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Ex Vivo Bioactivity and HIV-1 Latency Reversal by Ingenol Dibenzoate and Panobinostat in Resting CD4$^+$ T Cells from Aviremic Patients

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The human immunodeficiency virus type 1 (HIV-1) latent reservoir in resting CD4$^+$ T cells represents a major barrier to viral eradication. Small compounds capable of latency reversal have not demonstrated uniform responses across *in vitro* HIV-1 latency cell models. Characterizing compounds that demonstrate latency-reversing activity in resting CD4$^+$ T cells from aviremic patients *ex vivo* will help inform pilot clinical trials aimed at HIV-1 eradication. We have optimized a rapid *ex vivo* assay using resting CD4$^+$ T cells from aviremic HIV-1$^+$ patients to evaluate both the bioactivity and latency-reversing potential of candidate latency-reversing agents (LRAs). Using this assay, we characterize the properties of two candidate compounds from promising LRA classes, ingenol 3,20-dibenzoate (a protein kinase C agonist) and panobinostat (a histone deacetylase inhibitor), in cells from HIV-1$^+$ antiretroviral therapy (ART)-treated aviremic participants, including the effects on cellular activation and cytotoxicity. Ingenol induced viral release at levels similar to those of the positive control (CD3/28 receptor stimulation) in cells from a majority of participants and represents an exciting LRA candidate, as it combines a robust viral reactivation potential with a low toxicity profile. At concentrations that blocked histone deacetylation, panobinostat displayed a wide range of potency among participant samples and consistently induced significant levels of apoptosis. The protein kinase C agonist ingenol 3,20-dibenzoate demonstrated significant promise in a rapid *ex vivo* assay using resting CD4$^+$ T cells from treated HIV-1-positive patients to measure latent HIV-1 reactivation.

Durable blockade of viral replication by combinations of antiretroviral drugs has transformed human immunodeficiency virus type 1 (HIV-1) infection from an untreatable, lethal condition characterized by progressive immune deficiency into a chronic, manageable medical problem for the vast majority of patients with access to therapy (1). Despite the ability of antiretroviral therapy (ART) to block ongoing HIV-1 replication and allow for restoration of the circulating CD4$^+$ T cell population, HIV-1 eradication does not occur with these drugs due to the presence of long-lived viral reservoirs in resting memory CD4$^+$ T cells(2–4). ART can continuously suppress viral replication for years or even decades; however, patients who stop therapy will develop viremia within a matter of weeks and progress to overt immunodeficiency if ART is not resumed (5). This rebound viremia arises from a minority of cells among the resting memory CD4$^+$ T cell population harboring unexpressed HIV-1 proviral DNA that is stably integrated into the cellular genome (6).

The HIV-1 latent reservoir in patients on ART is stable over a period of many years and does not decay significantly during the life span of an infected patient (7). It is generally accepted that eradication of the virus will require elimination of this latent reservoir (8, 9). The absence of specific markers to distinguish latently infected cells from uninfected cells has led to the proposition that substances able to reverse the latent viral state should be used to "purge" the latent reservoir (10). Infected cells might then potentially be cleared via viral cytopathic effects or immune-mediated mechanisms (11).

The inability of currently available *in vitro* model systems to reliably predict latency reversal *ex vivo* (12) underscores the importance of evaluating candidate compounds using *ex vivo* techniques making use of cells obtained from HIV-1-infected patients on ART. The current technique using such cells is known as the viral outgrowth assay (VOA) (13). Because the VOA was designed to use serial dilutions of patient cells, this technique requires large numbers of cells that are best obtained via leukapheresis. In addition, the VOA relies on the patient's endogenous virus to spread to and replicate within indicator cells, a process that can take up to 10 days. To circumvent these limitations, we have optimized an assay that can be performed in 3 days and requires no more than 180 ml of peripheral blood, obtained via venipuncture. Rather than relying on virus spread, this procedure measures a burst in cell-free virus release that is detectable within 48 h of cell stimulation. This assay is ideal for evaluating the efficacy of candidate LRAs in preclinical studies. In this work, we evaluate representative candidates from two promising LRA classes, protein kinase C agonists (PKCa) (14–20) and histone deacetylase inhibitors (HDACi) (21–24).

MATERIALS AND METHODS

Participant involvement. Aviremic HIV-1-infected patients on ART were recruited for phlebotomy according to an approved institutional review board (IRB) protocol at the University of Utah. Inclusion criteria

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for this study required viral suppression (<50 HIV-1 RNA copies/ml) for a minimum of 6 months, ART initiation during chronic HIV-1 infection (>6 months since seroconversion), and compliance with a stable ART regimen for a minimum of 12 months per participant and provider reports. A healthy HIV-uninfected donor was recruited via a separate approved IRB protocol. Informed consents were obtained and phlebotomies were performed in the Center for Clinical and Translational Science Clinical Services Core at the University of Utah Medical Center.

