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The Histone Deacetylase Inhibitor ITF2357 Decreases Surface CXCR4 and CCR5 Expression on CD4+ T-Cells and Monocytes and is Superior to Valproic Acid for Latent HIV-1 Expression in Vitro

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Abstract

Objectives—Chromatin-associated repression is one mechanism that maintains HIV-1 latency. Inhibition of histone deacetylases (HDAC) reverses this repression resulting in viral expression from quiescently infected cells. Clinical studies with the HDAC inhibitor valproic acid (VPA) failed to substantially decrease the latent pool within resting CD4+ cells. Here we compared the efficacy of ITF2357, an orally active and safe HDAC inhibitor, with VPA for HIV-1 expression from latently infected cells in vitro. We also evaluated the effect of ITF2357 on the surface expression of CXCR4 and CCR5.

Methods—Latently infected cell lines were incubated with either ITF2357 or VPA and p24 levels were measured. Peripheral blood mononuclear cells of un-infected donors were treated with ITF2357 and HIV-1 co-receptors expression was assessed by flow cytometry.

Results—At clinically relevant concentrations, ITF2357 increased p24 by 15-fold in ACH2 cells and by 9-fold in U1 cells whereas VPA increased expression less than 2-fold. Analogues of ITF2357 primarily targeting HDAC-1 increased p24 up to 30-fold. In CD4+ T-cells treated with ITF2357, CXCR4 expression decreased by 54% ($P<0.001$).

Conclusion—ITF2357 is superior to VPA in inducing HIV-1 from latently infected cells. Safely used in humans, ITF2357 is an attractive candidate for HIV-1 clinical purging.

Keywords

HIV-1; latency; histone deacetylase inhibitors; CCR5; CXCR4

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Introduction

Since the introduction of highly active anti-retroviral therapy (HAART) more than a decade ago, HIV-1 infection can be well controlled, with HIV-1 viremia below detectable levels. However, eradication of HIV-1 infection with prolonged HAART therapy is still not feasible. Following discontinuation of HAART, rebound viremia occurs, typically after two weeks [1-4]. The source of viral re-emergence is from a long-lived pool, most likely the latently infected memory T-cell reservoir, harboring integrated HIV-1 proviral DNA [2]. Well established techniques are available to quantify the latent pool and it is estimated that one in every million memory cells of an HIV-1 positive patient bears a replication competent integrated provirus[5, 6].

The latent reservoir of HIV-1 within resting CD4+ cells is established early after acute infection and the initiation of HAART during this period does not prevent its establishment [6-8]. The latent pool is an extremely stable reservoir, having a half-life of 6-44 months even in treated patients who are continuously aviremic for long periods of time[6, 9-12]. Having this prolonged half-life, a complete decay of the reservoir is not expected before 70 years of treatment making eradication improbable. These time frames might be somewhat shorter by starting HAART early during acute infection or by intensifying HAART but not sufficient as a practical method for eradication [13, 14].

Mechanisms that maintain the proviral DNA transcriptionally inactive in the quiescent cells (see review in [15, 16]) include chromatin-associated regulation. In the latent cell, the integrated proviral DNA is densely organized in nucleosomes. The HIV-1 5′ long terminal repeat (LTR), containing the promoter and enhancer elements, binds several transcription factors and is arranged in two nucleosomes (nuc-0 and nuc-1)[17]. The NFκB p50 homodimer, as well as AP-4, YY1 and LSF1 recruit histone deacetylase (HDAC)-1 to the LTR, which in turn results in deacetylation of local histones, compaction of the chromatin and prevention of RNA polymerase-II binding[18-21]. In vitro studies have demonstrated that activation of the latent cell pool by different stimuli would reverse the repressive effect of the p50 homodimer-HDAC-1 complex by the binding of cytosolic NFκB p50-RelA heterodimer [19, 22]. This would enable the recruitment of histones acetyltransferase (HAT), acetylaton of the local histones, relaxation of the chromatin and initiation of transcription [19, 23, 24].

Inhibition of the enzymatic activity of HDAC-1 and likely HDAC-2 and 3 by synthetic inhibitors of HDACs (HDACi) leads to activation of the HIV-1 LTR and HIV-1 gene expression. Moreover, unlike cell activators of NFκB, such as IL-2, OKT3 or TNFα, HDACi facilitate gene expression without general activation cytokines and the T-cell[25, 26].

In vitro, various HDACi induce HIV-1 gene expression from latently infected cells line[27-29]. Valproic acid (VPA), a carboxilate HDACi prescribed for seizures and psychiatric disorders, has been combined with HAART in small clinical trials but without the desirable significant decrease of the latent reservoir [26, 30-32]. The studies with VPA, a non-specific weak HDACi, have not resolved the potential of HDACi to purge the virus.

ITF2357 is a hydroxamic acid-containing HDACi that has anti-inflammatory and anti-tumor properties in vitro and in vivo[33-36]. At therapeutic plasma levels of 125-250nM, there is no cell-toxicity in vitro and only minor reversible thrombocytopenia occurs in patients[37]. As an anti-inflammatory agent, twelve weeks of daily ITF2357 has been given to children with systemic onset juvenile idiopathic arthritis (SoJIA) with no safety issues and promising clinical improvement [38]. Since ITF2357 was shown to be a potent anti-inflammatory drug that is effective in nano-molar concentrations for cytokines suppression, we hypothesized it would be a potent stimulator of HIV-1 gene expression in latently infected cell lines. We

also examined the effects of three analogues of ITF2357, with a higher affinity and specificity for HDAC-1. Because of the importance of the chemokine co-receptors for HIV-1 cell entry, we evaluated surface expression of CCR5 and CXCR4 on primary human mononuclear blood cells.

