MAJOR ARTICLE

HIV-1 Expression Within Resting CD4$^+$ T Cells After Multiple Doses of Vorinostat

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Background. A single dose of the histone deacetylase inhibitor vorinostat (VOR) up-regulates HIV RNA expression within resting CD4$^+$ T cells of treated, aviremic human immunodeficiency virus (HIV)–positive participants. The ability of multiple exposures to VOR to repeatedly disrupt latency has not been directly measured, to our knowledge.

Methods. Five participants in whom resting CD4$^+$ T-cell–associated HIV RNA (rc-RNA) increased after a single dose of VOR agreed to receive daily VOR Monday through Wednesday for 8 weekly cycles. VOR serum levels, peripheral blood mononuclear cell histone acetylation, plasma HIV RNA single-copy assays, rc-RNA, total cellular HIV DNA, and quantitative viral outgrowth assays from resting CD4$^+$ T cells were assayed.

Results. VOR was well tolerated, with exposures within expected parameters. However, rc-RNA measured after dose 11 (second dose of cycle 4) or dose 22 (second dose of cycle 8) increased significantly in only 3 of the 5 participants, and the magnitude of the rc-RNA increase was much reduced compared with that after a single dose. Changes in histone acetylation were blunted. Results of quantitative viral outgrowth and other assays were unchanged.

Conclusions. Although HIV latency is disrupted by an initial VOR dose, the effect of subsequent doses in this protocol was much reduced. We hypothesize that the global effect of VOR results in a refractory period of ≥24 hours. The optimal schedule for VOR administration is still to be defined.

Keywords. vorinostat; HIV; latency; histone; acetylation; HDAC inhibitor.

Effective antiretroviral therapy allows human immunodeficiency virus (HIV)–infected individuals to live long and productive lives [1]. However, HIV infection remains incurable in part owing to the presence of quiescent, replication-competent provirus within a long-lived population of memory T cells, capable of reigniting new rounds of infection if therapy is interrupted. This latent pool of virus is established within days of infection and is refractory to the actions of the immune system or of current therapy [2–4]; thus, HIV infection will require lifetime treatment unless persistent, latent infection is purged.

After integration of HIV DNA into the cellular genome, the HIV long terminal repeat promoter exists in a nucleosome-bound conformation and is transcriptionally silent without stimulation of the infected cell. Studies by several laboratories have suggested that multiple mechanisms maintain the quiescent state of the HIV promoter [5–7]. Among the well-characterized factors that maintain latency are cellular histone deacetylases (HDACs) that deacetylate many cellular proteins, including histones. There are 4 classes of HDACs: class I includes HDACs 1, 2, 3 and 8; class IIa, HDACs 4, 5, 7 and 9; class IIb, HDACs 6 and 10; and class IV, HDAC 11 (class I is nuclear, and the

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others are cytoplasmic). The class III HDACs, sirtuins, are not regulators of HIV transcription and are not responsive to traditional inhibitors of HDAC activity [7, 8].

Multiple studies have shown that HDAC are recruited to the HIV promoter by transcription factor complexes and maintain the long terminal repeat in a transcriptionally silent state. HDACs 1, 2, and 3 mediate the repression of HIV transcription [9, 10]. When studied in cell line and primary cell models of HIV latency, HDAC inhibitors lead to acetylation of lysine residues on histone tails, an event associated with induction of transcription at the HIV promoter [11–13].

In a recent clinical trial, the administration of a single dose of the HDAC inhibitor vorinostat (VOR) to 8 participants receiving suppressive antiretroviral therapy resulted in an increase of HIV RNA within resting $CD4^+$ T cells, demonstrating for the first time disruption of HIV latency in vivo [14]. In this study design the pharmacokinetic profile of VOR was measured after an initial 400-mg dose, several weeks before a second, isolated dose of VOR. After this later dose, HIV RNA induction was specifically demonstrated within pools of resting, not total, $CD4^+$ T cells (hence the term *rc-RNA*). However, the ability of multiple doses of VOR to continuously disrupt HIV latency has not yet been clearly demonstrated.

In this study, we sought to determine whether administration of multiple doses of VOR would result in repeated induction of rc-RNA, in association with repeated increases in cellular acetylation, and whether such repetitive induction of bursts of viral gene expression for the latent proviral would be associated with a concurrent depletion of resting $CD4^+$ T-cell infection.