Resting CD4$^+$ T cell isolation and *ex vivo* culture conditions. Peripheral blood mononuclear cells were isolated from whole blood immediately after phlebotomy via density gradient centrifugation, followed by negative selection of resting CD4$^+$ (rCD4) T cells using magnetic bead separation (Miltenyi Biotec and StemCell Technologies). Aliquots of 5×10^6 resting CD4$^+$ T cells were cultured under multiple conditions: a negative control consisting of culture medium and dimethyl sulfoxide (DMSO) (compound solvent) alone, CD3/CD28 antibody-coated magnetic beads (Dynabeads human T-activator CD3/CD28 [Life Technologies]; positive control), and medium containing candidate latency-reversing agents (LRAs) at concentrations previously shown to induce viral reactivation (100 nM for both ingenol and panobinostat; compounds obtained from Martin Delaney Collaboratory of AIDS Researchers for Eradication [CARE], University of North Carolina, Chapel Hill, NC) (17, 18, 22). At 48 h, culture supernatant was collected for real-time quantitative PCR (qPCR) (described in detail below), and aliquots of 10^5 cells were obtained for flow cytometry in order to test for the presence of biomarkers of drug activity, cellular activation, and apoptosis (described in detail below).

Real-time quantitative PCR. Supernatant from each well was collected for quantification of cell-free virions using a two-step qPCR that makes use of a primer and probe set for conserved regions of the 3′-long terminal repeat (LTR) of HIV-1 mRNA using recently published methods (25).

Culture supernatant was subjected tot DNase treatment (Quanta Biosciences) followed by cDNA synthesis using qScript cDNA SuperMix containing oligo(dT) primers and random hexamers according to the manufacturer's protocol (Quanta Biosciences). RNA aliquots that did not contain reverse transcriptase (no-RT controls) were tested in parallel for every sample. Real-time quantitative PCR was subsequently performed in triplicate on cDNA and RNA (no-RT control) samples using TaqMan Universal Master Mix II (Applied Biosystems) on a Roche LC480 real-time PCR instrument. Primers and probe used were as follows: forward primer (5′ to 3′) CAGATGCTGCATATAAGCAGCTG, reverse primer (5′ to 3′) TTTTTTTTTTTTTTTTTTTTTTTTTTGAAGCAC, and probe (5′ to 3′) FAM-CCTGTACTGGGTCTCTCTGG-BHQ1. Cycling conditions were as follows: 50°C for 2 min followed by 95°C for 10 min for polymerase activation followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Serial 10-fold dilutions of a plasmid containing the HIV-1 3′-LTR (VQA plasmid; obtained from Greg Laird and Robert Siliciano) from 10^6 to 10^0 copies/well were amplified in triplicate along with unknowns in order to provide a standard curve and quantify cell-associated viral mRNA. This assay has been shown to be highly specific for the detection of HIV-1 mRNA with a lower limit of detection of 50 HIV-1 mRNA copies/ml (25).

Biomarker flow cytometry. After 48 h in culture, aliquots of 10^5 cells were fixed using BD Cytofix fixation buffer (50% by volume; BD Biosciences) for 10 min at 37°C. After incubation, DMSO (Fisher Scientific) was added to the sample to a final concentration of 10%, and the sample was frozen at −80°C. At the time of analysis, samples were thawed on ice and resuspended in 2 ml of phosphate-buffered saline (PBS). Aliquots of 500 μl were used for each staining and for the staining control. For acetyl-histone H3 analysis, samples were pelleted and resuspended in 100 μl of BD Phosflow Perm Buffer III (BD Biosciences) while vortexing and incubated on ice for 30 min. Cells were then washed with PBS and incubated in 100 μl of PBS plus 3% fetal bovine serum (FBS) containing 1 μl of acetyl-histone H3-phycoerythrin (PE) (Millipore) and 0.75 μl of cleaved caspase 3-AF488 (Cell Signaling Technology) for 1 h at room temperature protected from light. After incubation, cells were washed with PBS plus 3% FBS, and 100 μl of 2% paraformaldehyde (PFA) was added prior to flow cytometry acquisition. For CD69 analysis, samples were pelleted and resuspended in 100 μl of BD Cytofix/Cytoperm (BD Biosciences) and incubated at 4°C for 30 min. Cells were then washed with BD Perm/Wash (BD Biosciences) and incubated in 100 μl of Perm/Wash containing 1 μl of CD69-allophycocyanin (APC) (Invitrogen) and 0.75 μl of cleaved caspase 3-AF488 for 1 h at room temperature protected from light. After incubation, cells were washed with Perm/Wash buffer, and 100 μl of 2% PFA was added prior to flow cytometry acquisition.