Materials and Methods

Reagents and cell lines

ITF2357 and analogues were synthesized by the chemical department of Italfarmaco (Cinisello Balsamo, Italy). VPA and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). RPMI 1640, FCS and penicillin/streptomycin were purchased from Cellgro (Manassas, VA). ITF2357 and analogues were first dissolved in DMSO and then further diluted in RPMI (final concentration of DMSO was 0.01%).

The U1 and ACH2 cell lines were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD. Cells were cultured in flasks, washed in RPMI and resuspended in RPMI/10% FCS to a concentration of 2×10^6 cells/mL. 250μL of cells, 200μL of media and 50μL of HDACi/media containing 0.01% DMSO were aliquoted into 48-well polystyrene tissue culture plate (Falcon, Lincoln Park, NJ). After 24 hours of incubation at 37 °C/5%CO_2, 50μL of supernatant were removed for lactate dehydrogenase (LDH) cytotoxicity assay and Triton-X-100 (0.5% vol/vol final concentration) was added to each culture. p24 assays of lysates were done immediately.

Electrochemiluminescence (ECL) assays

p24 was measured using specific antibodies immobilized on magnetic beads as described previously [39].

Cytotoxicity assay

Acute cytotoxicity was determined using LDH Cytotoxicity Assay Kit II according to manufacturer's instructions (Vision, Mountain View, CA). In some experiments, cytotoxicity was verified using Cell-titer 96 Aqueous One Solution Cell Proliferation Assay kit according to manufacturer's protocol. (Promega, Madison, WI).

HDAC inhibition assay

Human recombinant enzymes were purchased from BPS Biosciences (Torrance, CA). Class I isoforms HDAC1, 2, 3; Class IIb HDAC6, 10 and Class IV HDAC11 were tested using the synthetic fluorogenic substrate Fluor-de-lys (Enzo Life Sciences, Plymouth Meeting, PA). Class IIa isforms were tested using the derivative of Boc-L-Lys-MCA (TFAL), described as specific substrate for these enzymes[40]. Assay of human recombinant HDAC8 was performed using HDAC8 Fluorimetric Drug Discovery Kit (Enzo) according to manufacturer's instructions. Each inhibitor was dissolved in DMSO and then further diluted in assay buffer. Concentrations of DMSO less than 0.5% did not affect the activity of the assay. The assays were performed by pre-incubating each enzyme with the inhibitors for 15 minutes at 37°C. The reaction was initiated by adding the substrate at 37°C and allowed to proceed for 60 minutes. The fluorescent signal was generated by adding 50μL of a 2-fold concentrated developing solution (Enzo) containing 4μM trichostatin A. The fluorescence generated was detected at wavelengths 355nm (excitation) and 460nm (emission).

Preparation of peripheral blood mononuclear cells (PBMC)

These studies were approved by the Colorado Multiple Institutional Review Board. Blood was taken from healthy HIV-1 negative human subjects according to previously described methods.[41]

Flow cytometry analysis of cell surface molecules

PBMC were incubated with ITF2357, VPA or vehicle media (containing the same concentrations of 0.01% DMSO). After incubation, cells were washed twice with PBS/1%BSA, incubated for 15 minutes with FcR-Binding Inhibitor (eBioscience, San Diego, CA), and incubated for 30 minutes at 4°C with monoclonal antibodies as follows: anti-CD4 (Per-CP; R&D Systems, Minneapolis, MN), anti-CD3 (FITC; R&D Systems), anti-CXCR4 (APC; BD, Franklin Lakes, NJ), anti-CCR5 (PE; BD), anti-CD14 (eFluor 450; eBioscience). Appropriate fluorescence minus one (FMO) (T-cells) or isotype antibodies (monocytes) were used in each experiment. Cells were fixed in 2% formaldehyde. Flow cytometric analysis was performed using LSR-II (BD). Lymphocyte gating was based on forward and side scatter and was further analyzed to identify CD3+ CD4+ cells on which CCR5 and CXCR4 expression were determined. Monocytes were identified by CD14+ expression. All experiments were done in duplicate and at least 200,000 events were collected. Whole blood was diluted 1:4 in RPMI and incubated with HDACi or media alone. After incubation blood was washed twice with PBS/1%BSA/0.02% sodium azide. 100μl of each sample was stained and fixed as described above. Data analysis was done using FlowJo (TreeStar, Ashland, OR).

RNA isolation and real-time polymerase chain reaction

RNA was isolated using mirVana kits (Ambion, Austin, TX), followed by determination of RNA concentrations using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). Reverse transcription and TaqMan real-time polymerase chain reactions (RT-PCRs) were then performed using the following primers and probes: CXCR4, β-Actin and GAPDH. All RT-PCR reagents and devices were by Applied Biosystems (Foster City, CA). Relative quantities were calculated by the ΔΔCT method as described previously [42].

Statistical analysis

Differences in the percent of cytotoxicity and co-receptors expression were analyzed using the non-parametric Mann-Whitney unpaired test. All reported *P* values were two tailed. Statistical analyses were conducted using GraphPad Prism software.

