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PII: S0166-3542(17)30436-9

DOI: 10.1016/j.antiviral.2017.08.013

Reference: AVR 4135

To appear in: *Antiviral Research*

Received Date: 12 June 2017

Revised Date: 18 August 2017

Accepted Date: 21 August 2017

Please cite this article as: Hayashi, T., Jean, M., Huang, H., Simpson, S., Santoso, N.G., Zhu, J., Screening of an FDA-approved compound library identifies levosimendan as a novel anti-HIV-1 agent that inhibits viral transcription, *Antiviral Research* (2017), doi: 10.1016/j.antiviral.2017.08.013.

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Title:

Screening of an FDA-Approved Compound Library Identifies Levosimendan as A Novel anti-HIV-1 Agent that Inhibits Viral Transcription

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Keywords: HIV cure; CD4+ T cell; transcriptional suppression; FDA-approved compound; levosimendan

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Abstract

Combination antiretroviral therapy (cART) has been proven to efficiently inhibit ongoing replication of human immunodeficiency virus type 1 (HIV-1), and significantly improve the health outcome in patients of acquired immune deficiency syndrome (AIDS). However, cART is unable to cure HIV-1/AIDS. Even in presence of cART there exists a residual viremia, contributed from the viral reservoirs of latently infected HIV-1 proviruses; this constitutes a major hurdle. Currently, there are multiple strategies aimed at eliminating or permanently silence these HIV-1 latent reservoirs being intensely explored. One such strategy, a recently emerged “block and lock” approach is appealing. For this approach, so-called HIV-1 latency-promoting agents (LPAs) are used to reinforce viral latency and to prevent the low-level or sporadic transcription of integrated HIV-1 proviruses. Although several LPAs have been reported, there is still a question of their suitability to be further developed as a safe and valid therapeutic agent for the clinical use. In this study, we aimed to identify new potential LPAs through the screening an FDA-approved compound library. A new and promising anti-HIV-1 inhibitor, levosimendan, was identified from these screens. Levosimendan is currently used to treat heart failure in clinics, but it demonstrates strong inhibition of TNFα-induced HIV-1 reactivation in multiple cell lines of HIV-1 latency through affecting the HIV-1 Tat-LTR transcriptional axis. Furthermore, we confirmed that in primary $CD4^+$ T cells levosimendan inhibits both the acute HIV-1 replication and the reactivation of latent HIV-1 proviruses. As a summary, our studies successfully identify levosimendan as a novel and promising anti-HIV-1 inhibitor, which should be immediately investigated *in vivo* given that it is already an FDA-approved drug.

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) infection causes the acquired immune deficiency syndrome (AIDS), which still remains a major public health concern. Combination antiretroviral therapy (cART), typically consisting of three or more antiretroviral (ARV) drugs, effectively blocks ongoing replication of HIV-1 to the undetectable level in peripheral blood. Due to the increased access to HIV-1 treatment globally, cART has significantly reduced the risk of HIV transmission and improved the life expectancy of HIV-infected patients. However, cART is unable to completely eliminate HIV-1, which exists in latent reservoirs that are the source of residual viremia, even in the presence of ARV drugs (Ruelas and Greene, 2013; Siliciano and Greene, 2011). Because of these reservoirs viruses would rebound if treatment were interrupted, patients are required to adhere to HIV-1 medicine for life (Garcia et al., 2012; Siliciano and Greene, 2011). Given that the development of a safe and effective HIV-1 vaccine still remains unsuccessful (Garcia et al., 2012; Pollara et al., 2017), a cure strategy to eliminate residual HIV-1 is attractive. Such a strategy would bypass the downside of cART and restore complete health.

Resting memory $CD4^+$ T cells are considered to be a major HIV-1 latent reservoir. In most activated $CD4^+$ T cells, HIV-1 infection causes a virus-induced cytopathic effect and cell apoptosis. However, some are survive and revert back to their resting state, forming the HIV-1 latent reservoirs (Ruelas and Greene, 2013; Siliciano and Greene, 2011). These cells harbor replication competent, but transcriptionally silent HIV-1 proviruses, which still retain the capability to produce infectious HIV-1 virions (Archin et al., 2014; Ruelas and Greene, 2013; Siliciano and Greene, 2011). Extensive efforts have been taken to explore the ‘‘shock and kill’’ cure strategy to purge latently infected HIV-1 proviruses in reservoir cells. This strategy relies on the use of latency reversing agents (LRAs) to reactivate latent HIV-1 proviruses in cART-treated AIDS patients, which would

theoretically eliminate latent reservoirs by virus-induced cell death and/or HIV-1-specific cytolytic T lymphocytes (CTLs) (Xing and Siliciano, 2013). Although numerous studies have shown that certain LRAs or LRA combinations effectively reverse HIV-1 latency in $CD4^{+}$ T cells isolated from HIV-positive, cART-treated AIDS patients *ex vivo* (Darcis et al., 2015; Laird et al., 2015), use of only LRAs does not lead to the killing of HIV-1 latently infected $CD4^{+}$ T cells. Additionally, HIV-1 antigen-specific stimulation of CTLs prior to HIV-1 reactivation is required (Shan et al., 2012). A recent clinical trial study also showed that administration of histone deacetylase inhibitors (HDACis) in HIV-positive, cART-treated AIDS patients fails to reduce the size of HIV-1 latent reservoirs although viral latency is successfully reversed (Archin et al., 2012). Combined these results suggest that the ‘‘shock and kill’’ cure strategy still faces major obstacles that are difficult to overcome.

