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The thioacetate-ω(γ-lactam carboxamide) HDAC inhibitor ST7612AA1 as HIV-1 latency reactivation agent

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ABSTRACT

Antiretroviral therapy (ART) is unable to cure HIV infection. The ability of HIV to establish a subset of latent infected CD4$^+$ T cells, which remain undetectable to the immune system, becomes a major roadblock to achieve viral eradication. Histone deacetylase inhibitors (HDACi) have been shown to potently induce the reactivation of latent HIV. Here, we show that a new thiol-based HDACi, the thioacetate-ω(γ-lactam carboxamide) derivative ST7612AA1, is a potent inducer of HIV reactivation. We evaluated HIV reactivation activity of ST7612AA1 compared to panobinostat (PNB), romidepsin (RMD) and vorinostat (VOR) in cell culture models of HIV-1 latency, in latently infected primary CD4$^+$ T lymphocytes and in PBMCs from HIV$^+$ patients. ST7612AA1 potently induced HIV-1 reactivation at submicromolar concentrations with comparable potency to panobinostat or superior to vorinostat. The presence of known antiretrovirals did not affect ST7612AA1-induced reactivation and their activity was not affected by ST7612AA1. Cell proliferation and cell activation were not affected by ST7612AA1, or any other HDACi used. In conclusion, our results indicate that ST7612AA1 is a potent activator of latent HIV and that reactivation activity of ST7612AA1 is exerted without activation or proliferation of CD4$^+$ T cells. ST7612AA1 is a suitable candidate for further studies of HIV reactivation strategies and potential new therapies to eradicate the viral reservoirs.

1. Introduction

Despite the success of antiretroviral therapy in controlling HIV replication, the virus remains hidden in cells as integrated provirus where it cannot be targeted by current drug regimens (Esté and Cihlar, 2010). The quest to affect long-term control of HIV-1 in the absence of HAART has led to numerous therapeutic approaches aimed at increasing host-mediated control of HIV and/or clearance of latent virus reservoirs (Ballana and Este, 2013; Chun et al., 1997).

Resting CD4$^+$ T cells harboring integrated proviruses do not actively produce viral particles, are phenotypically undistinguishable from uninfected CD4$^+$ T cells and have a relatively long half-life (Siliciano et al., 2003). Thus, they become a pool of long-lasting, latently infected cells that upon activation and proliferation produce new viral particles (Dahl et al., 2010; Finzi et al., 1997, 1999).

It is believed that HIV reactivation in the presence of antiretroviral therapy can cause death of infected cells whilst preventing reinfection of new cells, a strategy commonly referred to as *shock-and-kill* (Deeks, 2012). A number of pharmacological agents have been evaluated for their capacity to reverse HIV latency (Bruner et al., 2015), being histone deacetylase (HDAC) inhibitors the most widely studied latency reactivation agents (Barton et al., 2014). HDACs remove acetyl groups ($CO–CH_3$) from ε-N-acetyl lysine amino acids on histones that control chromatin condensation and DNA transcription (Jiang et al., 2007; Williams et al., 2006). HDACs have been implicated in maintaining HIV in a latent state and HDAC inhibitors have been show to initiate HIV transcription without inducing T cell proliferation. HDAC inhibitors approved for the treatment of different tumors, vorinostat (SAHA) (Archin et al., 2012), romidepsin (Wei et al., 2014), panobinostat (Rasmussen et al., 2015) or entinostat (Wightman et al., 2013) have been tested or are currently in clinical trials aiming at eradicating latent HIV infection. However their evaluation has led to the conclusion that alternative agents alone or in combination may be needed to effectively reduce the size of the latent reservoir (Cillo et al., 2014; Laird et al., 2015).

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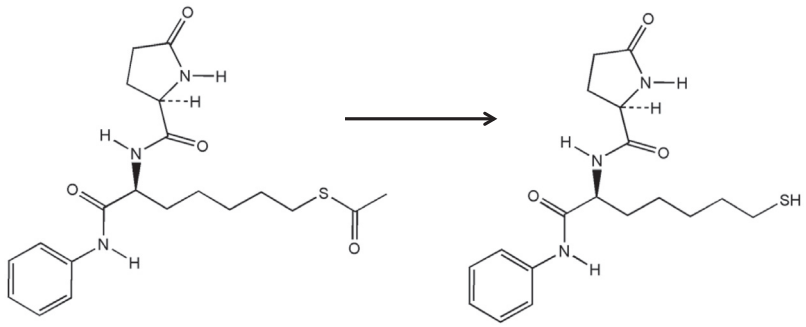

ST7612AA1
(pro-drug)

ST7464AA1
(drug)

Compound	IC_{50} (nM)						
	HDAC1	HDAC2	HDAC3	HDAC6	HDAC8	HDAC10	HDAC11
ST7464AA1	13	78	5	3	281	11	14

Fig. 1. Chemical structure of the pro-drug ST7612AA1 and its active drug ST7464AA1. The *in vitro* inhibitory activity (IC_{50}) of the active drug against different HDACs isoforms is shown (adapted from (Giannini et al., 2014; Vesci et al., 2015)).

ST7612AA1 (the pro-drug of ST7464AA1, Fig. 1) is a novel thioacetyl derivative shown to inhibit HDAC at a nanomolar range, demonstrating promising results in terms of *in vitro* profile, formulation, metabolic stability and cell permeability (Giannini et al., 2014). ST7612AA1 showed high cytotoxic activity on lung cancer NCI-H460 and colon cancer HCT116 cells and it induced significant anti-tumor effects in xenograft models of lung, colon, breast and ovarian carcinomas, leukemia and lymphoma (Vesci et al., 2015). ST7612AA1 has been selected as a drug candidate for further development.