Results

Comparison of VPA and ITF2357 in latently infected HIV-1 cell lines

We compared the ability of VPA and ITF2357 to induce the expression of HIV-1 in a dose response study that included the plasma concentrations of each HDACi achieved in humans. As shown in Figure 1A, after 24 hours of incubation, ACH2 cells responded to VPA with a doubling of p24 at 1mM and an 8.7-fold increase at 2mM; however, these plasma concentrations of VPA are often toxic in humans. Upon 24 hours of incubation of ACH2 cells with ITF2357, a two-fold increase was observed at 125nM whereas there was a 15-fold increase at 250nM. Unlike VPA, these levels of HIV-1 expression at 250nM ITF2357 are at concentrations sustained in humans without side effects. As shown in brackets of Figure 1A, a mean therapeutic concentration of ITF2357 is 200nM and 0.25-0.6mM (40-100 μg/mL) for VPA.

We then measured the effect of ITF2357 and VPA in U1 cells. As shown in Figure 1B, mean levels of p24 were 0.9, 1.3, 2.7 and 9.1-fold higher than control cultures at ITF2357 concentrations of 31, 62, 125, 250nM, respectively. VPA at 0.25, 0.5, 1, 2mM dose-dependently increased p24 production by 0.9, 1.2, 1.8 and 5.5 fold. Similar to the data in ACH2 cells, VPA at clinical relevant concentrations (indicated in brackets) did not double the levels of p24. In contrast, ITF2357 increased HIV-1 production by nearly 3-fold at 125nM and 9-fold increase was observed at 250nM.

In order to ascertain that the stimulation of HIV-1 expression by either ITF2357 or VPA was due to stimulation of HIV-1 expression by HDAC inhibitors and not due to cell stress, LDH cytotoxicity assays were performed. The numbers above each error bar in Figures 1A indicate the mean fold change in cell death compared to control cultures set as 1.0. In ACH2 cells, ITF2357 concentrations of 31, 62, 125 and 250nM increased LDH levels by 1.1, 1.0, 1.0 and 1.2 fold, respectively. At VPA concentrations of 0.25, 0.5, 1, and 2mM, the mean percent cytotoxicity was different by 1.2, 1.1, 0.9 and 1.2 fold, respectively. None of these values was significantly higher than the mean cell death of the control cultures. Similarly, levels of cell death in U1 cells were not significantly different from untreated cultures.

Comparison of time-dependent stimulation of HIV-1 by VPA and ITF2357 in ACH2 cells

In clinical trials, the total daily dose of ITF2357 is 1.5mg/kg administered in two divided oral doses; the daily dose of VPA is 15mg/kg in three divided oral doses. Therefore, we investigated the effect of ITF2357 and VPA at different time points. Cultures were incubated for either 6, 12 or 24 hours with either ITF2357 or VPA. As shown in Figure 2, after 6 hours of exposure to VPA, there was no induction of p24 at any concentration. On the other hand, ITF2357 at 250nM increased p24 levels 1.9-fold over control levels (set at 1 for 6 hours). After 12 hours of incubation, there was no increase with any concentration of VPA compared to a 3.4-fold increase at 250nM of ITF2357. Lower concentrations of ITF2357 did not show increase in p24 after 12 hours of incubation. By 24 hours, there were increases comparable to those shown in Figure 1A for ITF2357 and VPA.

Effect of ITF2357 analogues on HIV-1 expression

Since ITF2357 was more effective at inducing HIV-1 expression than VPA at clinically relevant concentrations, we evaluated three hydroxamic acid-containing analogues. Table 1 compares the mean concentration of ITF2357, three analogues of ITF2357 as well as VPA and suberoylanilide hydroxamic acid (SAHA), to inhibit 50% of the enzymatic activity of different HDACs. We next compared the effect of increasing concentrations of each analogue on p24 production from ACH2 cells. As shown in Figure 3A, each of the three analogues induced higher levels of HIV-1 compared to the same concentrations of ITF2357. For example at 125nM, there was a mean increase of 2-fold by ITF2357 whereas two analogues (ITFa and ITFc) resulted in nearly 10-fold increases. At 250nM, these two analogues induced 27 and 35-fold increases, respectively, compared to a 15-fold increase by ITF2357. The higher level of HIV-1 expression was observed with analogues that exhibited the greatest potency in inhibiting the Class-I HDAC inhibitors: HDAC-1, HDAC-2 and HDAC-3. As shown in Figure 3A, cell death as determined by LDH activity after 24 hours was not significantly different in cells exposed to the analogues compared to untreated cells.

In U1 cells, the three analogues induced similar increases in p24 to that of ITF2357. For example, as shown in Figure 3B, levels of p24 were 8.4-fold for ITFa at 250nM compared to 10.1-fold for ITF2357. At the same concentration, ITFb and ITFc yielded increases of 9.2 and 11.6 fold. As in ACH2 cells, cytotoxicity was measured in each experiment and is shown above error bars (Figure 3B). In fact, each analogue resulted in less cytototoxicity compared to ITF2357 but these differences did not reach statistical significance.

Expression of CXCR4 on CD4+ T-cells

PBMC from 7 healthy donors were incubated in the presence of ITF2357, VPA or media. After 4 and 24 hours, the surface expression of HIV-1 co-receptors was evaluated. We observed no changes in CCR5 surface expression on CD3+ CD4+ cells, as measured by both MFI as well as percent of positive cells. Moreover, as shown in Figure 4A and 4B, at therapeutically relevant concentrations of ITF2357 of 125nM and 250nM and of VPA at 0.5mM there was a dose-dependent decrease in the MFI of CXCR4. At 4 hours, a decrease of 47% ($P<0.001$) and 54% ($P<0.001$) was observed for 125nM and 250nM, respectively. At 24 hours, MFI ± SEM levels decreased by 32% (P=0.011) and 42% (P=0.002) for 125nM and 250nM of ITF2357, respectively. In unfractionated whole blood, we observed a similar trend in co-receptors expression in response to ITF2357 (data not shown). Consistent with the reduction of surface CXCR4 expression, steady-state mRNA levels of CXCR4 after 2 and 4 hours, as measured by RT-PCR, were reduced by 65% in cultures treated with 250nM of ITF2357 compared to control cultures (data not shown).