Since the ‘‘shock and kill’’ strategy still requires significant efforts to solve the above issues, other alternative strategies have been explored to reach an HIV-1 cure (Darcis et al., 2017; Mousseau et al., 2015b). One is to “block” the occasional reactivation of HIV-1 proviruses so that integrated proviruses are “locked” in a deep and permanent latency. The problem is that cART is unable to eliminate the low-level replication of HIV-1. Since no drug component in cART inhibits HIV-1 transcription, there is still sporadically reactivated HIV-1 expression (“blip”) that continues the replenishment of HIV-1 latent reservoir and maintains its persistence. For the “block and lock” strategy, latency-promoting agents (LPAs) will be complemented with cART regimen to suppress resurrected viral transcription and reduce the residual viremia (Darcis et al., 2017; Mousseau et al., 2015b). Such combination treatment will facilitate the decay of HIV-1 latent reservoir and significantly reduce its size (Darcis et al., 2017; Mousseau et al., 2015b). Once the viral load is under the control, LPAs alone would continue to reinforce the deep and irreversible latency of HIV-1 and the permanent silencing of HIV-1

proviruses even without cART, which would eventually lead to the elimination of HIV-1 latent reservoir and a functional cure of HIV-1. It would also allow the disruption of cART and the reduction of its side effects (Mousseau et al., 2015b). Recently several potential LPAs have been identified (Darcis et al., 2017). For example, one promising LPA is didehydro-Cortistatin A (dCA), which selectively inhibits HIV-1 Tat-mediated viral transcription (Mousseau et al., 2012). The dCA and cART combination is able to block the reactivation of latently infected HIV-1 proviruses in CD4^{+} T cells from HIV-positive, cART-treated AIDS patients (Mousseau et al., 2015a). However its long-term effect on reinforced and irreversible latency of HIV-1 proviruses still needs further investigation. Tat-mutated HIV-1 viruses may arise and cause drug resistance since dCA targets Tat, a viral protein. Expansion of new potential LPAs will benefit the further exploration of this “block and lock” cure strategy and facilitate the development of an effective therapy to eliminate HIV-1 residual viremia. In this study, we screened an FDA-approved compound library, composed of 978 unique small-molecule compounds, and identified Levosimendan as a novel and promising anti-HIV-1 inhibitor that warrants further investigation.

2. Material and methods

2.1. Cells. The following cell lines were kindly provided by the NIH AIDS reagent program: Jurkat Clone E6-1 (Cat. #177), J-LAT A2 (Cat. #9854), 6.3 (Cat. #9846) and 10.6 (Cat. #9849), and JLTRG (Cat. #11586). The HIV-1 latently infected T cell lines CA5 and EF7 were kindly provided by Olaf Kutsch (University of Alabama)(Duverger et al., 2014). All T cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS. Peripheral Blood Mononuclear Cells (PBMCs) from HIV-1 and HIV-2 negative healthy donors was purchased from Stemcell technologies (Cat. #70025) and were

cultured in complete media (RPMI 1640, 10% FBS, 1 x glutamine, 1 x MEM non-essential amino acid solution, 20 mM HEPES) containing 30 IU/ml IL-2.

2.2. Viruses and plasmids. HIV-1 IIIB wild type strain was kindly provided by the NIH AIDS reagent program. VSV-G pseudotyped HIV-1 NL4-3 luciferase virus (HIV-1 Luc) or retroviruses expressing HIV-1 Tat (pQCXIP-Tat) were created as described previously (Huang et al., 2015; Power et al., 2015).

2.3. Compounds and reagents. FDA approved compound screening library (978 compounds, 10 mM solution in DMSO, L1300) was purchased from Selleckchem. For the validation of the selected compounds, we separately ordered levosimendan (Cat. #S2446, SelleckChem; Cat. #L5545, Sigma), spironolactone (Cat. #S4054, SelleckChem; Cat. #S3378, Sigma), 9-aminoacridine (Cat. #92817, Sigma), and mycophenolic acid (Cat. #S2487, SelleckChem; Cat. #M3536, Sigma). Recombinant human TNFα (Cat. #BD 554618) was purchased from BD Bioscience. 3′-Azido-3′-deoxythymidine (AZT, Cat. #A2169) was purchased from Sigma. Raltegravir (RAL, Cat. #S2005) were purchased from SelleckChem. 3-Methyladenine (3-MA, Cat. S2767) was purchased from SelleckChem. Anti-human CD3 antibody (Cat. #16-0037-85) and anti-human CD28 antibody (Cat. #16-0289-85) were purchased from eBioscience.

2.4. Compound screen. An FDA approved compound screening library (978 compounds, 10 mM solution in DMSO) was used for the compound screening. Each compound was diluted to 500 μM solution in DMSO for the compound screen, or diluted to 50, 5, 0.5, 0.05, or 0.005 μM solution in DMSO for titration experiments of selected compounds. Compounds were kept at -80 ℃ before use. 384 well plates (Cat. #3712, Corning) were treated with 50μg/ml fibronectin (Sigma) in D-PBS for 2 hr at 37 ℃ to allow the floating cells to attach the surface of the wells (Yamazoe et al., 2009). The compounds (0.7 μL) or DMSO as a control were then added in 20 μL of RPMI-10% FBS

to fibronectin-treated wells. 50 µL of media containing J-LAT A2 cells (1 x 10^4 cells per well) and TNFα were then added. The final concentration of TNFα or DMSO was 10 ng/ml or 1%, respectively. Plates were then spun down, and incubated for 24 hr at 37℃. After 24 hr, cell nuclei were stained with D-PBS containing 2 µg/ml Hoechst® 33342 (Life technologies). The cells were imaged using a Cytation 5 Cell Imaging Multi-Mode Reader (BioTek) and the percentages of GFP positive cells and cell numbers in each well were determined by counting GFP and Hoechst positive cells using Gen5 Image+ software (BioTek).

2.5. Flow cytometry. HIV-1 latently infected cell lines (4 x 10^5 cells per ml) were co-treated with 10 ng/ml TNFα and indicated compounds. After 24 hr, GFP-positive cells were analyzed by flow cytometry (BD Accuri™ C6, BD). JLTRG cells (4 x 10^5 cells per ml) in 24 well plate were pre-treated with the indicated compounds for 6 hr. The cells were transduced with retroviruses expressing HIV-1 Tat (pQCXIP-Tat) or pQCXIP-empty for 48 hr before flow cytometry. The results were analyzed using FlowJo software (v10.0.7).