MATERIALS AND METHODS

Study Participants

All HIV-infected participants were receiving stable standard-of-care antiretroviral therapy with plasma HIV-1 RNA < 50 copies/mL and a $CD4^+$ cell count of >300/μL for ≥6 months before enrollment. Written informed consent was obtained from all participants before study enrollment. Approval was obtained from the University of North Carolina (UNC) institutional biomedical review board and the Food and Drug Administration, and the study was registered at ClinicalTrials.gov (NCT01319383). Five of the 8 participants described elsewhere [14] reenrolled in this follow-on study, in which they received 22 cyclical doses of VOR during a 12–16-week period.

The VOR dosing schema was empirically derived from oncology studies, with the goal of achieving maximal drug exposure with negligible I toxic effects. VOR is most often dosed at 400 mg/d, the dose that induced HIV expression in our original study [14]. Therefore, we elected to give daily VOR Monday through Wednesday for 4 weekly cycles, followed by a rest and evaluation period and then 4 more cycles.

Because of the limited number of leukapheresis evaluations that could be performed to precisely measure HIV RNA and replication-competent HIV within the circulating resting $CD4^+$ T-cell population, rc-RNA was assessed at the last dose of the fourth and eighth cycle of VOR administration, and the frequency of resting cell infection was reassessed at the end of the eighth cycle. Peripheral blood cells for research assays were obtained by leukapheresis performed at baseline before initiation of multiple-dose VOR administration and again 6 hours after doses 11 and 22 of VOR.

Analysis of Total Histone Acetylation

Peripheral blood mononuclear cells (PBMCs) were collected and viably frozen before and after VOR dosing. Cells were quickly thawed for batched assays by gentle addition of cold washing/staining solution (1× phosphate-buffered saline, 2% fetal bovine serum, and 0.09% sodium azide). Cells then washed and fixed with prewarmed BD Phosflow Fix Buffer (BD Biosciences; catalog No. 557 870) at 37°C for 10 minutes and then washed and permeabilized with ice cold BD Phosflow Perm Buffer II (BD Biosciences; catalog No. 558052) for 30 minutes. Cells were then washed and stained with anti-acetyl-histone 3 phycoerythrin-labeled antibody (Millipore; catalog No. FCABS325PE) or phycoerythrin-labeled rabbit immunoglobulin G antibody (Santa Cruz Biotechnology; catalog No. sc-3745) for 60 minutes, washed twice, and resuspended in 2% paraformaldehyde solution for analysis by flow cytometry (Cyan ADP Analyzer; Beckman Coulter) at the UNC Flow Cytometry Core Facility. Small lymphocytes, identified by forward and side scatter, were analyzed (Supplementary Figure 1).

Pharmacokinetic Analysis

Serum was collected for pharmacokinetic assays and stored at −70°C until analysis. The VOR concentrations were measured in human serum using a modification of the method of Musson et al [15]. Individual serum concentrations were used to estimate the following VOR pharmacokinetic variables: area under the curve for 0–24 hours, maximum concentration, and apparent terminal half-time. WinNonlin Phoenix (Certara) was used for the calculations. The apparent terminal half-time was estimated from the best fit of a single exponential to the log-linear portion of the serum concentration/time curve, using unweighted linear regression. The area under the receiver operating characteristic curve for 0–24 hours was calculated using the linear up/log down method. The maximum concentration and T_{max} were obtained by inspection of the concentration-time data [14].

Quantitative Viral Outgrowth Assay

Lymphocytes were obtained by continuous-flow leukapheresis and isolation of resting $CD4^+$ T cells. Recovery and quantification of replication-competent virus was performed as described elsewhere [16]. Briefly, approximately 50 million resting $CD4^+$ T cells

Table 1. Clinical Characteristics of Study Participants[a]

Characteristic	Patient 1	Patient 2	Patient 3	Patient 7	Patient 8
Treated during AHI	Yes	No			
Age, y	63	57	42	52	54
Duration of ART, y	6	10	3	13	27
Duration of viral suppression, y[b]	3	7	2	3	4
Lowest $CD4^+$ T-cell count recorded (cells/μL)	277	170	232	81	195
Peak plasma HIV-1 RNA recorded, copies/mL	84 545 454 (during AHI)	734	63 620	382 966	71 928
$CD4^+$ T-cell count at study entry (cells/μL)	705	473	476	747	1147

Abbreviations: AHI, acute HIV infection; ART, antiretroviral therapy; HIV, human immunodeficiency virus.