Expression of CCR5 on monocytes

As shown in Figure 5A and 5B, gating on CD14+ monocytes after 24 hours revealed a markedly decreased expression of CCR5 at both 125nM and 250nM of ITF2357 (MFI±SEM of 595 ±191, $P=0.02$ and 357 ±153 $P=0.007$, respectively). These changes were not observed with VPA at clinically relevant concentrations. We also studied the effect of ITF2357 on CCR5 gene expression in PBMC of three donors. PBMC were stimulated with LPS (100ng/mL) in the absence or presence of ITF2357 (100nM). After 4 hours, mRNA was obtained and gene expression was determined by Affimetrix chip analysis. Compared to LPS only, there was a 4.3-fold reduction in LPS-stimulated CCR5 gene expression ($P<0.01$).

To demonstrate that the decrease in co-receptor surface expression was not due to cell death, levels of LDH were measured in the supernatants of PBMC. There was no increase in LDH released from cultures exposes to ITF2357 or VPA after 4 and 24 hours compared to control. Moreover, Annexin V and PI staining of PBMCs or isolated monocytes treated with up to 300 nM of ITF2357 was not different to that of control cultures[36]. In addition, surface expression of CD3 and CD4 did not change in response to HDAC exposure. We conclude that the decrease in co-receptor expression is specific for ITF2357 and not due to cell stress.

Discussion

The reservoir of latently infected memory CD4+ T-cells represents a major obstacle in eliminating HIV-1 infection. Although consistent with reversing viral latency, HDAC inhibitors have not been fully exploited to purge the virus from the quiescent pool. In the present report, we demonstrate that ITF2357 exhibits a greater induction of HIV-1 than VPA, the only HDAC inhibitor that has been evaluated in vivo for HIV-1 purging. ITF2357 increased p24 production in a macrophage as well as T-cell line. Based on the present study, we propose that ITF2357 would be superior to VPA in clinical trials. Clearly, the next step is to compare viral induction of resting CD4+ T-cells from aviremic HIV-1 positive donors when exposed to ITF2357 or VPA in vitro.

There are three major advantages of ITF2357. First, at clinically achievable blood levels, ITF2357 induces at least a 10-fold increase in HIV-1 from latently infected cell in vitro compared to less than 2-fold for VPA. Second, oral ITF2357 is well tolerated in humans, including children[38]. Third, ITF2357 did not increase CCR5 surface expression on CD4+ T-cells, despite the fact that HDAC inhibitors often increase gene expression. It would be counter-productive if agents stimulating latent virus up-regulated CCR5 and CXCR4 surface expression. In fact, surface expression of CXCR4 on CD4+ T-cells and CCR5 on monocytes

was reduced by 50% by ITF2357. Although downregulation of CXCR4 by HDAC inhibitors has been observed in leukemic cells [43], this property of ITF2357 was unexpected in primary blood monocytes and T-cells from healthy donors. Consistent with decreasing co-receptors surface expression, ITF2357 reduced steady-state mRNA levels of CXCR4 and CCR5 in PBMC, as measured by RT-PCR and also by gene chip expression, respectively. To our knowledge, this is the first time that this inhibitory role of HDAC inhibitors on CD4+ T-cells and monocytes has been demonstrated

One possible explanation for the decreased co-receptor gene expression is that ITF2357 directly inhibits transcription. Another possibility is that ITF2357 up-regulates transcriptional repressors. Although several pro-inflammatory cytokines such as tumor necrosis factor-α, interferon-γ, and interleukin-1β negatively regulate CXCR4 transcription[44], a cytokine-mediated mechanism is unlikely since ITF2357 decreases the synthesis of these cytokines induced by Toll-like receptor ligands in PBMCs[36].

Clinically, the cell surface density of co-receptors has a major role in HIV-1 infection. CCR5 density on CD4+ T-cells is an important driver of R5 HIV-1 replication [45] and positively correlated with viral load [46]and disease progression[47]. The density of CCR5 can also determine the susceptibility of macrophages and monocytes to infection by R5 strains[48, 49]. The emergence of X4 strain is associated with disease progression and a rapid decline in CD4+ T-cells [50] and a link between CXCR4 density and X4 emergence has been suggested. There is an ongoing issue whether residual viremia in HIV-1 patients under HAART represents new cycles of viral replication[13] or virus output from stable reservoirs [51]. These studies are based on viremia under steady state conditions. However, although treated, HIV-1 patients often have transient rebounds of viremia. Therefore, the reduction in co-receptor surface expression by ITF2357 is valuable if added to the HAART regimen.

Attractive as the concept of HIV-1 purging by HDAC inhibitors is, clinical trials using VPA did not reduce the size of the latent pool within resting CD4+ T cells. As the probability of side effects and particularly severe thrombocytopenia increases significantly at VPA plasma concentrations above 110μg/mL in females and 135μg/mL in males (see [52]for review). To induce more viral expression with higher concentrations of VPA would be impractical. As shown in the present study, ITF2357 is significantly more effective than VPA in inducing HIV-1 production from U1 and ACH2 cells. Another hydroxamic acid containing HDACi, SAHA, is also more potent than VPA for HIV-1 induction in vitro[27]. The comparison of HDAC inhibition between ITF2357 and SAHA is shown in Table 1. In unpublished data from our laboratory, ITF2357 is 50-100 times more potent than SAHA in HIV-1 expression from U1 and ACH2 cells. Also, we describe here that two hydroxamic acid-containing analogues of ITF2357, ITFa and ITFc, at comparable nanomolar concentrations, induced p24 to higher levels compared to the parent compound.