2.6. Cell viability assay. Cell viability upon drug treatment was determined using CellTiter-Glo® Luminescent Cell Viability Assay (Promega) or LIVE/DEAD™ Fixable Green Dead Cell Stain Kit (Invitrogen).

2.7. Measurement of HIV-1 transcripts. Cells (4 x 10^5 cells per ml) were co-treated with 10 ng/ml TNFα and indicated compounds. After 24 hr, cells were collected and subjected to RNA extraction (RNeasy mini kit, QIAGEN), cDNA synthesis (iScript™ cDNA Synthesis Kit, Bio-Rad), and qPCR analysis using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad) as described previously (Huang et al., 2015; Zhu et al., 2012). We used initiation primers (Ini) targeting bp 10-59 of HIV-1 transcript, proximal primers targeting bp 29-180 of HIV-1 transcript, intermediate primers targeting bp 836-1015 of HIV-1 transcript, distal primers targeting bp 2341-2433 of HIV-1 transcript, and

GAPDH primers for normalization. Primer sequences were described previously (Zhu et al., 2012).

2.8. Measurement of HIV-1 late RT product. Jurkat cells (5 x 10^5 cells per ml) were pretreated with the indicated compounds for 6 hr, and then infected with HIV-1 IIIB virus (4.5 ng of HIV-1 p24 per 5 x 10^5 cells) for 18 hr. The cells were collected and subjected to DNA extraction (DNeasy Blood & Tissue Kit, QIAGEN) and qPCR analysis to measure late RT and mitochondrial DNA products using TaqMan® Universal PCR Master Mix (Life technologies) as described previously (Butler et al., 2001; Zhu et al., 2014).

2.9. Co-immunoprecipitation (Co-IP) and western blot. Co-immunoprecipitation and western blot analysis was performed as described previously (Huang et al., 2015; Power et al., 2015). Briefly, HeLa cells stably expressing Tat-Flag were treated with levosimendan (10 μM) for 24 hr. Total cell lysates were subjected to western blot analysis using Flag antibody (Cat. #14793, Cell signaling), PARP antibody (Cat. #9542, Cell signaling), or GAPDH antibody (Cat. #sc-47724, Santa Cruz). For Co-IP experiments, total cell lysates were immunoprecipitated using Protein A/G Magnetic Beads (Thermo scientific) with Flag antibody (Cat. #8146, Cell signaling). Immunoprecipitated proteins were subjected to western blot analysis using cyclin T1 antibody (Cat. #sc-10750, Santa Cruz).

2.10. Acute HIV-1 infection in primary CD4$^+$ T cells. CD4$^+$ T cells were isolated from healthy donor PBMCs by negative selection using CD4$^+$ T Cell Isolation Kit (Miltenyi Biotec). The cells were stimulated with anti-CD3/CD28 antibodies (2 μg/ml of each) for 3 days. The activated CD4$^+$ T cells were then infected with HIV-1 IIIB strain (10 ng of p24 per 10^6 cells) for 6 hr. The inoculum was removed, and the cells were washed once in D-PBS. The cells were cultured in complete media containing IL2 (30 UI/ml). Media and drugs were replaced every 3 days. On days 3, 6, 9 or 12 post infection, the

supernatant was harvested and subjected to HIV-1 p24 ELISA using HIV-1 p24 antigen capture assay kit (ABL_{inc}).

2.11. HIV-1 latency model using primary CD4$^+$ T cells. To determine the effect of the drugs on reactivation of latent HIV-1 *ex vivo*, we utilized Planelles' model with slight modifications (Bosque and Planelles, 2009, 2011). Briefly, CD4$^+$ T cells isolated from healthy donor PBMCs were stimulated with anti-CD3/CD28 antibodies (2 µg/ml of each) in the presence of TGF- β1 (10 ng/ml), anti-human IL-12 (2 µg/ml), and anti-human IL-4 (1 µg/ml) for 3 days. The activated cells were cultured in the presence of IL-2 (30 UI/ml) for 4 days. The activated CD4$^+$ T cells were then spinoculated with HIV-1 Luc (5 ng of p24 per 10^6 cells) at 1,741 x g for 2 hr at 37 ℃. Cells were then incubated in complete media containing IL-2 (30 UI/ml) for 7 days to establish HIV-1 latency. Finally, cells were stimulated with anti-CD3/CD28 antibodies (1 µg/ml of each) in the presence of LSM (10 µM) or SPR (10 µM) for 3 days. The level of HIV-1 reactivation was then determined by measuring luciferase activity using ONE-Glo™ Luciferase Assay System (Promega).

2.12. HIV-1 reactivation in CD4$^+$ T cells isolated from cART-treated, HIV-infected aviremic patients. PBMCs were isolated from fresh whole blood of cART-treated, HIV-infected aviremic patients as described previously (Huang et al., 2017). The CD4$^+$ T cells isolated from PBMCs (2 x 10^6 cells per well) were then stimulated with anti-CD3/CD28 antibodies (1 µg/ml of each) in the presence of LSM (10 µM) or SPR (10 µM). As a positive control, the cells were treated with cART (100 nM efavirenz [EFV], 180 nM zidovudine [AZT], 200 nM raltegravir [RAL]). On day 3, media and drugs were replaced. On day 6, the supernatant was harvested and subjected to RNA extraction followed by ultra-sensitive qPCR assay to quantify reactivated HIV viruses as described previously (Huang et al., 2017; Mousseau et al., 2015a). A serial dilution of HIV-1 IIIB RNAs with known copy numbers were used to create standard curve for calculation of copy number of HIV RNAs in supernatant.

2.13. HIV-infected subjects. cART-treated, HIV-infected aviremic patients were recruited from the AIDS clinic at the Strong Memorial Hospital of University of Rochester Medical Center (Rochester, New York). All patients signed informed consents to enroll in this study. All subjects were treated with cART for > 3 years, had an undetectable plasma HIV RNA level (< 50 copies/ml) for at least 6 months, and a normal $CD4^+$ T lymphocyte count (> 300 cells/mm^3) at the time of leukapheresis process. This study was approved by the University of Rochester Research Subjects Review Board with an assigned number (#RSRB00053667).