Biostatistics. Log response (CD69 mean intensity of fluorescence [MIF], percent active caspase 3, fold induction acetyl-histone H3) and log mRNA viral release were analyzed with a linear mixed-effects model. A fixed growth medium effect and a random subject effect were included in the predictor variables. For the response variables, mRNA and CD69, the response was log-transformed before analysis to account for larger variability in larger values. mRNA values below the limit of quantitation (LOQ) were imputed as 1/2 LOQ, i.e., 25 copies mRNA/ml. Data were analyzed using SAS version 9.3 PROC MIXED.

RESULTS

Participants. We recruited 12 HIV-1-positive, aviremic participants on ART (the participant characteristics are shown in Table 1). Two participants, H025 and H026, returned for a second phlebotomy during the study period. We therefore report the results of 14 independent *ex vivo* experiments using resting CD4$^+$ (rCD4) T cells from 12 individuals. The median age among our cohort was 49.5 years (range, 37-60 years). The median CD4$^+$ T cell count at the time of enrollment was 738 cells/μl (range 247 to 1,166 cells/μl) and time of viral suppression was 31.5 months (range, 7 to 55 months). All participants initiated ART during chronic infection. We recruited a healthy, HIV-1-uninfected participant for phlebotomy in order to isolate uninfected resting CD4$^+$ T cells as a negative control for the assay.

Rapid *ex vivo* evaluation of anti-latency activity. Reactivation of latent proviruses induces a burst of productive transcription as evidenced by the presence of cell-associated mRNA followed by release of HIV-1 virions into the culture medium. We reasoned that latently infected cells from the peripheral blood of aviremic patients would release a detectable quantity of virions into the medium upon stimulation. To detect the presence of such virions, we relied upon a recently published quantitative PCR protocol (25). This method uses primers that are specific for authentic viral mRNAs, including genomic viral RNA, and does not amplify products of aberrant read-through originating from adjacent cellular promoters (25).

We isolated between 15 and 60 million rCD4 T cells from 180 ml of peripheral blood by routine phlebotomy. We stimulated aliquots of 5 million rCD4 T cells per control or experimental condition. Based on previously described estimates of the frequency of replication-competent proviruses present in rCD4 T cells *in vivo* (3, 4, 14, 26), we estimated that 5-million rCD4 T cell aliquots would ensure the detection of virions released into the supernatant. The variable quantity of rCD4 T cells obtained from 180 ml of whole blood from participants limited our ability to test all conditions in all donors; ingenol was tested in all donors, and panobinostat was tested simultaneously in six donors.

The positive control, CD3/CD28 stimulation with antibody-coated magnetic beads, induced viral release in samples from all HIV-1-infected participants (Fig. 1) with a geometric mean of 3,868 copies of HIV-1 mRNA/ml (range, 1,030 to 14,518 copies/ml). Viral release in the absence of stimulation (culture medium

TABLE 1 Participant characteristics

Participant	Age (yr)	Gender	Race/ethnicity[a]	$CD4^+$ T cell count[b]	Duration of viral suppression[c]	ART regimen[d]
H007	60	Male	H	247	17	TDF/FTC/DRVr
H008	37	Male	H	439	18	TDF/FTC/EVGc
H010	43	Male	W	1,040	16	TDF/FTC/EVGc
H012	52	Female	W	621	31	TDF/FTC/RPV
H013	50	Male	W	976	32	TDF/FTC/EFV
H014	57	Male	W	813	24	TDF/FTC/EVGc
H018	45	Female	W	948	35	TDF/FTC/EFV
H020	55	Male	W	703	55	TDF/FTC/DRVr
H025	51	Male	W	398	7	TDF/FTC/EVGc
H026	47	Male	W	773	55	TDF/FTC/ATVr
H028	49	Male	H	687	32	TDF/FTC/EVGc
H029	42	Male	W	1,166	39	ABC/3TC/DTG

[a] H, Hispanic; W, non-Hispanic Caucasian.
[b] Absolute $CD4^+$ T cell count measured in cells/μl.
[c] Consecutive months of documented viral load (plasma HIV-1 RNA) suppression below the limit of clinical detection with ART.
[d] 3TC, lamivudine; ABC, abacavir; ATVr, atazanavir boosted with ritonavir; DRVr, darunavir boosted with ritonavir; DTG, dolutegravir; EFV, efavirenz; EVGc, elvitegravir boosted with cobicistat; FTC, emtricitabine; RPV, rilpivirine; TDF, tenofovir.

plus DMSO) was only detected in two aliquots and at very low levels (geometric mean, 52 copies/ml; range, 14 to 96 copies/ml) (Fig. 1). The ratio of means between our positive control and negative control was 74.1 (range, 11.4 to 481.3 with a probability of difference of $P < 0.0001$), representing the average fold increase in viral RNA copies detected between these conditions. Low-level viral release in negative-control samples may reflect suboptimal ART compliance or viral "blips" at the time of phlebotomy (27). The supernatant from the HIV-1-uninfected donor did not demonstrate any detectable viral mRNA from any condition. Viral release from rCD4 T cells exposed to CD3/CD28 antibodies was accompanied by induction of CD69 and a modest increase in histone acetylation measured by flow cytometry (Fig. 2 and 3, respectively).