[a] All patients were white and male.

[b] Viral suppression is defined as <50 HIV RNA copies/mL.

were plated in replicate limiting dilutions of 2.5 million (18 cultures), 0.5 million (6 cultures), or 0.1 million (6 cultures) cells per well, activated with phytohemagglutinin (Remel), a 5-fold excess of allogeneic irradiated PBMCs from a seronegative donor, and 60 U/mL interleukin 2 for 24 hours. Cultures were washed and cocultivated with CD8-depleted PBMCs collected from selected HIV-seronegative donors screened for adequate CCR5 expression. Culture supernatants were harvested and assayed for virus production by p24 antigen-capture enzyme-linked immunosorbent assay (ABL). Cultures were scored positive if p24 was detected at day 15 and was increased in concentration at day 19. The number of resting $CD4^+$ T cells in infected units per billion was estimated using a maximum likelihood method [17].

Measurement of HIV RNA

In vivo response to VOR dosing was measured as described elsewhere, with minor modifications [14]. Immediately after leukapheresis, resting $CD4^+$ T cells were isolated and plated at 1 million cells/well, pelleted, snap frozen, and stored at −80°C. Total RNA was isolated from 36 replicates of 1 million resting cells using the Magmax 96 Total RNA isolation kit (Life Technologies) and following the manufacturer's protocol. Complementary DNA (cDNA) was synthesized in duplicate from DNase-treated, isolated RNA using the SuperScript III First-Strand Synthesis SuperMix kit (Invitrogen), according to the manufacturer's procedures. Reverse transcriptase was omitted from duplicate wells of each treatment condition, which served as controls for DNA contamination. Duplicate polymerase chain reaction (PCR) amplification of duplicate cDNA was performed using the Biorad FX96 Real-Time PCR machine and primers and probe published elsewhere [18]. A standard curve was generated for each PCR reaction, as described elsewhere [12]. Results of the 4 PCR replicates representing each of the original 36 pools of RNA were averaged, and the standard deviation was determined for each condition. The Wilcoxon rank sum test was used to calculate the statistical significance of changes within each participant.

Single-copy assays of plasma HIV-1 RNA were performed as described elsewhere [19]. For droplet digital PCR assays, RNA and DNA were isolated from participant PBMCs collected at baseline and at doses 11 and 22, using the AllPrep DNA/RNA Mini Kit (Qiagen). DNA was assayed for HIV content using primer/probe sets in HIV pol and for cellular DNA content using a target in the 30-kDa subunit of RNAse P, as described elsewhere [20]. Extracted RNA was reverse transcribed using a Bio-Rad iScript cDNA kit and assayed in parallel for targets in HIV Gag (primers SK431/SK462 and probe SK102 [21] and in the 3′ long terminal repeat of polyadenylated HIV-1 transcripts [22]. Copy numbers of HIV RNA were normalized to the total RNA input to the cDNA reaction, measured by absorption at 260 nm with a Nanodrop 2000 spectrometer (Thermo Scientific).

RESULTS

Clinical Outcome of Multiple Doses of VOR

Previously, HIV latency was disrupted in vivo by administration of a single 400-mg dose of VOR [14]. Five of the 8 participants reported in that study subsequently reenrolled in this follow-on study, in which they received 22 cyclical doses of VOR during a 12–16-week period. Because of the limited number of leukapheresis evaluations that could be performed to precisely measure HIV RNA and replication-competent HIV within the circulating resting $CD4^+$ T-cell population, rc-RNA was assessed at the last dose of the fourth and eighth cycle of VOR administration, and the frequency of resting cell infection was reassessed at the end of the eighth cycle. All participants were male and white, with a mean age of 54 years. Participants were all virally suppressed (<50 copies/mL) for ≥2 years with a mean $CD4^+$ cell count of 709 at study enrollment (Table 1).