2.14. Data analysis and statistics. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test (GraphPad Prism 5.0 software). A p value of < 0.05 was considered statistically significant. Dose response curve was created by nonlinear regression model, and the 50% inhibitory concentration (IC_{50}) and the cytotoxic concentration (CC_{50}) was calculated using GraphPad Prism 5.0 software.

3. Results

3.1. A screen of FDA-approved compounds identifies new anti-HIV-1 inhibitor candidates. We screened an FDA-approved compound library to identify novel anti-HIV-1 agents, using the HIV-1 latency cell line, J-LAT A2, which contains an HIV-1 minigenome "LTR-tat-IRES-GFP", to determine the drug effect on TNFα-induced HIV-1 reactivation through the measurement of GFP-positive cell population (Huang et al., 2015; Jordan et al., 2003). J-LAT A2 cells were plated on fibronectin-treated 384 well plates and grown as a monolayer (Yamazoe et al., 2009), so that quantification of the GFP-positive cell population could be determined using fluorescence microscopy. Cells were treated with TNFα (10 ng/ml) and individual compounds (5 μM) for 24 hr. Nuclei were then stained using Hoechst® 33342 for quantification purposes (**Figure 1A**). TNFα

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induced the expression of GFP in the majority of J-LAT A2 cells (87.2%), whereas un-treated cells mostly remained GFP-negative (**Figure 1B**). We then determined the relative percentage of GFP-positive cells (% GFP-positive) and the relative total cell numbers (% cell numbers) by normalizing the results of compound-treated cells to DMSO-treated cells in each plate (**Table S1**). Screening results were plotted as % GFP-positive *vs* % cell numbers (**Figure 1C**). From this experiment we identified two FDA-approved compounds, levosimendan and 9-aminoacridine, which reduced the % GFP-positive by 50%, while the compound-treated cells remains % cell numbers over 50%. Levosimendan is a cardiostimulatory drug used for treatment of heart failure through increasing the sensitivity of troponin-C to calcium and enhancing the contractility of myocardium (Kasikcioglu and Cam, 2006; Nieminen et al., 2013). So far there is no reported antiviral effect of levosimendan. However, 9-aminoacridine is known to inhibit HIV-1 transcription through the Tat protein (Guendel et al., 2009). At the time that these initial screenings were completed, there was a timely report showing that spironolactone suppresses HIV-1 transcription. However there was no experimental evidence measuring the effect of spironolactone on HIV-1 latency (Lacombe et al., 2016). Although spironolactone only inhibits HIV-1 reactivation by 40%, we included spironolactone in further studies to evaluate its effect as an anti-HIV-1 inhibitor. Next, we performed titration experiments for all compounds that reduce the % GFP-positive by 50%, regardless of their cytotoxicity at lower concentrations, in a series of 10-fold dilution ranging from 0.05 to 500 nM, which was used to calculate the compound's IC_{50} and CC_{50} values (**Figure S1, Table S2**). Although cytotoxicity is reduced at lower concentrations for these compounds, most of their anti-reactivation effect is also abolished, making them less ideal as antiviral compound candidates. However, among these compounds we selected mycophenolic acid and its ester derivative, mycophenolate mofetil, as they potently inhibited HIV-1 reactivation in J-LAT A2 cells at

the concentration of 5 μM with moderate cytotoxicity, for further validation (**Table S1, Figure S1**). Mycophenolic acid is known to block HIV-1 reverse transcription through inhibition of the *de novo* synthesis of guanosine nucleotides (Chapuis et al., 2000; Ichimura and Levy, 1995).

3.2. Levosimendan inhibits HIV-1 reactivation in latency cell lines. Next we performed more profound experiments, titrating our five selected compounds (levosimendan, spironolactone, 9-aminoacridine, mycophenolic acid, and mycophenolate mofetil) in a series of 2-fold dilutions ranging from 0.625 to 20 μM, onto J-LAT A2 cells that were treated with TNFα to induce HIV-1 reactivation. GFP-positive cells were quantified using flow cytometry for this round of titration experiments. Treatment with levosimendan and spironolactone lead to a significant reduction of HIV-1 reactivation in a dose-dependent manner, while the cytotoxicity was subtle in all doses tested (**Figure 2A**). Treatment with 9-aminoacridine showed a significant anti-reactivation effect as well, however it was also more cytotoxic at higher doses. Compound's IC_{50} values were determined accordingly (levosimendan: 3.8 μM; spironolactone, 3.7 μM; 9-aminoacridine, 2.4 μM) (**Table S2**). Mycophenolic acid and mycophenolate mofetil demonstrated a very similar inhibitory effect in J-LAT A2 cells, with the same IC_{50} value of 0.6 μM (**Figure S2A, Table S2**). To confirm the identity of compounds within our library, we repeated tests using identical compounds from different sources; results in J-LAT A2 cells were comparable (**Figure S2B**). We further tested our anti-HIV-1 inhibitor candidates (levosimendan, spironolactone, 9-aminoacridine, mycophenolic acid) in multiple HIV-1 latency cell lines harboring HIV-1 proviral genomes. We used J-LAT 6.3 and J-LAT 10.6 cell lines harboring replication-incompetent, env-deleted HIV-1 NL4-3 GFP provirus (Jordan et al., 2003), and CA5 and EF7 cell lines which are latently infected with replication-competent HIV-1 NL4-3 GFP virus (Duverger et al., 2014). Treatment with levosimendan and spironolactone blocked

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the TNFα-induced HIV-1 reactivation in all tested HIV-1 latency cell lines in a dose-dependent manner (**Figure 2B-E**). However, treatment of 9-aminoacridine failed to block reactivation in these HIV-1 latency cell lines and even slightly enhanced HIV-1 reactivation in J-LAT 6.3 cells (**Figure 2B-E**). Although treatment of mycophenolic acid mildly blocked HIV-1 reactivation in J-LAT 6.3 cells, it showed no inhibitory effect on other cell lines (**Figure S3**). In parallel, we measured compound cytotoxicity in these HIV-1 latency cell lines, and all compounds showed minimal cytotoxicity at the tested concentrations in the presence of TNFα or HIV-1 Tat (**Figure S4A-E**). Likewise, there was no or minor cytotoxicity either in Jurkat cells that were only treated with the compound alone (**Figure S4F**). Collectively, these results suggest that levosimendan, similarly to spironolactone, is able to potently block reactivation of latent HIV-1 proviruses without severe cytotoxicity.