Here, we evaluate the effect of ST7612AA1 as an HIV latency reactivation agent and show that, similarly to other HDAC inhibitors, ST7612AA1 induced virus reactivation in cell culture models, in latently infected primary $CD4^+$ T cells and in PBMCs from HIV^+ patients.

2. Materials and methods

2.1. Cells

Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Paque density gradient centrifugation and used for fresh purification of naïve $CD4^+$ T lymphocytes by negative selection (StemCell Technologies). Purity of the populations was confirmed by flow cytometry. $CD4^+$ T lymphocytes were kept in complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Life Technologies). The work was approved by the scientific committee of Fundació IrsiCaixa. PBMC were isolated from 'buffy coats' of healthy blood donors. Buffy coats were purchased anonymously from the Catalan Banc de Sang i Teixits http://www.bancsang.net/en/index.html). The buffy coats received were totally anonymous and untraceable and the only information given was whether or not they have been tested for disease.

The human cell lines ACH-2 and MT-4 and Jurkat (J-Lat) clone 9.2 (Jordan et al., 2003) were obtained from AIDS Reagent Program, National Institutes of Health (Bethesda, MD). All cell lines were grown in RPMI 1640 medium, supplemented with 10% of heat-inactivated fetal calf serum (FCS, Gibco, Life Technologies, Madrid, Spain) and antibiotics 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies) and maintained at 37 °C in a 5% CO_2 incubator.

2.2. Compounds

ST7612AA1 was provided by Sigma-Tau Industrie Farmaceutiche Riunite SpA, (Roma, Italy) while other HDAC inhibitors were available on commercial catalogs, vorinostat (VOR) and romidepsin (RMD) were purchased from Prochifar srl (Italy) and panobinostat (PNB) was purchased from LC Laboratories. Antiretroviral agents 3′-azido-3′-deoxythymidine (zidovudine; AZT), nevirapine (NVP), tenofovir disoproxil fumarate (TDF) and efavirenz (EFV) and CXCR4 antagonist AMD3100 were obtained from the NIH AIDS Research and Reference Reagent Program. Abacavir (ABC) was purchased from Selleckchem (Munich, Germany). Integrase inhibitor raltegravir (RAL) was provided by Merck. All compounds were resuspended in DMSO and stored at −20 °C until use.

2.3. Viruses

The envelope-deficient HIV-1 NL4-3 clone encoding internal ribosome entry site (IRES)-green fluorescent protein (GFP) (NL4-3-GFP) was pseudotyped with vesicular stomatitis virus G protein (VSV-G) by cotransfection of HEK293T cells using polyethylenimine (Polysciences) as previously described (Pauls et al., 2013). The HIV-1 viral strain NL4-3 (X4-tropic) was obtained from the MRC Centre for AIDS Reagents (London, UK). NL4-3 strain was grown in lymphoid MT-4 cell line. Viral stocks were titrated for its use in MT-4 cells.

2.4. HIV reactivation in vitro in latently infected cell lines

Two latently infected J-Lat and ACH-2 cell lines were used to assess HIV reactivation induced by ST7612AA1. J-Lat cells, which harbor an HIV provirus containing the Green Fluorescent Protein (GFP) ORF instead of *nef* and a frame shift mutation in *env* (Jordan et al., 2003), were incubated for 48 h with different concentrations of all HDAC inhibitors tested in this study. Phorbol 12-myristate 13-acetate 7.5 nM (PMA, Sigma–Aldrich) and ionomycin (0.5 μM, Sigma–Aldrich) were used as controls for HIV-1 reactivation. Reactivation of HIV was monitored as the percentage of living GFP^+ positive cells according to forward and side laser light scatter flow cytometry analysis in a FACS LSRII flow cytometer (BD Biosciences). The data were analyzed using the FlowJo software.

Similarly, ACH-2 cells were cultured in the presence or absence of ST7612AA1, PNB, RMD and VOR for 48 h at 37 °C and 5% CO2. HDACi-induced HIV reactivation was measured by the production of HIV CA p24 antigen using Genscreen HIV-1 Ag ELISA (BioRad) according to manufacturer's instructions.

2.5. Ex vivo models of latency in primary CD4+ T cells

Latently infected CD4+ T cells were generated according to the cytokine-polarized primary T cells model of latency (Bosque and Planelles, 2011) with few modifications. Briefly, naïve CD4+ T cells were activated with αCD3/αCD28 antibodies (1 μg/ml each; BD, Madrid, Spain) and supplemented with TGF-β1 (10 μg/mL, Peprotech), αIL-12 (2 μg/mL) and αIL-4 (1 μg/mL, Peprotech). Medium supplemented with rIL-2 (30 IU/mL, Roche) was replaced every 3 days. Alternatively, latently infected CD4+ T cells were generated according to Saleh et al. (2007) based on activation with αCD3/αCD28 antibodies and supplemented with chemokines CCL19 (MIP-3-β) and CCL21 (Exodus-2, Peprotech) for 7 days prior to infection by VSV-NL43-GFP.