We observed a >100-fold variability between participants with regard to viral release in the CD3/28 antibody positive-control condition (Fig. 1). High variability in the magnitude of response to stimulation has also been observed in all published pilot clinical trials with LRAs to date (21, 28–30) and is likely to reflect underlying differences between individuals with respect to the viral reactivation threshold. This in turn is likely governed by a number of unique characteristics, including the overall reservoir size and the transcriptional regulatory state of these cells. Aliquots of 5 million purified rCD4 T cells cultured for 48 h consistently provided the dynamic range to test both latency reversal and bioactivity of candidate LRAs *ex vivo*. We have termed these combined protocols the rapid *ex vivo* evaluation of anti-latency assay (REVEAL).

We designed the REVEAL assay to amplify genomic RNA from virions in the culture supernatant. It has long been recognized that virions will pellet out of the supernatant when subjected to ultra-

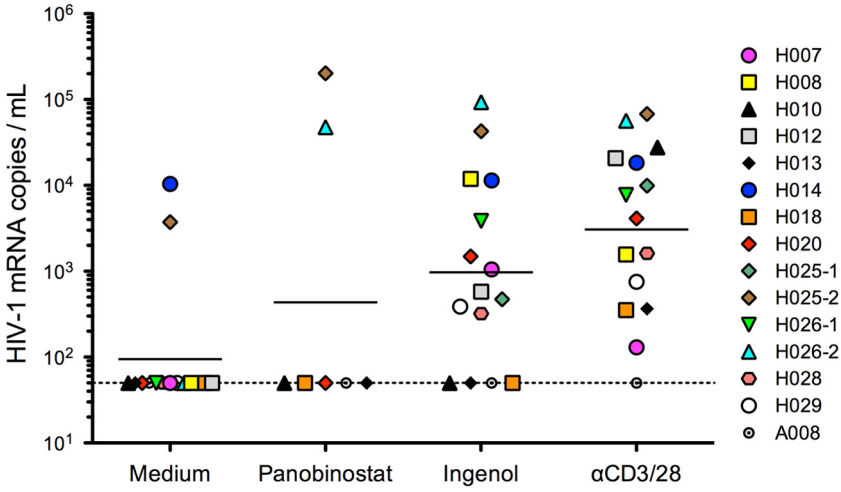

FIG 1 Quantification of HIV-1 viral release into culture supernatant after 48 h under four different conditions reveals that ingenol 3,20-dibenzoate has potency similar to that for CD3/28 stimulation. Aliquots of 5 million resting $CD4^+$ T cells from aviremic patients on ART were exposed to CD3/28 antibody-coated beads (positive control), medium and DMSO (negative control), 100 nM ingenol 3,20-dibenzoate, or 100 nM panobinostat for 48 h. Quantitative PCR was performed on culture supernatant to detect HIV-1 mRNA release. CD3/28 antibody stimulation resulted in widely ranging, although consistently detectable, HIV-1 mRNA release in all experiments. One HIV-1-uninfected donor (A008) was included to ensure the specificity of the assay, and HIV-1 mRNA was undetectable under all conditions. Ingenol demonstrated potency similar to that of the positive control with regard to viral release (ingenol mean of 811 copies/ml compared to 3,868 copies/ml for CD3/28; ratio of means of 4.77 with $P = 0.10$), while panobinostat exposure led to viral release in two of six experiments (mean, 266 copies/ml).