3.3. Levosimendan suppresses HIV-1 Tat-LTR mediated transcription. We further measured the effect of levosimendan and spironolactone on HIV-1 transcription as they were shown to potently block TNFα-induced HIV-1 reactivation. First we determined the effect of levosimendan and spironolactone on HIV-1 transcription by qPCR assay, using primers that measure HIV-1 transcripts at different sites. Both compounds potently inhibited transcriptional initiation and elongation to a similar extent in TNFα-treated J-LAT 10.6 and CA5 cells (**Figure 3A-B**). We next determined the effect of levosimendan and spironolactone on HIV-1 5' LTR promoter activity in the presence of HIV-1 Tat protein. We transduced the JLTRG cells, which harbor an integrated LTR-GFP construct, with a retroviral vector (pQCXIP) expressing FLAG-Tat to induce GFP expression. Both compounds reduced the percentage of GFP-positive cells, although the inhibitory effect of levosimendan seems greater than that of spironolactone and 9-aminoacridine, both of which were known to inhibit Tat-LTR mediated transcription (Guendel et al., 2009; Lacombe et al., 2016). However, both levosimendan and

spironolactone showed no inhibitory effect on HIV-1 reverse transcription (**Figure 3D**), suggesting that these compounds may specifically target the HIV-1 transcription step.

3.4. Levosimendan blocks HIV-1 replication and reactivation in primary CD4+ cells. As all of the above experiments were performed using Jurkat-derived cell lines, we chose to verify the compound's antiretroviral effect in more physiologically relevant, primary CD4+ T cells. We tested the cytotoxicity of levosimendan and spironolactone in primary CD4+ T cells. Levosimendan showed no cytotoxicity, while there was moderate cyotototoxicity of spironolactone on day 3 post-treatment (**Figure S4G**). We then verified the effect of levosimendan and spironolactone (10 μM) on acute replication of HIV-1 IIIB in activated primary CD4+ T cells isolated from 3 healthy donors. Levosimendan completely inhibited HIV-1 replication in all tested $CD4^+$ T cells (**Figure 4A**). Although spironolactone also showed the inhibitory effect, it was much weaker than that of levosimendan (**Figure 4A**). Next we tested the effect of levosimendan and spironolactone on HIV-1 reactivation in HIV-1-Luc latently infected primary CD4+ T cells, a cell model of HIV-1 latency that was established by Vicente Planelles' group (Bosque and Planelles, 2009, 2011). Treatment with levosimendan and spironolactone efficiently blocked anti-CD3/CD28-induced HIV-1 reactivation in all tested $CD4^+$ T cells isolated from the three healthy donors comparably (**Figure 4B**). We further evaluated the effect of levosimendan and spironolactone on HIV-1 reactivation in primary CD4+ T cells that were isolated from 3 cART-treated, HIV-infected aviremic patients. Both levosimendan and spironolactone significantly inhibited anti-CD3/CD28-induced HIV-1 reactivation (**Figure 4C**). Although levosimendan consistently showed no obvious cytotoxicity, spironolactone still caused moderate cytotoxicity (**Figure 4D**).

3.5. Levosimendan inhibits HIV-1 reactivation through phosphoinositide 3-kinase (PI3K) pathway. As the next step, we would have liked to understand the molecular mechanism that underlines the inhibitory effect of levosimendan on HIV-1

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transcription. Since HIV-1 Tat protein plays a key role in viral transcriptional elongation through the interaction with positive transcription elongation factor b (P-TEFb) complex, composed of cyclin T1 and cyclin-dependent kinase 9 (Kamori and Ueno, 2017), we first determined the effect of levosimendan on Tat protein level and Tat-P-TEFb protein interaction in Tat-expressing HeLa cells. Neither the Tat protein level nor the Tat-P-TEFb protein interaction was affected by the treatment of levosimedan (**Figure 5A-B**). We also ruled out the anti-HIV effect of levosimendan is through the induction of cell apoptosis. Treatment of levosimendan in Tat-expressing HeLa cells is unable to induce the cleavage of PARP, a hallmark of cell apoptosis (**Figure S5**). A previous study showed that overexpression of constitutively active PI3K reduced HIV-1 transcription, whereas PI3K inhibitors enhance it, suggesting that PI3K pathway negatively regulates HIV-1 transcription (Cook et al., 2002). Interestingly, it has also been reported that levosimendan inhibits oxidative injuries, caused by hydrogen peroxide, in rat cardiac cells, and PI3K inhibitor, 3-MA, compromised such effect (Uberti et al., 2011). These previous findings led us to hypothesize that levosimendan may activate PI3K pathway to suppress HIV-1 transcription. Indeed, our results showed that treatment of 3-MA is able to restore the reactivated HIV-1 expression that was suppressed by levosimendan in a dose-dependent manner in J-LAT 10.6 cells (**Figure 5C**).

