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Current latency reversing agents reactivate a small fraction of latent proviruses

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Abstract

Background: Small molecule latency reversing agents (LRAs) are currently under intense investigation for their ability to reactivate latent proviruses, but the fraction of all inducible proviruses that can be reactivated by LRAs is unknown.
Methods: Resting CD4+ T (rCD4) cells were isolated by negative selection from participants on long-term ART. The frequency of infected rCD4 cells was evaluated by qPCR targeting HIV-1 *pol*. rCD4 cells were pulse-treated with LRAs of interest (Table) at pharmacologically achievable concentrations, and then serially diluted and cultured for 7 days. Separate serially diluted rCD4 cells were treated with medium control or anti-CD3/CD28 as negative and positive controls, respectively. Virion production (HIV-1 RNA in supernatants) was measured after 7 days by qRT-PCR (LOD 40 cps/ml). Maximum likelihood estimates were used to quantify the fraction of proviruses that were reactivated to produce virions (Cillo, et al. PNAS 2014).
Results: rCD4 cells were isolated from leukapheresis products from 8 donors on suppressive ART for an average of 9 years (range: 3-17), and pulsed with 40 nM RMD for 4 hours, 17.5 nM PAN for 30 minutes, 25 uM JQ1 for 30 minutes, or 10 nM BRY for 1 hour. Pulses with 2 LRAs were at the same concentration and duration as single LRAs. Median fold-increase in virion production over medium control was 5-fold for RMD, 1-fold for PAN, 3-fold for JQ1, 1.7-fold for BRY, 19-fold for BRY+RMD, and 22-fold for RMD+JQ1 compared with 592-fold for anti-CD3/CD28. Despite increases in virion production, only a small fraction of proviruses (between 0.012% and 0.12%) were reactivated with any LRA or combination of LRAs, compared to 3.7% of proviruses reactivated with anti-CD3/CD28. When the fraction of proviruses that were reactivated is expressed as a percent of the anti-CD3/CD28 positive control (set at 100%), only 1.4% of inducible proviruses were reactivated with RMD, 0.48% for PAN, 0.26% for JQ1, 0.31% for BRY, 3.3% for RMD+JQ1, and 1.8% for RMD+BRY.
Conclusions: Although latency reversing agents targeting HDAC, PKC, and bromodomain and extraterminal domain (Brd4) can stimulate virion production from resting CD4 cells, only a very small fraction of proviruses (<<1%) are reactivated to produce virions. Combinations of latency reversing agents increased the fraction of proviruses reactivated, but only to a few percent of that achieved with T cell activation. The "shock and kill" strategy will require latency reversing agents with much greater activity.

Introduction

- **Pharmacologic reversal of proviral latency has been proposed to eliminate latently infected cells in patients[1-3]**
- **The proportion of latent proviruses that are reactivated with putative latency reversing agents is unknown[4]**

Objectives

- **Assess the potency of proposed latency reversing agents to reactivate proviruses in resting CD4+ T cells**
- **Evaluate latency reversal by both virion production in the supernatant and the fraction of proviruses reactivated in limiting dilution cultures**

Materials and Methods

Study Population and Entry Criteria: Consecutive HIV-1 positive participants on suppressive ART for ≥3 year from the University of Pittsburgh Center for AIDS Treatment clinic were selected as volunteers to undergo leukapheresis.
Isolation and culture of rCD4 cells and quantification of HIV-1 DNA: Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-hypaque density gradient centrifugation. PBMC were frozen as cell pellets for quantitation of cellular HIV-1 DNA by qPCR, normalizing for cell number by CCR5 gene as previously described[4]. rCD4 cells were isolated by negative selection using a depletion cocktail (Stemcell Technologies). Following isolation, rCD4 cells were serially diluted in 3-fold dilutions from 667,000 cells/well to 152 cells/well, with 5 replicate wells at each dilution. Diluted rCD4 cells were then cultured for 7 days with either anti-CD3/CD28 activation beads (3 bead/cell), medium control or following pulse treatment with LRAs as described in Table 2. All cultures included 300nM efavirenz to prevent viral spread following proviral reactivaiton. After 7 days, supernatants were removed, spun at 500xg for 10 minutes, and HIV-1 RNA levels were quantified by Roche COBAS AmpliPrep/TaqMan v2.0. The vast majority (>95%) of nucleic acid detected was virion-associated HIV-1 RNA.
Statistical Analyses: The limiting dilution cell culture format, and the frequency of infected cells per million rCD4 cells, were combined in a maximum likelihood analysis[5] to ascertain the number of proviruses that were reactivated in any given treatment condition. To determine whether levels of virion production or the fraction of proviruses that were reactivated were statistically significant, linear mixed effects analysis was performed, followed by paired T-tests if the linear models were significant. Statistical analysis was carried out in R, and figures were generated in PRISM (version 7).