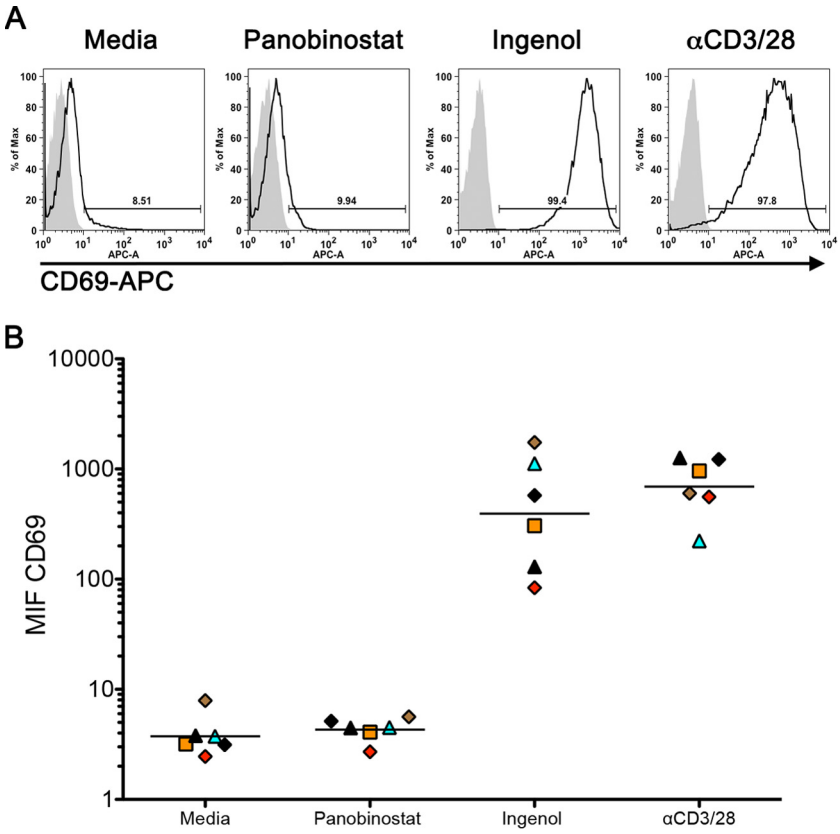

FIG 2 Resting CD4$^+$ T cells from aviremic patients exposed to 100 nM ingenol 3,20-dibenzoate demonstrated an increase in the mean intensity of fluorescence (MIF) of CD69 to levels similar to those for CD3/28 antibody exposure (ingenol mean of 336 compared to 577 for CD3/28; ratio of means of 1.7 with $P = 0.239$). Panobinostat at 100 nM induced no change in CD69 fluorescence compared to medium alone (MIF 4.27 versus 1.39; $P = 0.063$). (A) Representative flow cytometry histograms for the four conditions tested from a single experiment. The solid gray histogram represents the MFI of the isotype control, and the black outline histogram represents the MFI for each condition. (B) Results from six independent experiments.

centrifugation, while naked mRNA will not (31). To verify that the RNA detected in the supernatants was pelletable, the CD3/CD28 condition of one donor was subjected to ultracentrifugation followed by qPCR. The initial REVEAL analysis in unconcentrated supernatant measured a total of 1,500 mRNA copies. Ultracentrifugation of this supernatant led to a 125-fold volume concentration with detection of 875 total mRNA copies in the pellet, representing 58% recovery of the RNA signal. Therefore, the RNA detected in the supernatant is pelletable. We also performed a p24 enzyme-linked immunosorbent assay (ELISA) per the manufacturer's instruction (HIV-1 p24 antigen ELISA 2.0; ZeptoMetrix Corp), in an attempt to detect Gag protein secreted to the culture supernatant at 48 h. Only samples treated with the positive control (CD3/28 antibodies) showed positive p24 values, although these were below the quantitative threshold of the assay (3.9 pg/ml; data not shown). None of the negative-control treatments resulted in positive ELISA values. These data further support the viral RNA and not the p24 ELISA as representing a sensitive and quantitative readout for the REVEAL assay. Attempts to coculture virus with indicator cells resulted in easily detectable viral replication after 14 days in culture by p24 ELISA for the CD3/CD28 condition but not for the medium/DMSO condition. However, sufficient numbers of lymphocytes to allow for a quantitative, limiting-dilution-based assay are not typically available from 180-ml blood draws and require leukapheresis. These experiments provide further evidence that stimulation of aviremic patient cells results in release of replication-competent virus.

Reactivation and bioactivity of ingenol 3,20-dibenzoate. We have recently observed that ingenol 3,20-dibenzoate, a protein kinase C agonist, induced viral reactivation reproducibly and with potency similar to that of CD3/28 stimulation in a primary central memory T cell model of latency (32). Therefore, it was compelling to further test ingenol 3,20-dibenzoate in aviremic patient cells. Ingenol at 100 nM induced viral release from resting CD4$^+$ T cells at levels close to those following T cell receptor stimulation in 11 of 14 independent experiments (Fig. 1). The geometric mean for CD3/28 stimulation among all participants was 3,868 HIV-1 mRNA copies/ml (range, 1,030 to 14,518 copies/ml) compared to 811 copies/ml for ingenol (range, 216 to 3,044 copies/ml). Using the linear mixed-effects model for comparison, we determined that the relative fold increases in viral RNA induced by CD3/28 versus ingenol treatments were not statistically significant ($P = 0.10$).