After 7 days of activation, CD4+ T cells were infected with VSV-NL43-GFP by spinoculation (1200×g, 1 h 30 min at 37 °C). Three days after infection, latently infected/GFP negative cells were sorted using a FACSAria II flow cytometer (BD Biosciences) and incubated for 4 h with ST7612AA1, PNB, RMD and VOR. Subsequently, cells were washed with PBS and kept in fresh media containing rIL-2 for 3 days at 37 °C and 5% CO_2. HIV reactivation by HDAC inhibitors was measured the percentage of GFP+ cells using a FACS LSRII flow cytometer (BD Biosciences). The data were analyzed using the FlowJo software.

2.6. Ex vivo activation of HIV transcription in HIV+ patients

HIV-infected patients from the HIV unit of the Hospital Universitari Germans Trias i Pujol were included in the study. Patients were selected on the basis of at least having a CD4 cell count of 200 cells/μL and viral load around 30,000 RNA copies/ml (see Supplementary Table 2). All patients who participated in the study provided informed consent, and protocols were approved by the Scientific Committee of Fundació IrsiCaixa. Blood samples were processed in accordance with current procedures.

To assess HIV reactivation induced by ST7612AA1, PNB, RMD or VOR, 6.5×10^5 PBMCs/well were plated in 24-well plates in 1 mL of total media supplemented with the antiretroviral drugs raltegravir (1 μg/mL) and efavirenz (0.1 μg/mL) and rIL-2 (30 U/mL) for the entire duration of the assay. Control without drugs (ND) and with αCD3/αCD28 were included to assess cell activation. PBMCs were incubated with HDACi at specified concentrations and time of exposure. Briefly, high concentration for each HDACi (4 μM for PNB, ST7612AA1 and VOR; 4 nM for RMD) was used for a 4 h-pulse treatment and subsequently, cultures were washed twice with PBS. Alternatively 1 μM of HDACi (nM for RMD) was used for a continuous exposure for 6 days. After 6 days of HDACi treatment, 0.6 mL of culture supernatant was collected and stored at −80 °C until analysis.

To assess HIV-1 reactivation, total RNA was isolated using Purelink Viral RNA/DNA kit (Life technologies) according to manufacturer's instructions. RNA was treated with DNA-free™DNase Treatment and Removal Reagents (Ambion, Life technologies) prior to cDNA synthesis by High-capacity RNA-to-cDNA kit (Life Technologies). Controls lacking reverse transcriptase were included to assess DNA contamination. Quantification of HIV-1 reactivation was determined using a two-step quantitative polymerase chain reaction (qPCR) as described by Shan et al. (2013) with few modifications. Briefly, samples were run in triplicate on cDNA using TaqMan Universal Master Mix II (Applied Biosystems) on a 7500 Real Time PCR System (Applied Biosystems) Real-Time PCR instrument. Controls without cDNA and RNA without RT treatment were included as controls. We used the following primer and probe set for conserved regions of the 3′LTR of HIV-1 mRNA: Forward primer (5′–3′) CAGATCCTGCATATAAGCAGCTG; reverse primer (5′–3′) TTT TTTTTTTTTTTTTTTTTTTTGAAGCAC and probe (5′–3′) FAM-CCTGTACTGGGTCTCTCTGG-TAMRA. 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. A standard curve from 10^6 to 10^2 copies of HIV-1 3′LTR was performed using ACH-2 and run in parallel with samples.

2.7. Cell activation and proliferation analysis

For cell activation studies, CD4+ T cells isolated from healthy donors, were cultured in the presence or absence of tested HDAC inhibitors for 48 h and incubated with αCD4-APC-Cy7, αCD25-PE, αCD38-PE, αCD69-APC and HLA-DR-PerCP. All antibodies were purchased from BD Biosciences.

Cell proliferation was measured by intracellular Ki-67 staining as previously described (Pauls et al., 2014a,b). Briefly, cells were fixed for 3 min with fixation buffer (FIX & PERM; Life Technologies) before adding precooled 50% methanol for 10 min at 4 °C. Cells were washed in PBS with 5% FBS and incubated for 30 min with the Ki-67 FITC (1:10; clone B56; BD Biosciences) Antibodies were diluted in permeabilization buffer. Flow cytometry was performed in a FACS LSRII flow cytometer (BD Biosciences). The data were analyzed using the FlowJo software.

2.8. Anti-HIV and cytotoxicity assays

Activity of antiretroviral agents with or without ST7612AA1 was determined on HIV-1 infection based on viability of MT4 cells infected or not with the viral strain NL4-3 at multiplicity of infection (MOI) of 0.003 (100 $CCID_{50}$ in 30,000 cells). HIV-1 infection was quantified by a tetrazolium based colorimetric method [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium, MTT method] (Moncunill et al., 2008) commonly used in the evaluation of anti-HIV active drugs. Anti-HIV activity determinations were performed in triplicates and data calculated from three independent experiments.

2.9. Statistical analyses

Experimental data are presented as mean ± SD. One-way ANOVA test was used for comparison between groups, using the GraphPad Prism software (GraphPad Software, San Diego, California, USA). A p-value of 0.05 was considered to be statistically significant.