HDACs are divided into 3 major classes. Class I is comprised of HDAC-1, 2, 3 and 8. Class II is comprised of HDAC 4, 5, 6, 7 and 10. HDAC-11 has properties of both class I and class II. The class III HDAC are not affected by HDAC inhibitors. Various pharmacological agents such as pyrrole-imidazole polyamides, trichostatin A, SAHA or phorbol esters effectively prevent the recruitment of HDAC-1 to the HIV-1 promoter allowing hyperacetylation of H3 and H4[23, 53, 54]. In addition, a link between HDAC-2 and HDAC-3 and the HIV-1 LTR has been suggested[55, 56]. As shown in Table 1, we compared the ability of four hydroxamic acid-containing HDAC inhibitors to reduce the enzymatic activity of HDACs by 50%. In addition, the ability of VPA to inhibit the same HDACs was determined in the same assays. In each case, the IC50 of each the four hydroxamic acid-containing HDAC inhibitors and VPA were compared to induction of HIV-1 in U1 and ACH2 cells.

In general, there was greater induction of HIV-1 expression with analogues with the lowest IC50 for Class I HDACs 1, 2 and 3. These observations are consistent with the molecular association between the HIV-1 LTR and HDACs 1, 2 and 3. In contrast, VPA is impressively a weak inhibitor of each HDAC compared to ITF2357 and analogues (Table 1). In fact, the IC50 for VPA for HDAC inhibition is not achievable clinically and therefore not surprisingly, VPA failed to efficiently induce HIV-1 expression in vitro in the present study. Thus testing the hypothesis of HDAC inhibition for HIV-1 purging from latently infected cells should be re-visited using HDAC inhibitors such as ITF2357 or its analogues with specificity and potency for class I HDACs.

Consistent with the present observations, Archin et al demonstrated that HDAC inhibitors specifically targeting class I HDACs induced HIV-1 gene expression from cell lines and resting CD4+ T cells of aviremic patients[57]. An additive effect was seen with inhibition of HDAC-6. Our findings support those data regarding the selectivity of the class I HDACs 1, 2 and 3 inhibition and the potency of induction of HIV-1 gene expression. For example, in ACH2 cells, ITFc, a specific inhibitor of HDAC-1, 2 and 3 was twice as active in inducing p24 (35 fold) compared to ITF2357 (15-fold). However, inhibiton of HDAC-6 is inconsistent with the effectiveness of the three analogues, which were poor inhibitors of HDAC-6, particularly ITFc. Importantly, preserving the deacetylated status of HDAC-6 might protect CD4+ cells from HIV-1 infection and Env-mediated syncytia formation[58].

In the study by Archin et al, out of four HDAC inhibitors that were active in inducing HIV-1 gene expression in cells lines, only two recovered replication competent virus from resting CD4+ T cells of infected patients. We propose that from the data of the present study, ITF2357 or its analogues would be effective inducers of virus outgrowth from resting CD4+ cells of aviremic HIV-1 infected patients. The analogues have minor antiproliferative properties, which Archin et al suggested may interfere with maintaining the cell line in vitro potency in resting CD4+ of infected patients. However, since cell lines do not reflect the status HIV-1 of resting CD4+ T cells, measuring recovery of replication competent virus from these cells would clearly be necessary.

Safety is always a consideration when evaluating a drug in a disease with no immediate danger to the patient. In testing the hypothesis that HDAC inhibition will purge the latent pool of HIV-1, VPA falls short due to a low level of expression compared to ITF2357. Oral ITF2357 is safe and effective in humans. In healthy human subjects in a Phase I trial, a single dose of ITF2357 of 1.5mg/Kg resulted in a peak plasma level of 200nM [37]. In a Phase II trial in children with active SoJIA, a daily oral dose of ITF2357 at 1.5 mg/kg for 12 weeks exhibited no organ toxicity and achieved significant ($p<0.01$) reduction in parameters of systemic disease as well as the number of painful joints [38].

In conclusion, this present study demonstrates that at clinically relevant doses, ITF2357 induces HIV-1 production from latently infected cell lines, and at the same time reduces the MFI of HIV-1 entry co-receptors. For targeting clearance of HIV-1 infection, ITF2357 might be an effective addition to the HAART regimen of HIV-1 patients.

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SM., CAD, PM, GF and MFN designed the study. SM, GF, AF, AK and MFN performed the experiments. SM, CAD, PM, and GF wrote the manuscript. BEP, SM and AK analyzed the flow cytometry.

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SM and AF performed the statistical analysis.