More than 6 months after the single doses for pharmacokinetics and rc-RNA [14], a new baseline rc-RNA was measured and then participants self-administered 400 mg of VOR every 24 hours for 3 days, followed by 4 days with no VOR. This weekly cycle was repeated 4 times except on the 4th week

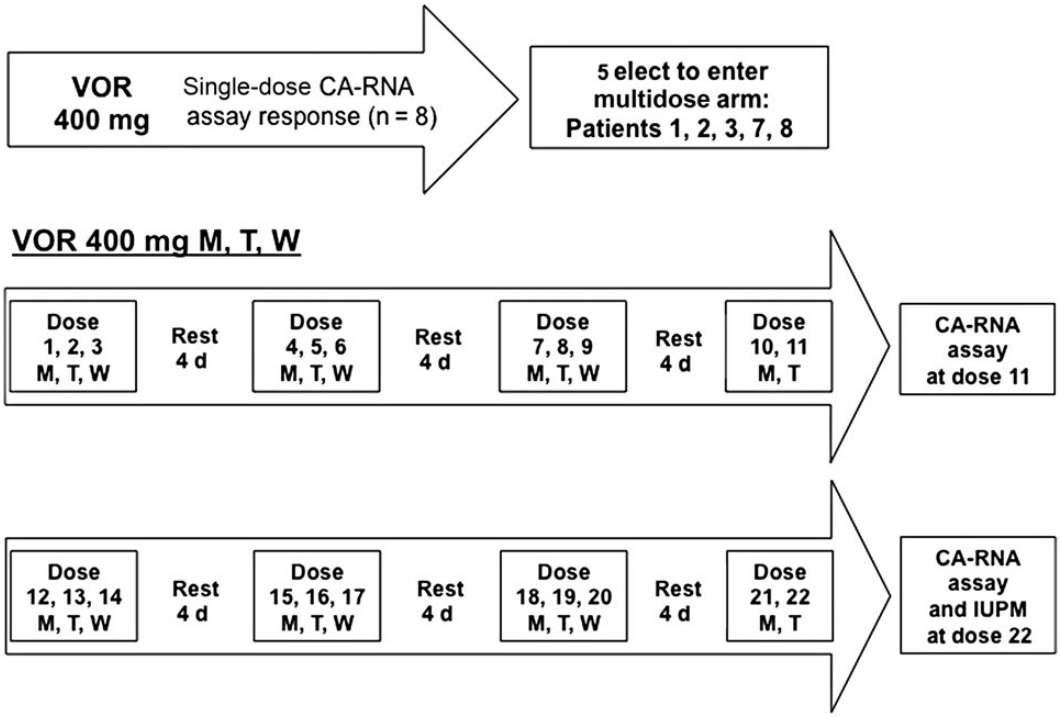


Figure 1. Protocol schema for multiple-dose vorinostat testing. Abbreviations: CA-RNA, cell-associated HIV RNA; IUPM, infected units per million; M, Monday; T, Tuesday; VOR, vorinostat; W, Wednesday.

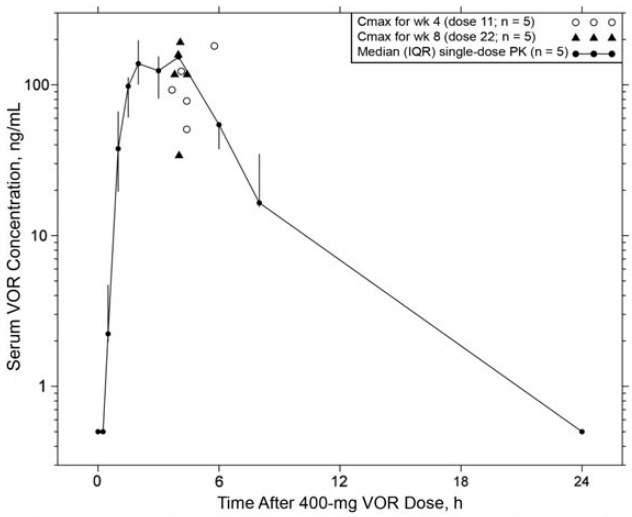
only 2 doses were given, for a total of 11 doses. Leukapheresis was performed 6 hours after the 11th dose. This was followed by a 4–8-week interval, and then identical subsequent cycles of VOR Monday through Wednesday with 4-day rest periods for an additional 11 doses (Figure 1). Leukapheresis was performed for the second time 6 hours after the 22nd dose. Antiretroviral therapy was never interrupted.