4. Discussion

In this study, we screened an FDA-approved compound library and successfully identified levosimendan as a novel and promising anti-HIV-1 inhibitor. We found that this drug suppresses HIV-1 reactivation in all tested HIV-1 latency cell lines, primary CD4$^+$ T cell models of HIV-1 latency, as well as the primary CD4$^+$ T cells that were isolated from cART-treated, HIV-infected aviremic patients (**Figures 2, 4B-C**). Levosimendan is a calcium-sensitizing positive inotropic drug used for treatment of acutely decompensated

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heart failure (Kasikcioglu and Cam, 2006; Nieminen et al., 2013; Pathak et al., 2013). This drug has multiple mechanisms of action, including 1) enhanced cardiac contractility by increasing the sensitivity of troponin-C to calcium without affecting the intracellular calcium level, 2) vasodilation, through opening the ATP-sensitive potassium (KATP) channel, 3) phosphodiesterase (PDE) III inhibition activity, and 4) cardioprotection possibly through the anti-inflammatory and anti-apoptotic effects (Kivikko et al., 2003; Nieminen et al., 2013; Pathak et al., 2013). Whether these functions of levosimedan associate with inhibition of HIV-1 reactivation remains unclear at this stage. Interestingly, our screening results also showed that pimobendan, a known calcium sensitizer and PDE III inhibitor, does not efficiently suppress TNFα-induced HIV-1 reactivation in J-LAT A2 cells (**Table S1**) (Pollesello et al., 2016). We further titrated pimobendan with a series of 2-fold dilutions ranging from 0.625 to 20 μM in J-LAT A2 cells. Results showed that there was only subtle inhibition of TNFα-induced HIV-1 reactivation even at a high concentration of 20 μM (**Figure S2C**). This suggests that levosimendan's suppressive effect on HIV-1 reactivation could be independent of its calcium sensitizing and PDE III inhibitory activities.

Our studies suggest that levosimendan specifically suppresses HIV-1 transcription since it has no effect on HIV-1 reverse transcription (**Figure 3D**). We further confirmed that levosimendan blocks HIV-1 Tat-LTR mediated transcription (**Figure 3C**). However, our results showed that the inhibitory effect of levosimendan on HIV-1 transcription and reactivation is not through the direct reduction of either Tat protein level or the Tat-P-TEFb protein interaction (**Figure 5A-B**). It is possible that the inhibitory effect of levosimendan relates to its interference with the TNFα-mediated NF-κB pathway, which is required for HIV-1 transcription. A previous study has shown that levosimendan suppresses NF-κB-mediated transcription, although the inhibitory effect seems to be

moderate (approximately 30% reduction) even when levosimendan was used at a high concentration (10 μM) (Sareila et al., 2008). However, a PI3K inhibitor, 3-MA, is sufficient to overcome the inhibitory effect of levosimendan and restore the HIV-1 reactivation in a dose-dependent manner (**Figure 5C**), suggesting that levosimendan may activate the PI3K pathway to suppress HIV-1 transcription and reactivation. Further investigations are needed to further unravel the mechanism(s) relevant to the antiretroviral effect of levosimendan.

A pharmacological study of levosimendan in patients with heart failure showed that maximum plasma concentration (C_{max}) is reached at approximately 0.43 μM after intravenous administration of levosimendan (Kivikko et al., 2003). The IC_{50} of levosimendan determined in J-LAT A2 cells is approximately 9 times higher than the C_{max} *in vivo*, although the effective concentration in cell culture *in vitro* cannot be simply compared with the *in vivo* situation (Blaauboer, 2008). Further *in vivo* studies are required to determine the optimal concentration of levosimedan that is able to effectively block HIV-1 reactivation in the clinical setting. It has been reported that levosimendan, within the range from 1 nM to 10 μM, increases calcium sensitization in a dose-dependent manner in primary guinea pig myocytes (Szilagyi et al., 2005; Szilagyi et al., 2004). In contrast, a higher concentration of levosimendan is required to exert its cytoprotective effect against the apoptosis in primary rat cardiac fibroblasts (30 - 100 μM) or to activate the KATP channel in rat ventricular cells (EC_{50} = 4.7 μM) (Okada and Yamawaki, 2015; Yokoshiki et al., 1997). Therefore, a higher concentration of levosimendan might be needed to result in other actions, which could be apart from its calcium sensitizing effect.

In addition to levosimendan, our screen also identified spironolactone as an anti-HIV-1 agent. Spironolactone is currently used as a potassium-sparing diuretic, in clinics, to treat hypertension, hyperaldosteronism, and heart failure (Lainscak et al., 2015). Very

recently, this drug has been reported to inhibit acute HIV-1 infection through the inhibition of Tat-mediated HIV-1 transcription (Lacombe et al., 2016). However, the effect of spironolactone on HIV-1 latency has never been tested. For the first time, our results show that spironolactone potently blocks HIV-1 reactivation in HIV-1 latency cell lines and primary $CD4^+$ T cells harboring latently infected HIV-1 proviruses (**Figures 2, 4B-C**). Spironolactone, together with levosimendan, could be immediately moved to clinical studies for further investigation since they are FDA-approved compounds.

Acknowledgements

This work is supported by grants from NIH (R01GM117838 and R33AI116180) to J.Z., and supported in part by the UR CFAR grant from NIH (P30AI078498). We acknowledge Drs. Stephen Dewhurst and Sanjay Maggirwar (URMC) for helpful discussions and preparation of the manuscript.

Author contributions

JZ conceived the project. JZ and TH designed the study and wrote the paper. TH conducted the experiments. TH, NS, and JZ analyzed the results. MJ, HH, and SS provided the reagents and advised the study. All authors reviewed the results and approved the final version of the manuscript.

Competing interests

The authors declare no competing financial interests.

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Figure legends:

Figure 1. Screening for FDA-approved compounds that inhibit reactivation of latent HIV-1. (A) Schematic representation of the compound screening. J-LAT A2 cells were co-treated with compounds (5 μM) and TNFα (10 ng/ml) for 24 hr. Cells were then stained with Hoechst® 33342 followed by imaging analysis. The assay was performed in quadruplicate. (B) Fluorescence images showing the reactivation (GFP, green) and cell nucleus (blue) in J-LAT A2 cells treated with TNFα for the indicated compounds. Values represent the mean ± s.d. of the % GFP-positive cells (n ≥ 4). (C) Scatter plot of the % GFP-positive cells *vs* % cell numbers for all tested compounds. Results were normalized to DMSO control. LSM, levosimendan; SPR, spironolactone; 9AA, 9-aminoacridine.