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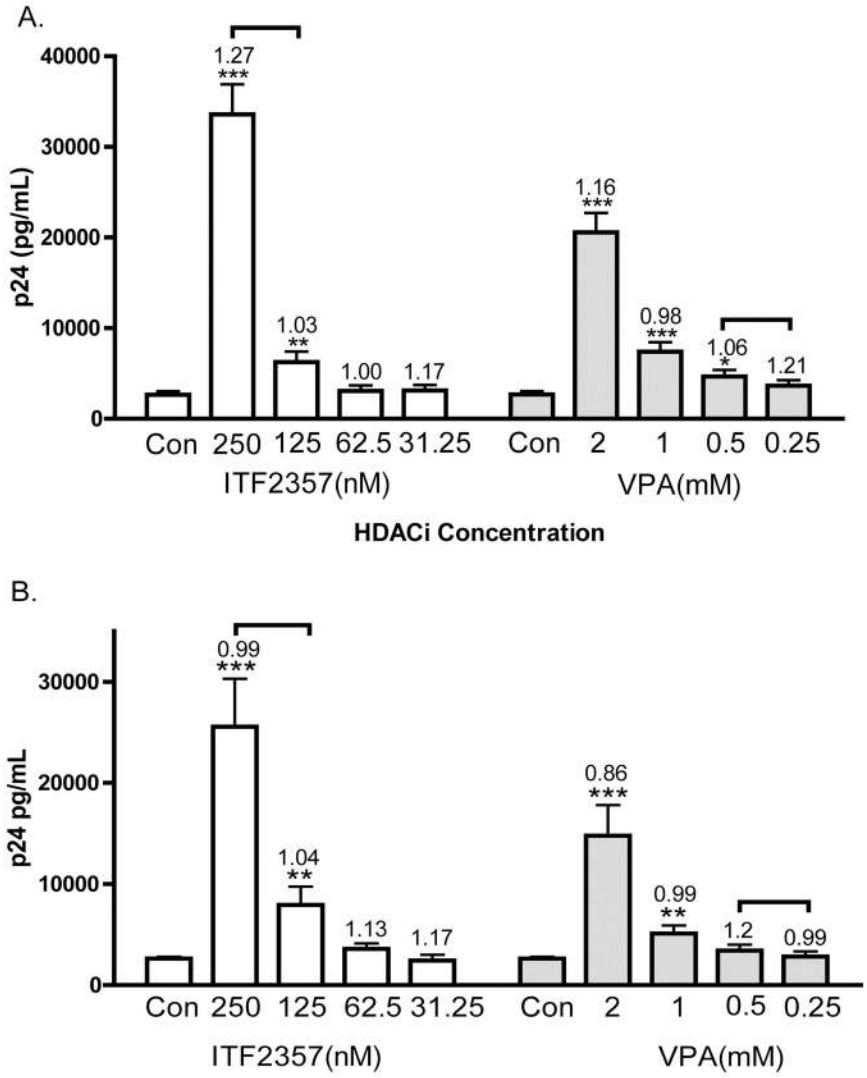

Figure 1. HIV-1 expression in ACH2 and U1 cells stimulated by ITF2357 or VPA

(A) Mean ±SEM p24 pg/mL in ACH2 cells of 20 separate experiments. (B) Mean ±SEM p24 pg/mL in U1 cells of 22 separate experiments. Numbers above error bars indicate the mean fold change of cell death as determined by LDH cytotoxicity assay. The levels of LDH for each experiment without HDAC inhibitors were set at 1.0 and fold increases calculated. The brackets above the error bars indicate the range of therapeutic plasma levels for each HDAC inhibitors.

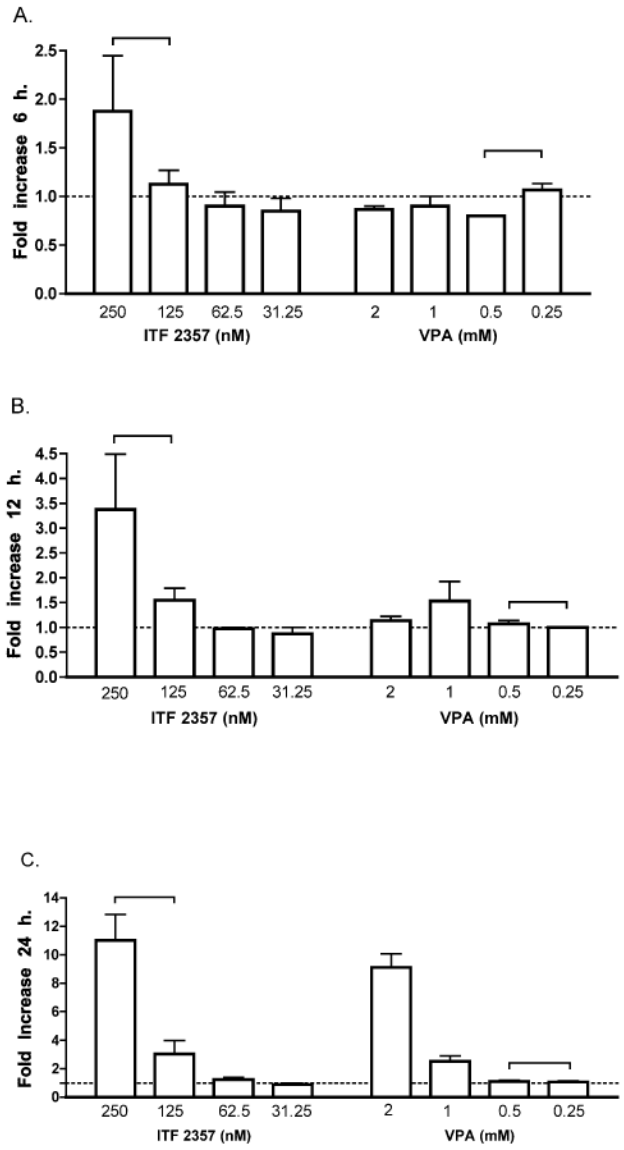

Figure 2. Time-dependent HIV-1 production in ACH2 cells exposed to VPA or ITF2357

Mean ±SEM fold-increase in levels of p24. The level of p24 for each experiment without HDAC inhibitors was set at 1.0 and fold increase values calculated. All experiments were performed in triplicate in three independent experiments. Dashed line represents control cultures set at 1.0. The brackets indicate the range of therapeutic plasma levels for each HDAC inhibitor. (A) p24 fold increase after incubation of 6 hours with either ITF2357 or VPA. (B) p24 fold increase after incubation of 12 hours. (C) p24 after 24 hours of incubation.