The dosing regimen was well tolerated by all participants except for some mild, transient gastrointestinal symptoms and headache, which did not approach grade I severity levels. All participants exhibited a 15%–35% decline in platelet counts by the fourth cycle of dosing, which recovered during the 4–8-week rest interval, and recurred during the 7th or 8th cycle of dosing. However, this transient thrombocytopenia reached grade I toxicity level during only a single visit for a single study participant. No other clinical events or drug-related toxic effects were observed.

Owing to the theoretical concern that systemic disruption of latent HIV infection might induce CNS toxic effects, resulting from an inflammatory or neurotoxic response in the brain, a neurocognitive battery of tests was administered just before multiple VOR dosing and at the end of the study. No significant changes were measured.

Multiple-Dose VOR Pharmacokinetics

To verify that drug exposure after multiple doses of VOR was uniform, as has been reported in oncology studies [23], pharmacokinetic analysis was performed on serum samples collected 4 and 24 hours after administration of dose 11 and 22 of VOR, and values compared to steady state levels obtained after administration of a single dose. As shown in Figure 2, exposure to VOR



	Patient 1	Patient 2	Patient 3	Patient 7	Patient 8
AUC, ng/mL <times> h	759	859	1190	614	808
C_{max}, ng/ML	215	245	301	273	243

Figure 2. Exposures to vorinostat (VOR) at weeks 4 and 8 are similar to single-dose exposure. Abbreviations: AUC, area under the receiver operating characteristic curve; C_{max}, maximum concentration; IQR, interquartile range; PK, pharmacokinetics.

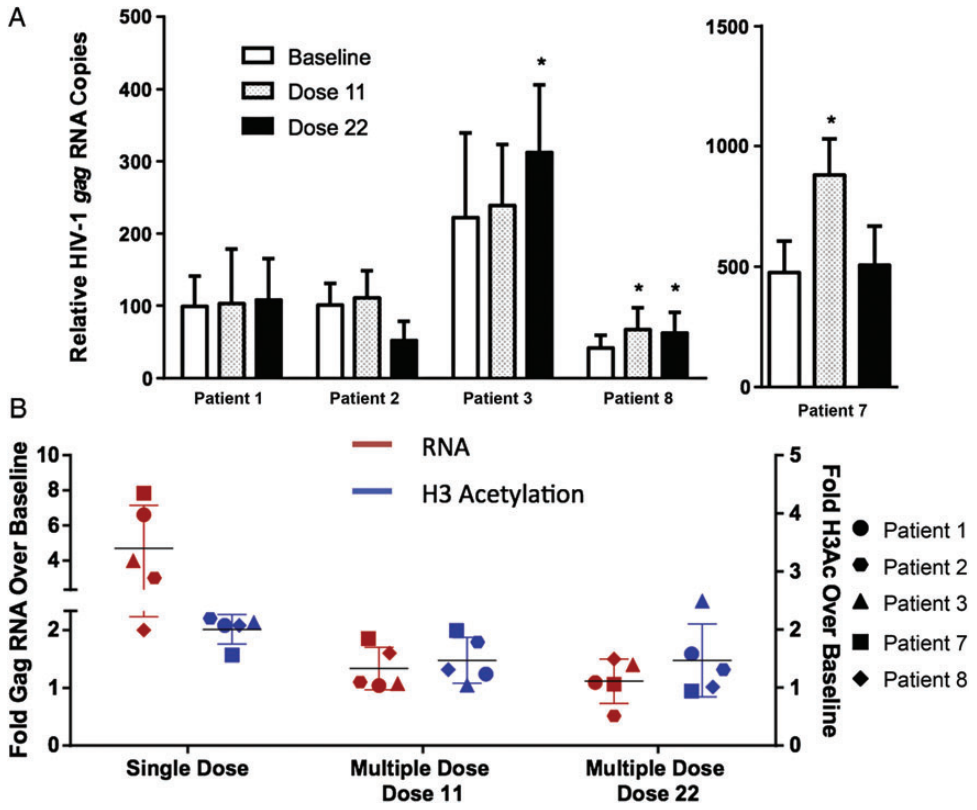


Figure 3. *A*, Relative human immunodeficiency virus (HIV) 1 *gag* RNA copies per million resting CD4$^+$ T cells at study baseline and after doses 11 and 22. *$P < .05$ (significant increase from baseline; Mann–Whitney test). *B*, Fold change from baseline of relative HIV-1 *gag* RNA copies per million resting CD4$^+$ T cells (*blue*) and total H3 acetylation in small lymphocytes (*red*) at baseline and after doses 11 and 22 (single-dose data from Archin et al [14]).

after multiple doses was similar to that observed after a single dose. Most of the observed concentrations after multiple dosing fell within the inter-quartile range of the first dose; not markedly dissimilar from values seen after single isolated doses (Figure 2).