Figure 2. Effect of anti-HIV-1 inhibitor candidates on viral reactivation in HIV-1 latency cell lines. (A) J-LAT A2 cells were co-treated with compounds at the indicated concentrations and TNFα (10 ng/ml) for 24 hr. The GFP-positive cells were analyzed by flow cytometry. Cell viability was measured using CellTiter-Glo assay. The % of GFP-positive cells and % cell viability were plotted for each compound. Results were normalized to DMSO control. (B-E) The % of GFP-positive cells upon compound treatment were determined for the indicated HIV-1 latency cell lines (B. J-LAT 6.3; C. J-LAT 10.6; D. CA5; E. EF7). Results were normalized to DMSO control. Values represent the mean ± s.d. (n = 4-6). * $p < 0.05$, one-way ANOVA followed by Tukey's multiple-comparison test.

Figure 3. Levosimendan inhibits HIV-1 Tat-LTR mediated transcription. (A, B) Indicated HIV-1 latency cells (A. J-LAT 10.6; B. CA5) were treated with levosimendan or spironolactone (5, 10, or 20 μM) in combination with TNFα (10 ng/ml). After 24 hr, mRNA was extracted, and reverse transcribed to cDNA for measurement of HIV-1 initiated or elongated (proximal [Pro], intermediate [Int], and distal [Dis]) transcripts by qPCR. Results were normalized to DMSO control. Values represent the mean ± s.d. (n =

4-6). (C) JLTRG cells were pre-treated with indicated compounds for 6 hr and transduced with either pQCXIP empty or pQCXIP-Tat for 48 hr. The % of GFP-positive cells was determined by flow cytometry. Results were normalized to DMSO control (Tat+). Values represent the mean ± s.d. (n = 4-6). (D) Jurkat cells were pre-treated with indicated compounds (10 μM) for 6 hr, then infected with HIV-1 IIIB viruses for 18 hr. HIV-1 reverse-transcriptase inhibitor (AZT, 25 μM) and integrase inhibitor (raltegravir [RAL], 1 μM) were also used. The DNA was extracted for measurement of late RT products by qPCR. Results were normalized to DMSO control. Values represent the mean ± s.d. (n = 9). * $p < 0.05$, one-way ANOVA followed by Tukey's multiple-comparison test. NS, not significant ($p > 0.05$).

Figure 4. Levosimendan inhibits HIV-1 replication and reactivation in primary CD4+ T cells. (A) Primary CD4$^+$ T cells were isolated from three healthy donors and activated using anti-CD3/CD28 antibodies. Cells were then infected with HIV-IIIB in the presence of indicated compound (10 μM). At each time point, supernatant was harvested and subjected to HIV-1 p24 ELISA assay. (B) Isolated primary CD4$^+$ T cells from donors were infected with HIV-1 Luc followed by long-term culture to allow for the establishment of HIV-1 latency (the Vicente Planelles' model). HIV-1 latently infected cells were then stimulated with anti-CD3/CD28 antibodies in the presence of indicated compound (10 μM) for 3 days. Luciferase activity was measured and normalized to the DMSO control. (C) CD4$^+$ T cells isolated from 3 cART-treated, HIV-infected aviremic patients were stimulated with anti-CD3/CD28 antibodies in the presence of levosimendan or spironolactone (10 μM) for 6 days. cART (100 nM efavirenz [EFV], 180 nM zidovudine [AZT], 200 nM raltegravir [RAL]) was used as a positive control. HIV-1 RNAs in the supernatant were quantified by ultra-sensitive qPCR. (D) The viability of cells in (C) was measured using the LIVE/DEAD™ Fixable Green Dead Cell Stain Kit.

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Results of (C) and (D) were normalized to DMSO control. $*p < 0.05$, one-way ANOVA followed by Tukey's multiple-comparison test).

Figure 5. Levosimendan does not reduce Tat protein level or Tat-P-TEFb interaction but PI3K inhibiton rescues its effect. (A, B) HeLa cells stably expressing Tat-Flag were treated with levosimendan (10 μM) for 24 hr. (A) Total cell lysates were subjected to western blot to detect Tat-Flag or endogenous GAPDH protein level using Flag antibody or GAPDH antibody, respectively. (B) Total cell lysates were immunoprecipitated using Flag antibody followed by western blot to detect endogenous cyclin T1 or Tat-flag protein level using Cyclin T1 or Flag antibody, respectively. (C) J-LAT 10.6 cells were pre-treated with 3-MA at indicated concentrations for 6 hr, then co-treated with levosimendan (10 μM) and TNFα (10 ng/ml) for 24 hr. The GFP-positive cells were analyzed by flow cytometry. Results were normalized to DMSO control. Values represent the mean ± s.d. (n = 3). $* p < 0.05$, one-way ANOVA followed by Tukey's multiple-comparison test.

Supplementary Materials:

Figure S1. Titration of the effect for the selected 30 compounds in J-LAT A2 cells. J-LAT A2 cells were co-treated with compounds at indicated concentrations and TNFα (10 ng/ml) for 24 hr. Cells were then stained with Hoechst® 33342 followed by imaging analysis. The assay was performed in quadruplicate. Black bars or green dots indicate the % GFP-positive cells or % cell numbers. Results are normalized to DMSO control. Values represent the mean ± s.d. (n = 4).

Figure S2. Validation of the selected compounds in J-LAT A2 cells. J-LAT A2 cells were co-treated with compounds purchased from different resources at indicated concentrations and TNFα (10 ng/ml) for 24 hr. The % GFP-positive cells were

determined and normalized to the DMSO control. MPA, mycophenolic acid; LSM, Levosimendan; SPR, spironolactone.

Figure S3. Validation of mycophenolic acid's effect in HIV-1 latency cell lines. Different cell lines (J-LAT 6.3, J-LAT 10.6, CA5, EF7) were co-treated with mycophenolic acid at the indicated concentrations and TNFα (10 ng/ml) for 24 hr. The % GFP-positive cells were determined and normalized to the DMSO control. The values represent mean ± s.d. (n = 4). MPA, mycophenolic acid.