3. Results

3.1. ST7612AA1 induces HIV reactivation in two in vitro models of latency

Assessment of ST7612AA1 ability to promote HIV transcription in latently infected cells was first studied in lymphoid latently infected Jurkat cells (J-Lat) measured as the percentage of GFP+ cells induced by ST7612AA1 comparing to the different HDAC inhibitors used as controls (panobinostat; PNB, romidepsin; RMD and vorinostat; VOR) (Fig. 2A). ST7612AA1 induces potent *in vitro* HIV reactivation at submicromolar concentrations in a similar manner compared to PNB, leading between 52–60% of GFP+ cells at the highest concentrations tested, respectively (Fig. 2A). In contrast,

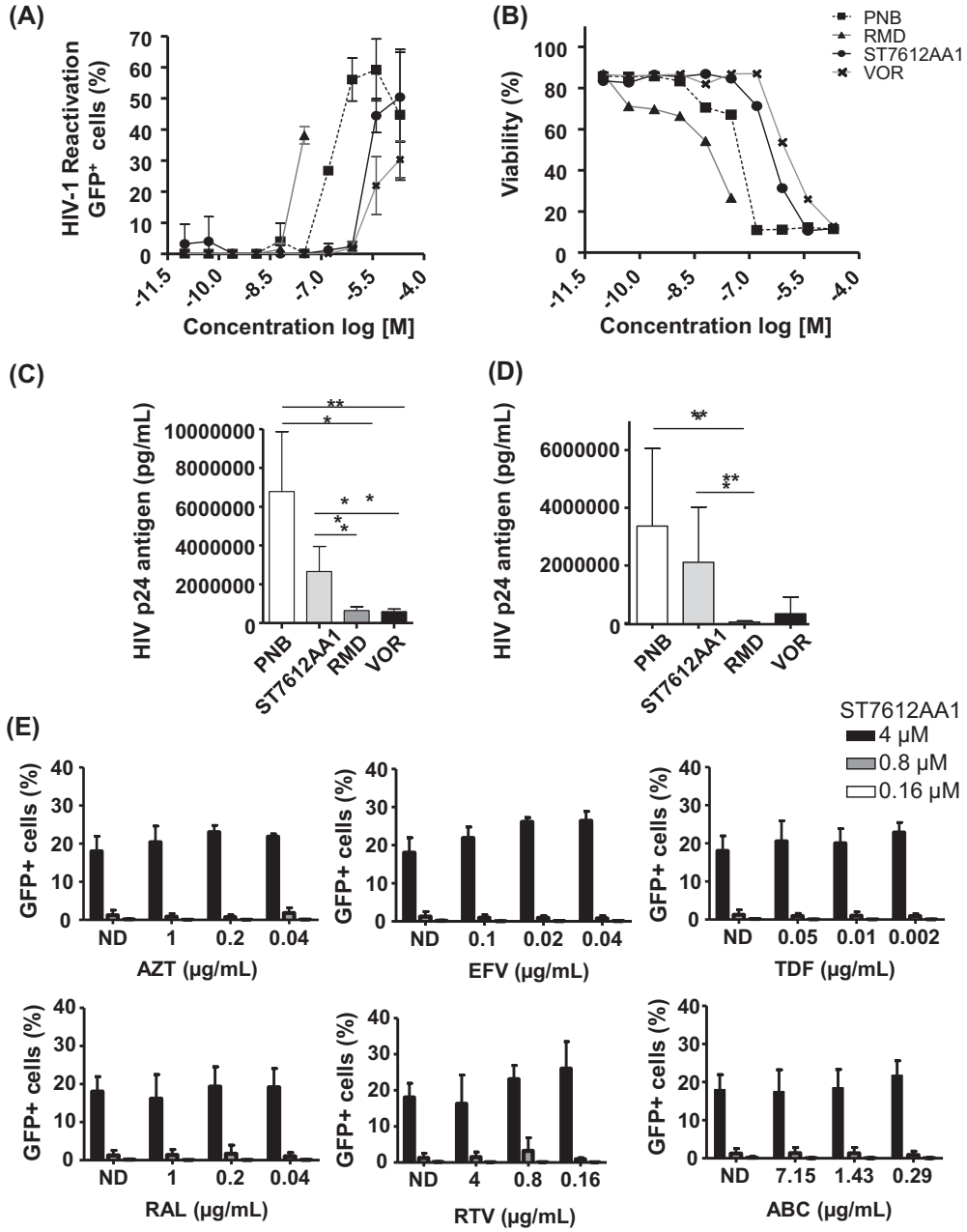

Fig. 2. *In vitro* reactivation of HIV-1 by HDAC inhibitors in latently infected cell lines. (A) HIV reactivation induced by HDAC inhibitors in lymphoid Jurkat (J-lat) cells. Activity of panobinostat (PNB), romidepsin (RMD), ST7612AA1 and vorinostat (VOR) was determined by the quantification of GFP$^+$ cells (%) after culturing J-lat with HDACi at 4 and 0.8 µM (except for RMD at 4 and 0.8 nM) for 48 h. (B) Toxicity of HDACi in J-Lat cells measured as percentage of viable cells by flow cytometry. (C) and (D) HIV-1 activation *in vitro* induced by ST7612AA1 in ACH-2 latency model. Incubation of HIV-1 latently infected lymphoid ACH-2 cells for 48 h in the presence of HDACi inhibitors PNB, RMD, ST7612AA1 and VOR at 4 µM (C) and 0.8 µM (D) (except for RMD at 4 and 0.8 nM). HIV reactivation is determined by HIV-1 p24 antigen production in the supernatant of infected cells. (E) Reactivation ability of ST76122A1 is not affected by the coculture with increasing concentrations of antiretrovirals AZT, efavirenz (EFV), tenofovir (TDF), raltegravir (RAL), ritonavir (RTV) or abacavir (ABC). Mean ± SD of three independent experiments is shown. $^*p < 0.05$; $^{**}p < 0.01$.