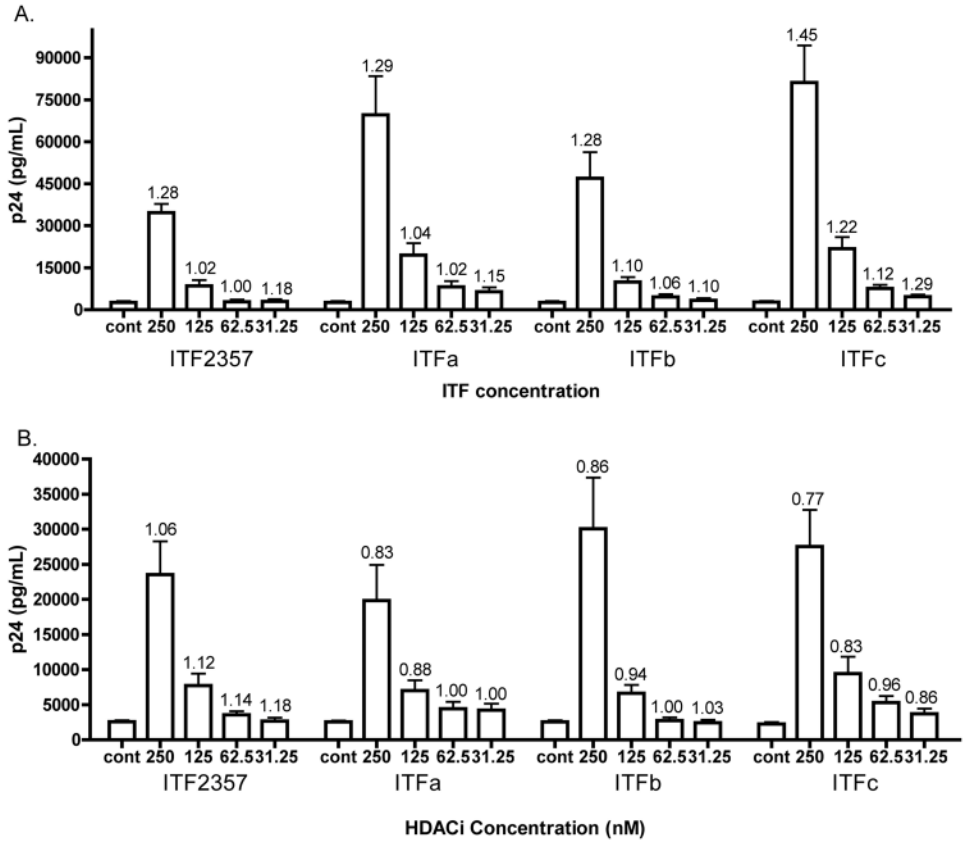

Figure 3. HIV-1 expression in ACH2 and U1 cells by ITF2357 and three analogues

p24 (A) Mean ±SEM p24 in pg/ml/mL in ACH2 cells of 17 separate experiments. (B) Mean ±SEM p24 in pg/ml/mL in U1 cells of 16 separate experiments. Numbers above error bars indicate the mean fold change of cell death as determined by LDH cytotoxicity assay. The levels of LDH for each experiment without HDAC inhibitors were set at 1.0 and mean fold increases calculated.