PKC agonists are known to upregulate cell surface expression of CD69 (33), an early marker of T cell activation. Cells exposed to ingenol upregulated CD69 to levels approximating T cell receptor stimulation by flow cytometry (Fig. 2), with a geometric mean intensity of fluorescence (MIF) for ingenol of 336 (range, 176–643) compared to 577 for CD3/28, the antibody positive control (range, 302–1,104). These means were not statistically significant ($P = 0.24$) by the linear mixed-effects model analysis. The CD69

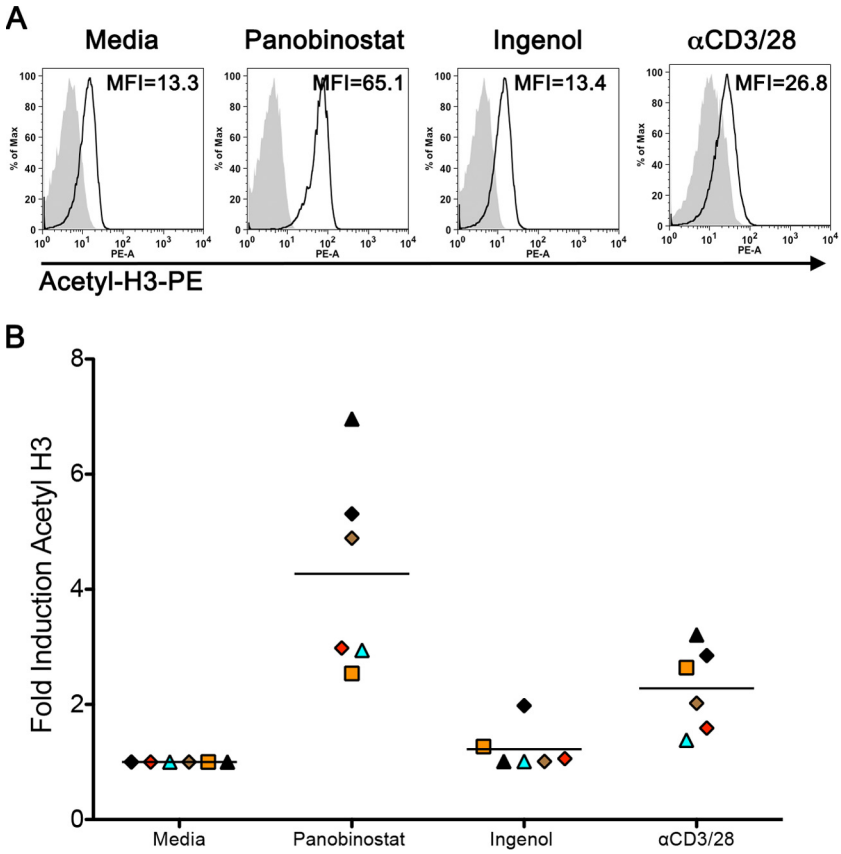

FIG 3 A 48-h exposure to 100 nM panobinostat leads to a mean 3.57-fold increase in intracellular acetylated histone 3 (Acetyl H3) compared to that for the negative control. (A) Representative flow cytometry histograms from a single experiment measuring the mean florescence intensity (MFI) of intracellular acetylated histone 3 under four different conditions. The solid gray histogram represents the MFI of the isotype control, and the black outline histogram represents the MFI for each condition. (B) Panobinostat, a histone deacetylase inhibitor (HDACi), at 100 nM increased acetylation at histone 3 an average of 3.57-fold in six independent experiments compared to that for medium and DMSO alone (negative control; $P < 0.0001$). Ingenol 3,20-dibenzoate at 100 nM did not result in any significant change in acetyl H3 from baseline.

MIF for ingenol was significantly higher than that for the medium-alone condition (MIF, 1.4; range, 0.73–2.7) with a ratio of 241 (range, 96–604) ($P < 0.0001$).

Reactivation and bioactivity of panobinostat. Panobinostat, a histone deacetylase inhibitor, at 100 nM induced viral reactivation from cells in a minority of participants, with viral release detected from cells of two of six individuals tested (Fig. 1). The geometric mean of viral release from aliquots exposed to panobinostat was 266 copies/ml (range, 38–1,845 copies/ml) with a statistically significant ratio of means between panobinostat and the CD3/28 antibody-positive control of 14.5 (range, 1.39–151.6) ($P = 0.03$).

Panobinostat demonstrated inhibition of histone deacetylation at 100 nM in all participant samples tested (Fig. 3) independent of latency reversal. The mean fold change in histone 3 acetylation was 3.57 (range, 2.78 to 4.58) compared to that with medium alone. Unlike ingenol, panobinostat did not induce expression of CD69 (MIF, 4.3; range, 1.6–11.5) (Fig. 2) to levels that differed from those with medium alone (ratio of means, 3.1; range, 0.94–10.03; $P = 0.06$).

Induction of programmed cell death. Activation of intracellular caspase 3 is an early and reliable biomarker of apoptotic cell death (34) and is the mechanism of apoptosis induced by ingenol compounds (19, 35). Cells exposed to 100 nM ingenol demonstrated a modest but statistically significant decrease in the percentage of cells undergoing early apoptosis compared to that with medium alone (6.03% versus 12.62%, $P = 0.0001$) (Fig. 4). Conversely, 100 nM panobinostat induced apoptosis in a significant percentage of cells compared to that with medium alone (30.75% versus 12.62%; $P < 0.0001$) (Fig. 4) in these culture conditions. Cell viability, measured by forward and side scatter gating, is decreased among cells exposed to panobinostat but not under other conditions compared to that with medium alone (Fig. 4C). The lack of apoptosis induction by ingenol 3,20-dibenzoate may reflect the relatively low concentration of ingenol (100 nM) and the fact that our cells are not transformed in contrast to those in previous studies (19, 34).