HIV reactivation induced by RMD and VOR resulted in a lower percentage of J-Lat GFP$^+$ cells (30–40%) at their respective highest concentrations (Fig. 2A). All HDAC inhibitors tested in this study, which were first developed as anti-tumor compounds, showed significant cell toxicity (Fig. 2B). We observe that toxicity related to RMD is 100-fold higher (CC_{50} = 3.95 nM) comparing to PNB, ST7612AA1 and VOR (Fig. 2B).

Activity of ST7612AA1 as an HIV reactivation agent was confirmed in latently infected ACH-2 cells according to the production of HIV antigen p24 in the supernatant of cells cultured in the presence of all HDACi tested. HIV reactivation induced by ST7612AA1 at 4 and 0.8 µM induced p24 antigen production in a similar manner as PNB (Fig. 2C and D). ST7612AA1 (4 µM), as well as PNB, promotes significant ($p < 0.05$) higher HIV reactivation in ACH-2 cells comparing to RMD (4 nM) and VOR (4 µM) (Fig. 2C). p24 production was between 2.4-fold and 2.8-fold higher in ST7612AA1 treated cells compared to RMD and VOR. The lack of activation of HIV with RMD as measured by p24 (Fig. 2C and D) is likely caused by excessive cytotoxicity of RMD during continual 48-h incubation. It has been shown that for RMD, a drug that is administered by IV infusion and has very short elimination half-life *in vivo*, short pulse treatment followed by incubation in the absence of the com-

Table 1
Anti-HIV activity of antiretrovirals in the presence of ST7612AA1.

Compound		+ST7612AA1		+ST7612AA1
	EC_{50}	EC_{50}	CC_{50}	CC_{50}
AZT	0.001 ± 0.001	0.001 ± 0.0003	>1	>1
AMD3100	0.001 ± 0.001	0.002 ± 0.001	>1	>1
EFV	0.035 ± 0.004	0.031 ± 0.02	>0.1	>0.1
TDF	0.035 ± 0.01	0.045 ± 0.02	>0.06	>0.06
RAL	0.001 ± 0.001	0.001 ± 0.0001	>1	>1
RTV	0.19 ± 0.07	0.35 ± 0.05	>4	>4
ABC	0.31 ± 0.17	0.45 ± 0.13	>7.15	>7.15

Anti-HIV-1 activity of AZT, AMD3100, EFV, TDF, RAL, RTV and ABC determined in the absence or presence (+) ST7612AA1 at 0.04 μM in lymphoid MT4 cells. Data represent Mean ± SD of three independent experiments.

pound substantially reduced the compound cytotoxicity and thus improve the virus activation effect (Wei et al., 2014). HIV-1 reactivation was also confirmed by cell toxicity. Cell death is associated to HIV-reactivation in ACH-2 cell line (Klase et al., 2014). In that sense, viral reactivation mediated by PNB and ST7612AA1 activity results in a higher cell toxicity compared to RMD and VOR (Supplementary Fig. 1).

3.2. Antiretroviral drugs do no interfere with HIV reactivation activity induced by ST7612AA1

Potential interactions between the recently developed HDAC inhibitor ST7612AA1 and commonly used anti-HIV compounds, including reverse transcriptase inhibitors (AZT, EFV, TDF, ABC), integrase inhibitors (RAL) and protease inhibitor (RTV), was evaluated in latently infected J-Lat cells incubated with or without increasing concentrations of ST7612AA1 (4, 0.8 and 0.16 μM) and different concentrations of antiretroviral drugs AZT, EFV, TDF, RAL, RTV and ABC. We observe that none of the anti-HIV compounds modifies the activity of ST7612AA1 as a reversal agent of HIV latency in J-Lat cells (Fig. 2E). ST7612AA1 (4 μM) induced HIV-reactivation of up to 25% of GFP^+ cells in the presence or absence of anti-HIV compounds (Fig. 2E). No synergistic or antagonist effects were observed when co-culturing cells at any concentration of ST761AA1 or antiretroviral compounds (Fig. 2E). In addition, cell viability was not impaired by the addition of any of anti-HIV compounds (Supplementary Fig. 2).

3.3. ST7612AA1 does not modify the anti-HIV activity of antiretroviral compounds

Anti-HIV activity of antiretroviral compounds targeting at different steps of HIV-1 replication cycle, including reverse transcriptase inhibitors (AZT, EFV, TDF and ABC), the integrase inhibitor (RAL) and the protease inhibitor (RTV) was determined in MT4 infected with HIV-1 NL4-3 and incubated with the highest subtoxic concentration of ST7612AA1 (0.04 μM) when left in the cell culture during 5 days (Supplementary Table 1). Anti-HIV activity and cell viability, calculated as the EC_{50} and CC_{50} values respectively, were