Effect of Multiple Doses of VOR on rc-RNA and Histone Acetylation

To assess the efficacy of multiple doses of VOR on the CD4$^+$ T-cell reservoir, we measured the quantity of HIV-1 *gag* RNA in 36 pools of 1 million freshly isolated resting CD4$^+$ T cells at a baseline time point and after doses 11 and 22 of VOR, in parallel with flow-based measures of total cellular histone acetylation (Figure 3*A* and 3*B*). In contrast to the uniform increase of rc-RNA in each participant after a single isolated dose for VOR in the previous [14], rc-RNA was increased in this study only after both doses 11 and 22 for participant 8 and after 1 of the 2 doses evaluated in participants 3 and 7. Although rc-RNA did increase at ≥1 time point in 3 of 5 participants and at 4 of the 10 time points evaluated, in only 1 case was the magnitude of rc-RNA induction comparable to that seen in the previous study [14], when the dose evaluated was not closely preceded by an earlier VOR exposure. A signed rank test of the log-fold change of HIV RNA from baseline to dose 11 and from baseline to dose 22 for these 5 study participants showed a trend toward an increase at dose 11, which did not achieve statistical significance ($P = .06$), but no such trend at dose 22 ($P = .625$). However, the increase was >1.5-fold at only a single time point in a single participant (Figure 3*B*), whereas rc-RNA uniformly increased 1.5–5-fold in all participants after the initial doses of VOR [14]. In agreement with this observation, the fold induction of total cellular H3 acetylation was also reduced after doses 11 and 22, compared to that seen in the previous study [14].

As another measure of persistent HIV infection, we also measured total PBMC-associated HIV DNA and PBMC-associated HIV RNA by a droplet digital PCR method. Because these studies were performed in total PBMCs and not in sorted resting CD4$^+$ cells, and because of the different amplification techniques used, we could not directly compare these measures and resting CD4$^+$ T-cell rc-RNA values (given above). In PBMCs, however, no significant change was observed in HIV DNA or RNA from baseline to either dose 11 or 22 (Table 2).

Single-copy plasma HIV-1 RNA assays did not show increases in plasma viremia in temporal association with VOR dosing in most patients, but levels of viremia were low, and any changes could not be distinguished from biological or PCR variation in this small cohort (Supplementary Table 3). We also we

Table 2. Droplet Digital Polymerase Chain Reaction Assays of HIV RNA and DNA

Patient and Assay Timing	HIV-1 RNA, HIV Copies/µg Total RNA		HIV-1 DNA, HIV Copies/1 Million Cells
	PolyA Primers	Gag Primers	Pol Primers
Patient 1			
Baseline	23.6	31.6	135.3
Dose 11	17.1	22.5	140
Dose 22	13.4	14.6	116.8
Patient 2			
Baseline	14.5	9.4	12.2
Dose 11	23.6	28.4	7.5
Dose 22	23.1	30.6	21.2
Patient 3			
Baseline	4.1	24.8	290.8
Dose 11	8.6	33.5	182.9
Dose 22	6.3	24.6	285.5
Patient 7			
Baseline	73.9	108.8	575.2
Dose 11	98.4	219.2	314.5
Dose 22	79.7	136.8	788.9
Patient 8			
Baseline	0	16.7	36.6
Dose 11	0	20.3	45.7
Dose 22	0	19.6	57.8

Abbreviation: HIV, human immunodeficiency virus.