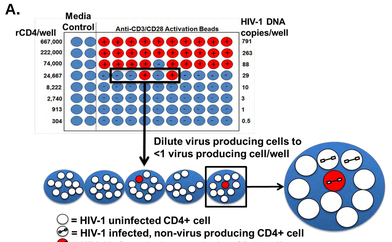
Results

Table 1. Study participant characteristics

Participant ID	Sex	Race	Age	Current CD4+ count (cells/mm³)	Resting CD4+ T cell vDNA (cps/10⁶ cells)	ART Regimen	Years aviremic
1	Male	Caucasian	51	ND	3597	3TC/ABC/RAL	17
2	Female	Caucasian	45	ND	428	EFV/FTC/TDF	3
3	Female	African American	36	809	1545	FTC/RPV/TDF	10
4	Female	African American	57	380	3207	ATV/FTC/r/TDF	5
5	Male	Caucasian	51	603	985	EVG/c/FTC/TDF	7
6	Male	Caucasian	46	714	822	3TC/ABC/EFV	5
7	Female	African American	57	1462	1752	FTC/RPV/TDF	15
8	Female	African American	62	1033	3589	DTG/FTC/TDF	10
Median (IQR)	**N/A**	**N/A**	**51 (46-57)**	**762 (631-977)**	**1649 (944-3302)**	**N/A**	**8 (5-11)**

IQR = interquartile range; ND = Not done; N/A = Not applicable

Figure 1. Experimental design to assess fractional provirus expression



Adapted from Cillo et al, PNAS 2014

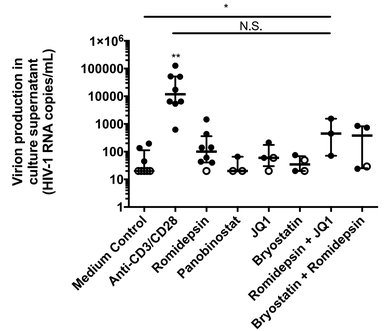
Proviruses per well	Number of Replicate Wells	Number of Wells with HIV-1 RNA >20 copies
791	10	10
263	10	10
88	10	9
29	10	2
10	10	0
3	10	0
1	10	0
0.5	10	0

Experimental design for fractional provirus expression (fPVE). **A.)** Resting CD4+ T cells are isolated for leukaphersis product,serially diluted and treated with LRAs of interest. Efavirenz (300nM) is included to prevent viral spread. **B.)** HIV-1 RNA is quantified in individual wells by Roche COBAS AmpliPrep TaqMan and results are tabulated **C.)** fPVE is then calculated by maximum likelihood estimate using R.

Table 2. Proposed mechanisms of action of LRAs

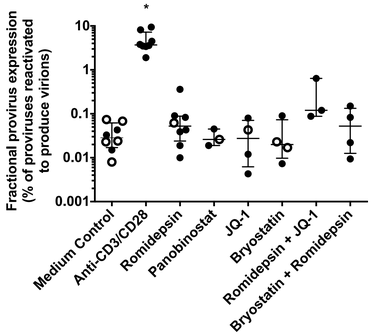
Compound	General compound class	Putative mechanism of latency reversal	Concentration and duration of *ex vivo* treatment
Romidepsin	Histone deacetylase inhibitor	Relaxation of chromatic structure surrounding HIV-1 long terminal repeat	40 nM, 4 hours
Panobinostat			17.5 nM, 30 minutes
JQ1	BRD4 inhibitor	Free P-TEFb for interaction with HIV-1 trans-activator of transcription	25 uM, 30 minutes
Bryostatin	Protein kinase C agonist	Activation of NF-kB and interaction with HIV-1 long terminal repeat	10 nM, 1 hour
Romidepsin + JQ1	Histone deacetylase inhibitor + BRD4 inhibitor	Relaxation of chromatic and free P-TEFb	40 nM, 4 hours + 25 uM, 30 minutes
Bryostatin + Romidepsin	Protein kinase C agonist + histone deacetylase inhibitor	Interaction of NF-kB with long terminal repeat and open chromatin strcuture	10 nM, 1 hour + 40 nM, 4 hours

Figure 2. Virion production following treatment with anti-CD3/CD28 and LRAs



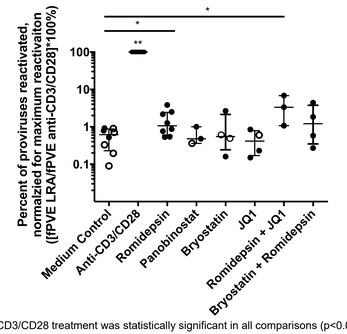
Anti-CD3/CD28 treatment led to significantly (p<0.025) more virions compared with all other treatments. Romidepsin + JQ1 led to significantly higher levels of virion production compared with medium control (p=0.042), and no other comparisons were significant. Open circles represent undetectable virion production.

Figure 3. Fraction of proviruses that have been reactivated to produce virions following treatment with anti-CD3/CD28 or LRAs



Anti-CD3/CD28 treatment led to a significant (p<0.013) increase in the fraction of proviruses responsible for virion production; not other comparisons were significant. Open circles represent samples with no detectable virion production; the limit of detection varies based on the number of proviruses present in the resting CD4+ T cell population.

Figure 4. Fraction of proviruses reactivated to produce virions, normalized by maximum fractional provirus expression



Anti-CD3/CD28 treatment was statistically significant in all comparisons (p<0.002). Both romidepsin treatment alone and romidepsin + JQ1 reactivated a significantly larger fraction of proviruses compared with medium control (p=0.05 and p=0.012, respectively); no other comparisons were significant. Open circles represent samples with no detectable virion production; the limit of detection varies based on the number of proviruses present in the resting CD4+ T cell population.

Conclusions

- **Fractional provirus expression can be used to determine the proportion of proviruses that have been reactivated with LRAs**
- **Current LRAs at clinically achievable concentrations can induce virus release, but reactivate only a small fraction of proviruses (<5%) compared with maximum stimulation (anti-CD3/CD28)**
- **More potent and effective LRAs are needed to reactivate a larger fraction of all inducible proviruses in resting CD4+ T cells from patients on long-term ART**

References

1. Archin *et al*, *Nature*, 2012.
2. Bullen *et al*, *Nature Medicine*, 2014.
3. Laird/Bullen *et al*, *Journal of Clinical Investigation, 2014.*
4. Cillo *et al*, *PNAS*, 2014

Acknowledgements

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