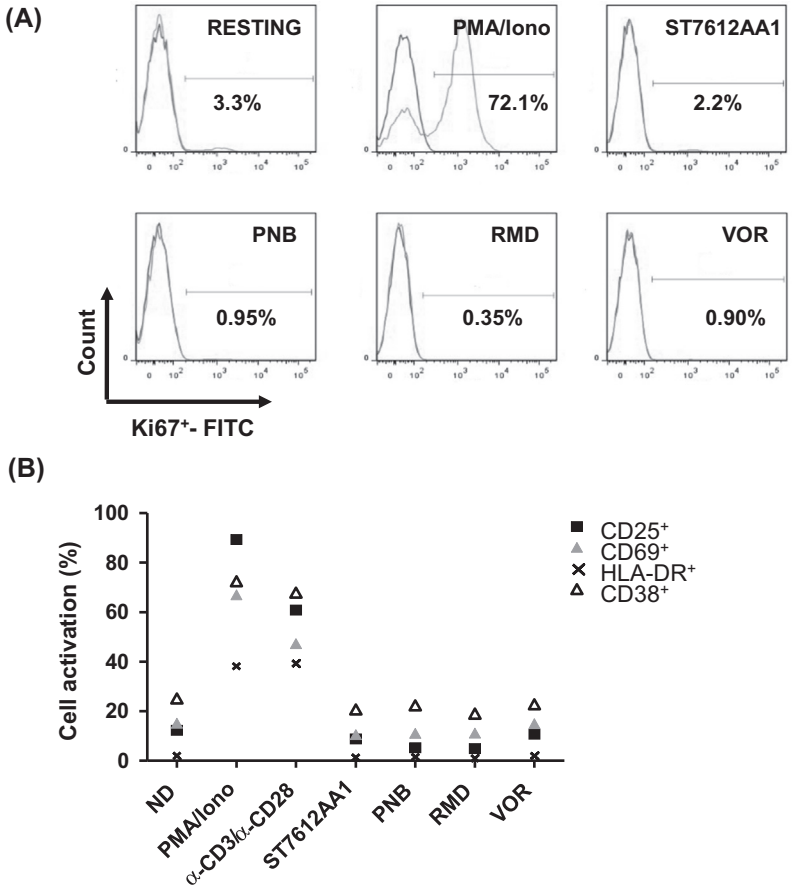

Fig. 3. Evaluation of cell proliferation and activation of $CD4^+$ T lymphocytes treated with HDAC inhibitors. (A) Incubation of $CD4^+$ T cells at subtoxic concentrations of PNB, RMD, ST7612AA1 and VOR did not modify Ki67 intracellular staining determined by flow cytometry. Resting and $CD4^+$ T activated with PMA (7.5 nM) and ionomycin (0.5 μM) were used as controls. (B) HDAC inhibitors do not induce $CD4^+$ T lymphocytes cell activation at subtoxic concentrations. Cells were treated with PNB, RMD, ST7612AA1 and VOR at 0.8 μM (except for RMD at 0.8 nM) and global cell activation was measured as by the expression of cell surface markers CD25, CD69, HLA-DR and CD38 by flow cytometry. Data represents the percentage of positive cells of one representative donor.

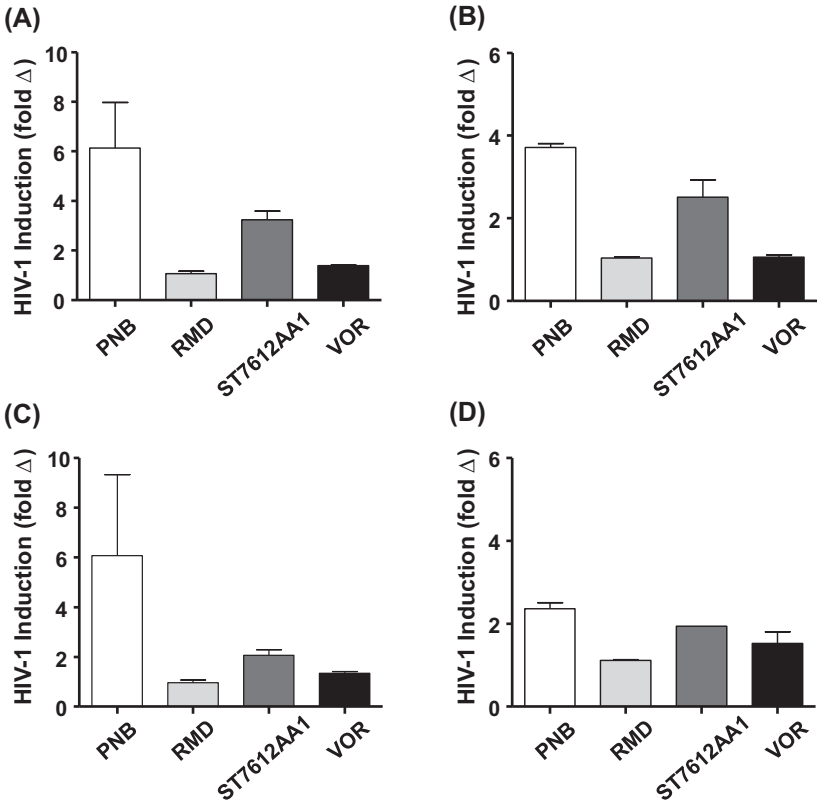

Fig. 4. ST7612AA1 induces HIV-1 reactivation in two *ex vivo* models of latency. (A) and (B) HIV reactivation in the cytokine-polarized model of latency using TGFβ in the presence of 4 μM (A) or 0.8 μM (B) of HDACi (nM for RMD). (C) and (D) HIV reactivation in the CCL19/CCL21 model of latency in the presence of 4 μM (C) or 0.8 μM (D) of HDACi (nM for RMD). HIV-1 reactivation responses to ST7612AA1, PNB, RMD and VOR were measured as by the increase of GFP^+ cells in both models. Data represent HIV-1-fold induction compared to a non-treated control from one representative experiment.

not significantly modified for none of the antiretroviral agents in the presence of the HDAC inhibitor ST761AA1 (Table 1).