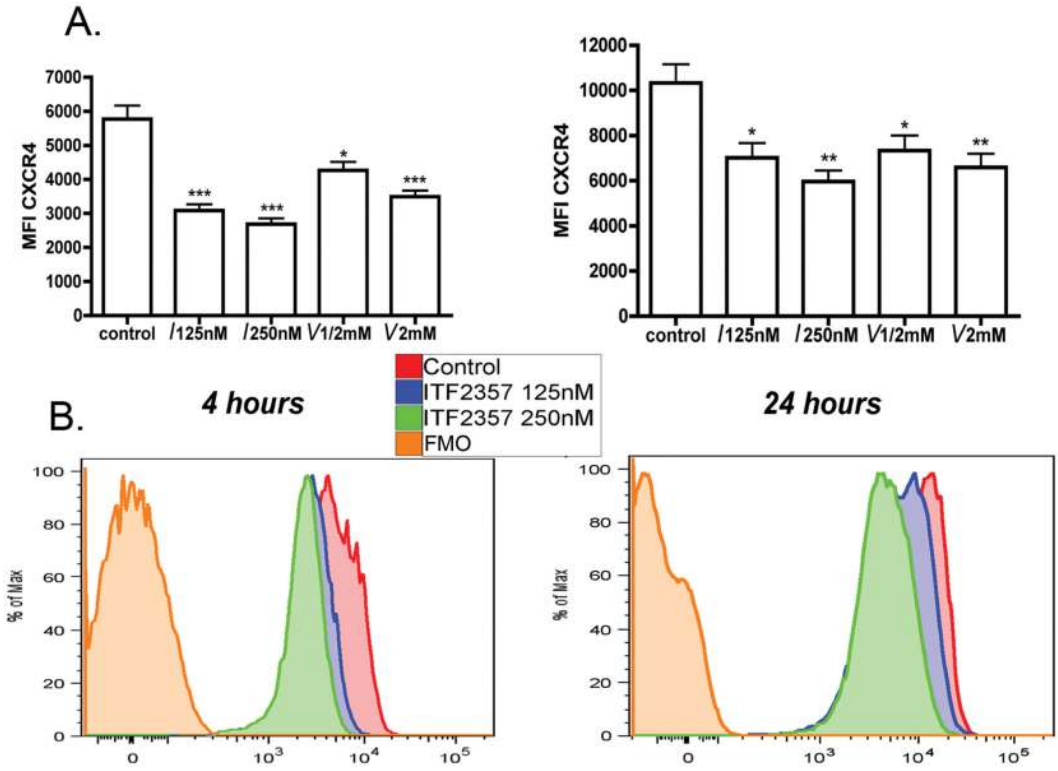

Figure 4. CXCR4 expression on peripheral blood lymphocytes

PBMC were treated with ITF2357 and VPA for either 4 or 24 hours. Percent of CXCR4 expressing cells was determined by four-color flow cytometry. The concentration for each HDACi are shown under each bar (*I*- ITF2357; *V*- VPA) . (A) Mean MFI ± SEM of CXCR4 on CD4+ T-cells after 4 hours (left) and 24 hours (right) incubation. The data are from seven different donors. (B) A representative experiment showing percent of maximal CXCR4 MFI of control cultures compared to 125nM and 250nM of ITF2357 at 4 (left) and 24 hours. * $P<0.05$; ** $P<0.01$. *** $P<0.001$ compared to control expression level.

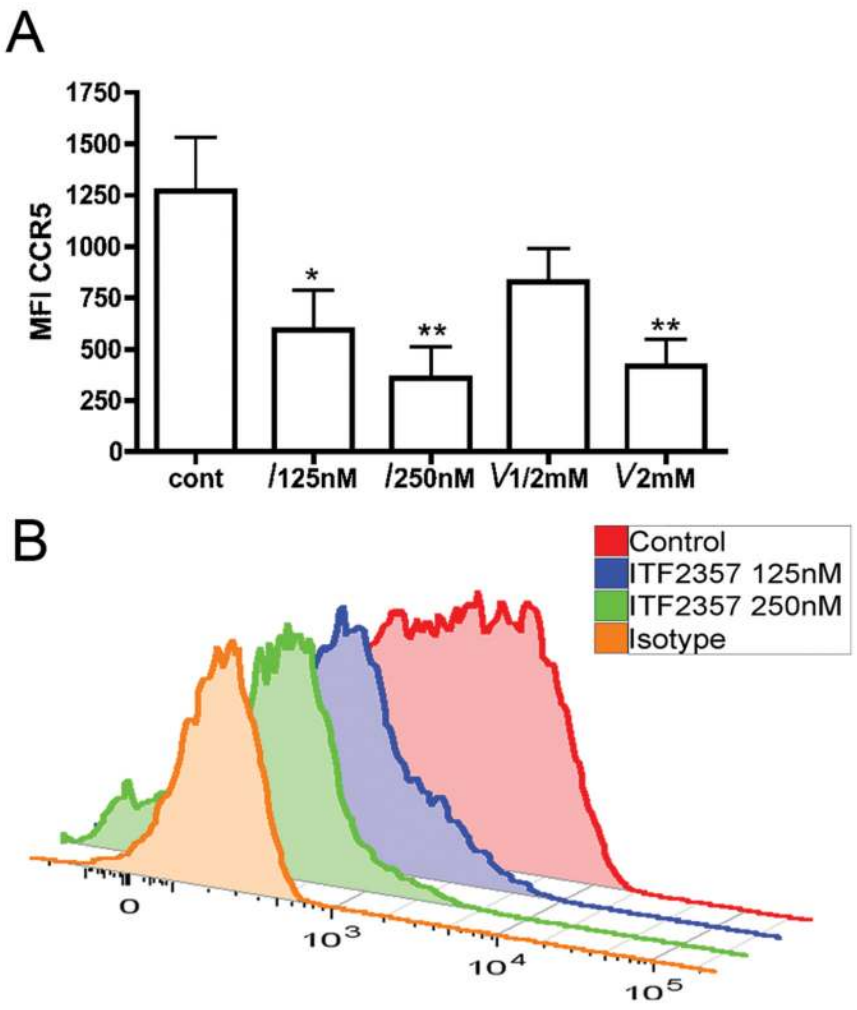

Figure 5. Effect of HDACi on MFI of CCR5 in mononcytes

PBMC were treated with ITF2357 and VPA for 24 hours and then subjected to flow cytometry for CCR5 expression. The concentration of each HDACi are shown under each bar (*I*- ITF2357; *V*- VPA). (A) MFI ±SEM in 7 donors. (B) A representative experiment showing MFI of CCR5 in isotype control, control culture, ITF2357 125nM and 250nM. * $P<0.05$. ** $P<0.01$

Table 1

IC50 (in nM) of different human HDAC inhibitors[1]

	Class I	Class I	Class I	Class I	ClassIIa	ClassIIa	ClassIIa	ClassIIa	ClassIIb	ClassIIb	Class IV
Agent	**HDAC-1**	**HDAC-2**	**HDAC-3**	**HDAC-8**	**HDAC-4**	**HDAC-5**	**HDAC-7**	**HDAC-9**	**HDAC-6**	**HDAC-10**	**HDAC-11**
ITF2357	133	293	136	837	1059	532	524	512	312	331	287
ITFa	81	72	117	144	>10000	nd	7372	>10000	469	260	314
ITFb	62	29	269	438	4541	nd	1728	4109	2751	125	412
IFTc	16	59	32	8583	1885	nd	1635	1033	1725	436	125
VPA	3940	>5000	>5000	1590	>5000	>5000	>5000	>5000	>5000	nd	nd
SAHA	89	707	182	1494	>10000	nd	>10000	>10000	358	143	175

[1] Mean values of at least three separate determinations.

[2] nd denodes not done.