liponucleotides in S. cerevisiae and E. coli”
2nd LIPID MAPS Annual Conference, San Diego, CA. 2010

“Artifactual formation of N-acylphosphatidylethanolamine and related molecules during chloroform-methanol based lipid extractions”
1st LIPID MAPS Annual Conference, San Diego, CA. 2009

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The John Hopkins University School of Medicine

Pollard Scholar 2012

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The Johns Hopkins University School of Medicine