3.4. Cell activation and proliferation of primary $CD4^+$ T cells is not modified by ST7612AA1

Compounds with HDAC inhibitory activity and therefore potential HIV latency reversing agents, should reactivate HIV transcription without inducing a global cell activation (Wei et al., 2014). We observe that ST7612AA1, as well as PNB, RMD and VOR, did not induce cell proliferation, determined by the intracellular marker Ki67, compared to the control of proliferating cells induced by PMA (7.5 nM) and ionomycin (0.5 nM) (Fig. 3A) at a concentration with minimum observed cytotoxicity. $CD4^+$ T cells cultured in the presence of 0.8 μM of ST7612AA1 showed similar percentage of $ki67^+$ cells compared to the non-proliferating control of resting $CD4^+$ cells (Fig. 3A).

Moreover, ST7612AA1, as well as PNB, RMD and VOR, does not lead to changes of cell surface markers related to cell activation. CD25, CD38, CD69 and HLA-DR remained in similar values in ST7612AA1 treated cells comparing to resting $CD4^+$ T cells and to PNB, RMD and VOR controls (Fig. 3B).

3.5. HIV reactivation in primary latently infected $CD4^+$ T lymphocytes

Assessment of ST7612AA1 as an HIV reactivation agent was evaluated in latently infected primary $CD4^+$ T lymphocytes generated *in vitro* according to previous published protocols (Bosque and Planelles, 2011; Saleh et al., 2007). In the model based in the use of TGFβ, we observed that HIV-reactivation mediated by PNB was higher than the others HDACi, including ST7612AA1. Highest HIV reactivation activity was observed for PNB (6-fold increase at 4 μM) while ST7612AA1 (4 μM) achieved up to 4-fold increase related to the untreated control (Fig. 4A and B). In the CCL19/CCL21 model of latency, PNB retained significant HIV reactivation activity comparing to the previous model. However, slight HIV reactivation was observed for ST7612AA1 (2-fold increase) at 4 and 0.8 μM (Fig. 4C and D). Both HDAC inhibitors, PNB and ST7612AA1, showed higher HIV reactivation activity compared to RMD and VOR at the different concentrations and models tested in our study. RMD and VOR lead up to 1.5-fold increase related to the non-induced control in both models of latency (Fig. 4). Cytotoxicity of HDAC inhibitors under these cell culture conditions was calculated at 75.7%, 47.2%, 65.5% and 53.6% at 4 μM concentration for PNB, RMD (4 nM), ST762AA1 and VOR, respectively.

3.6. ST7612AA1 induces HIV reactivation ex vivo in PBMCs from HIV^+ patients

ST7612AA1 activity as a reversal agent of HIV latency was confirmed in latently infected PBMCs from HIV^+ patients (described in Supplementary Table 2).

HIV reactivation was measured as HIV-RNA copies in the supernatant of PBMCs cultured or not with different concentrations of HDAC inhibitors ST7612AA1, PNB, RMD and VOR. PBMCs cultured without drugs (ND) or with antibodies α-CD3/α-CD28 were included as controls of HIV-reactivation. Differential sensibility was observed among tested patients according to different sort of stimulus and time of exposure. In that sense, one patient (patient number 4) showed spontaneous reactivation in the ND condition while patients 1–3 required the α-CD3/α-CD28 activation to effectively induce HIV transcription (Fig. 5).

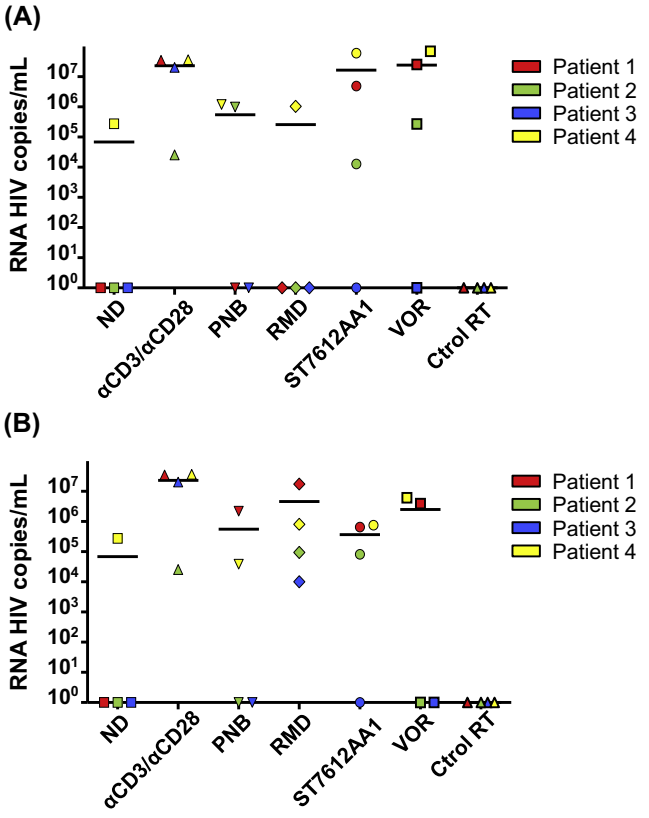

Fig. 5. *Ex vivo* response to ST7612AA1 in PBMCs from HIV$^+$ patients. HIV-RNA copies was determined in the cell culture supernatant from cells of HIV$^+$ individuals incubated for a 4 h pulse at 4 μM of each HDACi (except for RMD at 4 nM) (A) or to a continuous exposure of 1 μM of each HDACi (except for RMD at 4 nM) (B). Data represent the HIV-RNA value for each patient after 6 days since the incubation with PNB, ST7612AA1, RMD or VOR. Control with αCD3/αCD28 was continued for the whole duration of the experiment.