DISCUSSION

We describe a rapid *ex vivo* system making use of resting $CD4^+$ T cells from aviremic HIV-1-positive participants on stable ART to estimate the reactivation potential, bioactivity, and toxicity of candidate LRAs, which we have named the REVEAL assay. After 180 ml of whole blood is obtained by phlebotomy, resting $CD4^+$ T cell isolation and 48 h of cell culture are followed by standardized qPCR (25) and flow cytometry techniques. This assay produces reproducible results characterizing the behavior of candidate

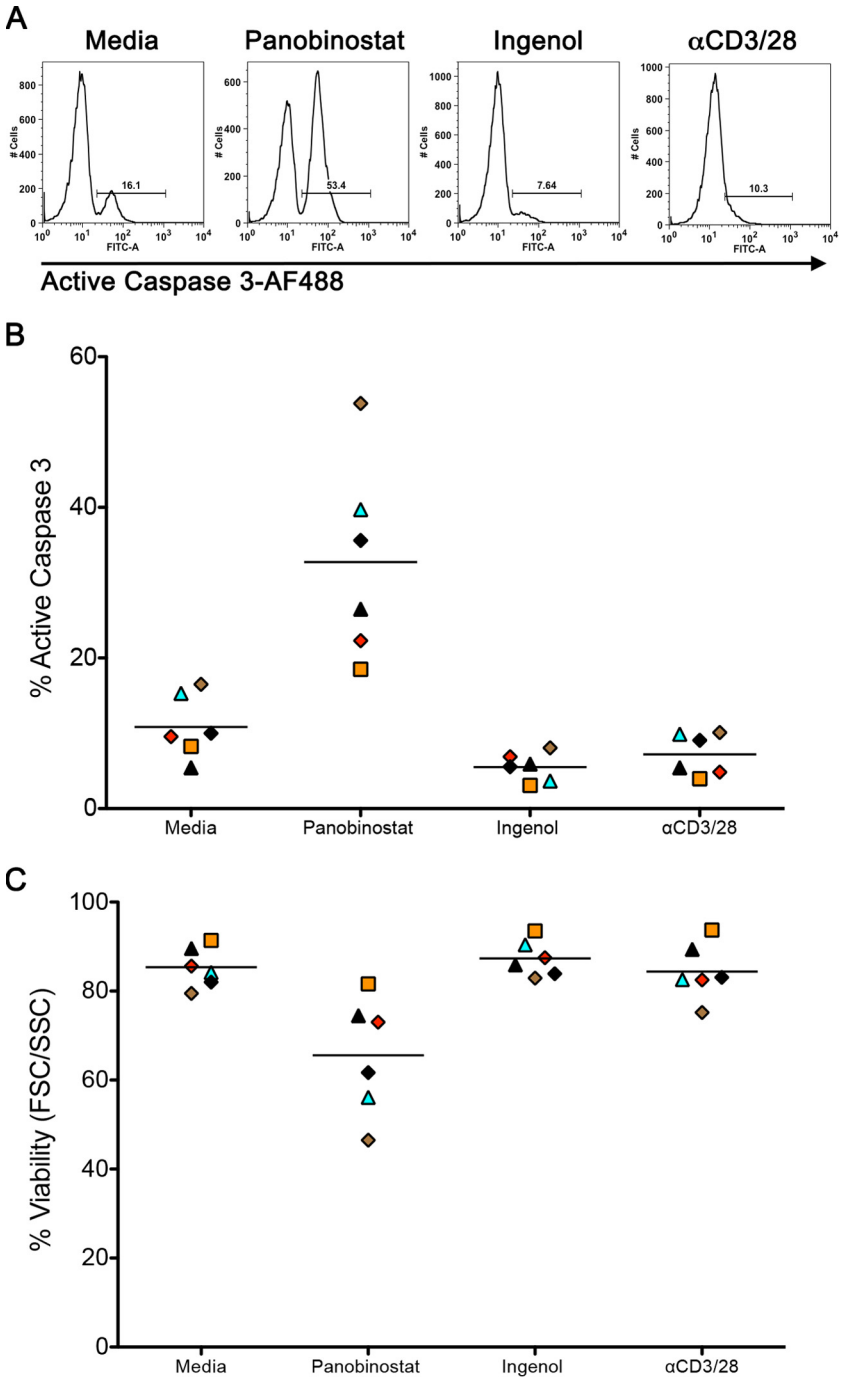

FIG 4 A 48-h exposure to 100 nM panobinostat resulted in a significantly higher percentage of resting $CD4^+$ T cells expressing activated caspase 3, an early marker of apoptosis, than for the negative control (30.75% compared to 12.62%; $P < 0.0001$). (A) Representative panel of flow cytometry histograms from a single experiment demonstrating a 3-fold increase in activated caspase 3 expression in cells exposed to panobinostat compared to that for the medium-alone condition. The horizontal bars indicate the percentages of cells expressing activated caspase 3. FITC, fluorescein isothiocyanate. (B) Panobinostat led to increased expression of activated caspase 3 in six independent experiments compared to that for the negative control. Cells exposed to 100 nM ingenol 3,20-dibenzoate and CD3/28 antibody stimulation demonstrated modest but statistically significant decreases in activated caspase 3 expression compared to that for medium alone (6.03% for ingenol and 7.18% for CD3/28; $P = 0.0001$ and $P = 0.001$, respectively). (C) Cell viability measured by forward (FSC) and side scatter (SSC) gating for each condition is decreased in the panobinostat condition but not for ingenol or anti-CD3/28 exposed cells.

LRAs in patient cells *ex vivo* within 3 days. An assay using the same RNA PCR output measurement and a similar culture protocol has recently been reported (20). Laird et al. observed that PKC agonists (including bryostatin-1 and prostratin, but not ingenol) reliably induce proviral transcription and viral release from patient cells *ex vivo* alone and in combination with HDAC inhibitors, including panobinostat. Panobinostat alone did not induce significant viral reactivation (20).

Using the REVEAL assay, we have observed that ingenol 3,20-dibenzoate activates patient resting $CD4^+$ T cells as measured by upregulation of CD69 and reactivates latent virus to levels that approximate those of T cell receptor stimulation. Minimal apoptosis was observed after 48 h in culture. Our results are in keeping with recently published reports of the latency-reversing potential and safety of ingenol derivatives in cell lines (18, 36) and an *in vivo* nonhuman primate simian immunodeficiency virus (SIV) model (37). PKC agonists are recognized as a promising class of latency-reversing compounds (14, 15, 18, 20, 36), and ingenol derivatives in particular deserve further preclinical characterization.