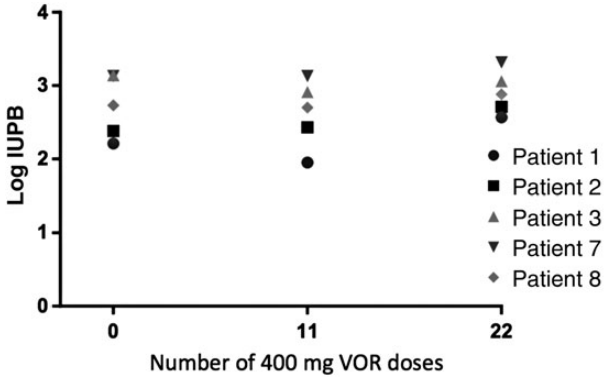


Figure 4. The frequency of replication-competent human immunodeficiency virus infection within resting CD4$^+$ T cells was measured at baseline and after doses 11 and 22. Abbreviations: IUPM, infectious units per billion; VOR, vorinostat.

evaluated the frequency of replication-competent latent HIV infection by performing quantitative viral outgrowth assay on resting CD4$^+$ T cells obtained at baseline and after doses 11 and 22 (Figure 4). No significant change in this measurement was observed.

DISCUSSION

Archin et al [14] first reported the disruption of HIV latency as measured by increased levels of resting CD4$^+$ T-cell–associated HIV RNA (rc-RNA) immediately after a single dose of VOR. Preliminary reports have been published of the effect of 14 daily doses of VOR [24] and thrice-weekly, every-other-week dosing of panobinostat [25], although the cell populations studied and assay methods differ. Given the strong evidence that VOR can disrupt HIV latency, our current study focused on measurements of the response to VOR after multiple prior VOR doses. Because of the resources necessary for this study, only 3 leukapheresis-based evaluations could be performed in each participant. In addition to assessing HIV RNA expression in resting CD4$^+$ cells, we also sought evidence of depletion of persistent HIV infection after durable VOR exposure, mindful of the evidence in a laboratory model system suggesting that the disruption of HIV latency might not lead to immediate clearance of the substantial majority of latently infected cells [26], as measured by currently available assays.

When used in oncology, VOR is generally dosed daily; twice-daily dosing is used in some settings [27]. Because total cellular histone acetylation had returned nearly to baseline levels in our initial study [14], we tested daily dosing with VOR, with rest intervals each week to minimize toxic effects. Participants received 8 weekly cycles of VOR, with a 4–8-week interval between cycle 4 and 5, to reduce the inconvenience of frequent required evaluations and to distribute the blood loss incurred by required phlebotomy. VOR given in this setting was very well tolerated, with only mild and reversible declines in platelet counts seen by cycles 4 and 8. No significant gastrointestinal toxic effects were seen. Although the evaluation is preliminary, we saw no effect of VOR on neuropsychiatric measures [28].

In contrast to the changes seen after an initial dose of VOR, in 5 participants who received a total of 22 400-mg doses of VOR during a 4-month period, cellular acetylation and rc-RNA were increased only minimally when measured shortly after doses 11 and 22. Given the complex modulation of numerous cellular genes by VOR and the fact that cellular acetylation remains modestly up-regulated 24 hours after VOR exposure, compensatory cellular effects that follow VOR administration may blunt subsequent response to additional VOR doses during the 24-hour dosing interval tested. We hypothesize that a longer interdose interval may be required to allow cellular mechanisms to be resensitized to HDAC inhibition and to observe robust and reproducible induction of latent HIV proviral expression. This result was unexpected, but there has been surprisingly little examination of the kinetic effects of HDAC inhibitors on cellular gene expression and protein modification or after multiple HDAC inhibitor exposures [29, 30].

A complex, multiphasic cascade of host gene expression is triggered after exposure to inhibitors. In primary $CD4^+$ T cells studied ex vivo after a single exposure to VOR, modest up-regulation was observed for 1000 genes, as well as down-regulation of 847 genes [29]. The downstream effects of these changes and how they affect the cellular and proviral response to a second exposure to VOR have not been studied. For example, one recent hypothesis holds that VOR induces HIV expression via the release of pTEF-b [31]. If this is so, pTEF-b mobilized by an initial exposure to VOR might not reaccumulate in time to allow a response to a second exposure within 24 hours.

HDAC inhibitors are currently leading candidates for use as therapeutic agents to attack latent HIV infection. There is a widely held expectation that eradication of HIV infection will require the use of combination therapy to efficiently disrupt latent infection and the use of vaccines or immunotherapy to assist in clearing latent HIV infection [32–34]. Our current findings illustrate that careful study of the effects of each intervention, and the interactions of these interventions, will be required to advance toward the ultimate goal of therapies that can cure HIV infection.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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