Regarding the activity of HDAC inhibitors at high concentration (4 μM) for a 4 h-pulse period, PNB, ST7612AA1 and VOR induced reactivation in 2/4 and 3/4 patients respectively (Fig. 5A). No significant differences in the HIV-reactivation potency of PNB, ST7612AA1 and VOR were observed (Fig. 5A). However, HIV-reactivation mediated by RMD was observed only in one patient, probably due to the low concentration (4 nM) used of this agent for such a short exposure.

Longer incubation at lower concentrations of HDACi (1 μM for all except 1 nM for RMD) showed HIV reactivation in 2/4 patients for PNB and VOR and 3/4 patients for ST76122A1 treatment at micromolar level. RMD induced HIV reactivation in all patients at nanomolar concentrations (Fig. 5B).

Of note, patient number 3, HIV reactivation was only achieved with 1 nM of RMD but remained insensitive to PNB, ST7612AA1 and VOR (Fig. 5A and B).

4. Discussion

The persistence of the latent reservoir is a major roadblock to eradicate HIV from infected patients. In latently infected cells, the proviral DNA is integrated in the host's genome but it does not actively replicate, becoming invisible to the host immune system and unaffected by existing antiviral drugs. Rebound of viremia and recovery of systemic infection that follows interruption of therapy or treatment failure, necessitates life-long treatments with problems of compliance, toxicity, and costs. Thus, extensive research efforts have led to the proposal of HDAC inhibitors, as well as many other agents including cytokines and chemokines, DNA methyltransferase inhibitors (DNMTI), protein kinase C (PKC) activators and P-TEFb, as potential strategies for targeting the hidden forms of HIV provirus (Shang et al., 2015). However, overall, eradication strategies have had, so far, limited clinical success while posing several risks for patients.

Recently, a new HDAC inhibitor, currently in preclinical phase, has proved to be a potent inhibitor against multiple HDAC isoforms (Giannini et al., 2014) and therefore, based on data from other well characterized HDAC inhibitors it was expected to induce the activation of HIV from latently infected cells. In accordance, in cell culture models of stable cell lines containing a single integrated copy of HIV, ST7612AA1 induced potent *in vitro* HIV reactivation at submicromolar concentrations and its reactivation activity was not affected by antiretroviral drugs commonly used in the treatment of HIV infection. In addition, the antiviral activity of common antiretroviral drugs was not affected by the presence of ST7612AA1, similar to previously reported data for romidepsin (RMD) and vorinostat (VOR) (Lan et al., 2014).

Interestingly, ST7612AA1 did not affect cell activation or proliferation markers in primary CD4$^+$ T cells. Certainly, absence of cell activation or proliferation mediated by ST7612AA1 is comparable to previous described results for RMD (Wei et al., 2014) but is remarkable that ST7612AA1 induced lower cell toxicity compared to RMD, suggesting that ST7612AA1 could represent an alternative agent for HIV intervention strategies aiming at purging HIV out of latently infected cells and reducing the viral reservoir in patients under antiretroviral therapy.

The potency of ST7612AA1 as a reactivation agent varies depending on the model used, being relatively more potent in ACH-2 cells than in J-Lat cells. In primary cells, ST7612AA1 showed a significant effect in two models of latently infected primary CD4$^+$ T cells generated *in vitro* but more importantly, ST7612AA1 was able to reactivate virus in PBMCs from HIV$^+$ patients. Stable cell systems are useful in determining the possible mechanism of action of a given protein or pathway but they do not necessarily represent an ideal model system for evaluating potential therapeutic interventions (Klase et al., 2014). Recently, it has been demonstrated that freshly isolated latently infected cells from infected individuals may respond differently to latency reactivation agents than cellular models in conditions where PMA/ionomycin were able to induce virus production (Bullen et al., 2014). Conversely, romidepsin, a potent natural HDAC inhibitor has been demonstrated to induce reactivation of latent HIV in resting and memory CD4$^+$ T cells infected *in vitro* or isolated from HIV-infected patients (Wei et al., 2014), indicating that cell culture conditions and models provide an inaccurate evaluation of the capacity of any given agent to induce virus reactivation. In this sense, ST7612AA1 showed robust results across all models, albeit with potency differences depending on the cell model or between patients. Inter-individual differences in reactivation in HIV$^+$ individuals may be consequence of different responses related to a diverse sort of stimuli, latency reversal agent or cell culture conditions, ranging from spontaneous HIV transcription in absence of any stimuli to lack of response in presence of diverse sort of HDAC inhibitors, as previously reported (Bullen et al., 2014).

While the number of agents active *in vitro* in disrupting latency is increasing, their validation in primary cell models and cells from patients as well as the elucidation of their mechanisms of action have to be assessed before their designation as candidates for employment *in vivo*. Importantly, no latency reactivation agent has been shown yet to reduce the size of the latent reservoir in infected individuals (Wei et al., 2014). Ongoing clinical trials should help clarify this issue. However, it has to be taken into account that overall establishment and maintenance of latency is a dynamic process regulated by accumulation of different events

and, once established, the expression of the proviral promoter is tightly restricted at several levels, all of which need to be overcome to restart viral production as part of the "shock and kill strategy". Combinatorial approaches that can effectively induce reactivation of the latent reservoirs and enhance specific immune responses to control virus replication and eliminate virus-infected cells should be considered. Similarly to epigenetic approaches that have been borrowed from cancer therapy (Mitsuyasu, 2013), approaches already used in cancer therapy may provide an interesting approach to combat HIV infection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2015.09.004.

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