Panobinostat was recently tested in an unblinded clinical trial as a latency reactivation agent in 15 aviremic patients on ART (30). The results from this study demonstrate mean increases in cell-associated viral RNA and plasma viremia in this cohort, with significant variations in individual responses. We have observed an analogous phenomenon by exposing patient rCD4 T cells to 100 nM panobinostat with a minority of participants exhibiting viral release. Laird et al. described similar results with panobinostat alone (20) as did Cillo et al. using the HDAC inhibitor vorinostat (38). Regardless of viral transcription, panobinostat demonstrated marked inhibition of histone deacetylation in cells from all patients tested at this concentration (Fig. 3B). This activity is associated with induction of activated caspase 3, an early marker of apoptosis, in our system (Fig. 4B).

There are a number of limitations to the *ex vivo* patient cell assay described here. In an attempt to maintain the baseline quiescent state of the resting $CD4^+$ T cells isolated from participants and to minimize background viral reactivation, no cytokines (including interleukin 2) are added to cell cultures. While the lack of exogenous cytokines improves the signal-to-noise ratio with regard to viral reactivation in the REVEAL assay, it likely comes at the cost of higher induction of apoptosis (12.62% caspase 3 positive in medium alone at 48 h) (Fig. 4B). The significantly lower percentage of cells undergoing activation of caspase 3 under ingenol and αCD3/28 conditions (Fig. 4B) may reflect the initiation of endogenous interleukin 2 production as these cells enter an activated state.

The quantity of resting $CD4^+$ T cells obtained from a single phlebotomy allows for relative quantification of LRA responses relative to T cell receptor (TCR) stimulation; however, not enough cells are obtained to simultaneously measure the size of the inducible reservoir for each participant (13). The dynamic range of the REVEAL assay is likely to be partially dependent on the reservoir size for each participant. However, the assay is designed with internal positive and negative controls that allow for direct comparison between these controls and experimental LRA treatments.

The REVEAL assay isolates resting $CD4^+$ T cells in order to evaluate latency reversal and does not currently account for the potential effects of candidate LRAs on other immune cells, including $CD8^+$ T cells, NK cells, and monocytes. These cells may play an important role in latent reservoir eradication *in vivo*, and the effect of LRAs on these cell types deserves further exploration. Additional areas of active study include dose-response relationships and the potential for synergy between candidate LRAs as recently described by Laird et al. (20). The REVEAL assay described here allows for rapid, reproducible evaluation of candidate LRAs and provides an accessible platform to inform pilot clinical trials aimed at HIV-1 latent reservoir eradication.

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We declare no conflicts of interest.

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