Figure S4. Cell viability assay for the selected compounds in the cells used for this study. (A-F) The HIV-1 latency cell lines (J-LAT 6.3, J-LAT 10.6, CA5, EF7) were co-treated with compounds at the indicated concentrations and TNFα (10 ng/ml) for 24 hr. JLTRG cells were pretreated with the compounds at the indicated concentrations for 6 hr and then transduced with Tat expressing retroviruses for 48 hr. The Jurkat cells were treated with the compound alone at the indicated concentrations for 24 hr. The % cell viability was measured and normalized to the DMSO control. Values represent the mean ± s.d. (n ≥ 3). (G) $CD4^+$ T cells isolated from 3 cART-treated, HIV-infected aviremic patients were treated with levosimendan or spironolactone (10 μM) for 3 days. Cell viability was measured using LIVE/DEAD™ Fixable Green Dead Cell Stain Kit. Results were normalized to DMSO control.

Figure S5. Levosimendan does not induce cell apoptosis. (A) HeLa cells stably expressing Tat-Flag were treated with levosimendan (10 μM) for 24 hr. Taxol (500 nM) was used as a positive control. Total cell lysates were subjected to immunoblotting using an anti-PARP antibody that detects both the full length and the cleaved PARP, or an anti-GAPDH antibody. LSM, Levosimendan.

Table S1. Complete compound screening results. J-LAT A2 cells were treated with each compound (5 μM) and TNFα (10 ng/ml). The % GFP-positive cells and % cell

numbers for each compound were determined and normalized to the DMSO control. Values represent the mean ± s.d. (n = 4).

Table S2. Effect (IC_{50}, CC_{50}) of 30 tested compounds in J-LAT A2 cells. IC_{50} and CC_{50} values were calculated based on the titration experiments for each compound in J-LAT A2 cells. Flow cytometry data was used for Levosimendan, Spironolactone, 9-aminoacridine, Mycophenolic acid, and Mycophenolate mofetil (Figure 2A and S2A). Immunostaining data was used for other 25 compounds (Figure S1 and Table S1). IC_{50}; 50% inhibitory concentration, CC_{50}; 50% cytotoxic concentration.

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Figure 1

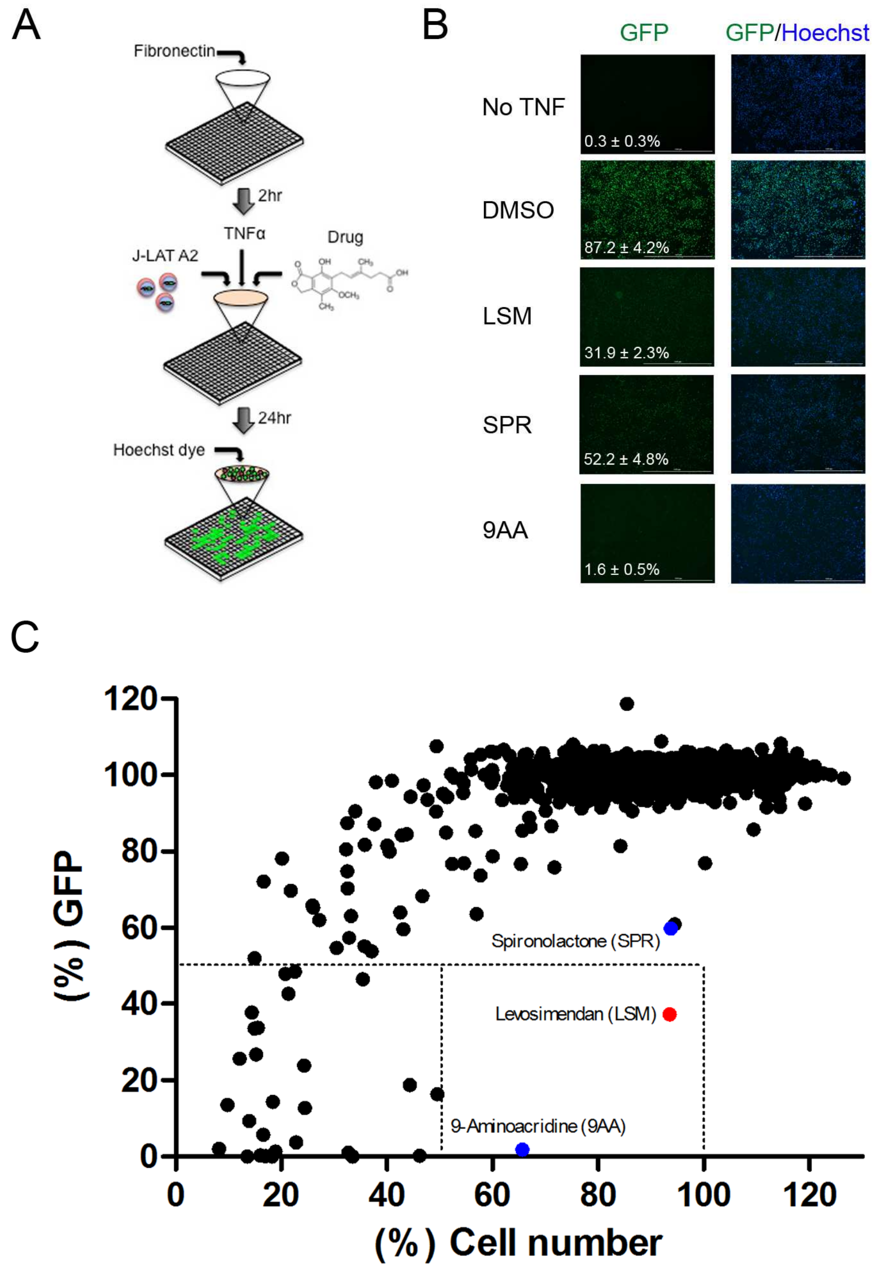

Figure 2

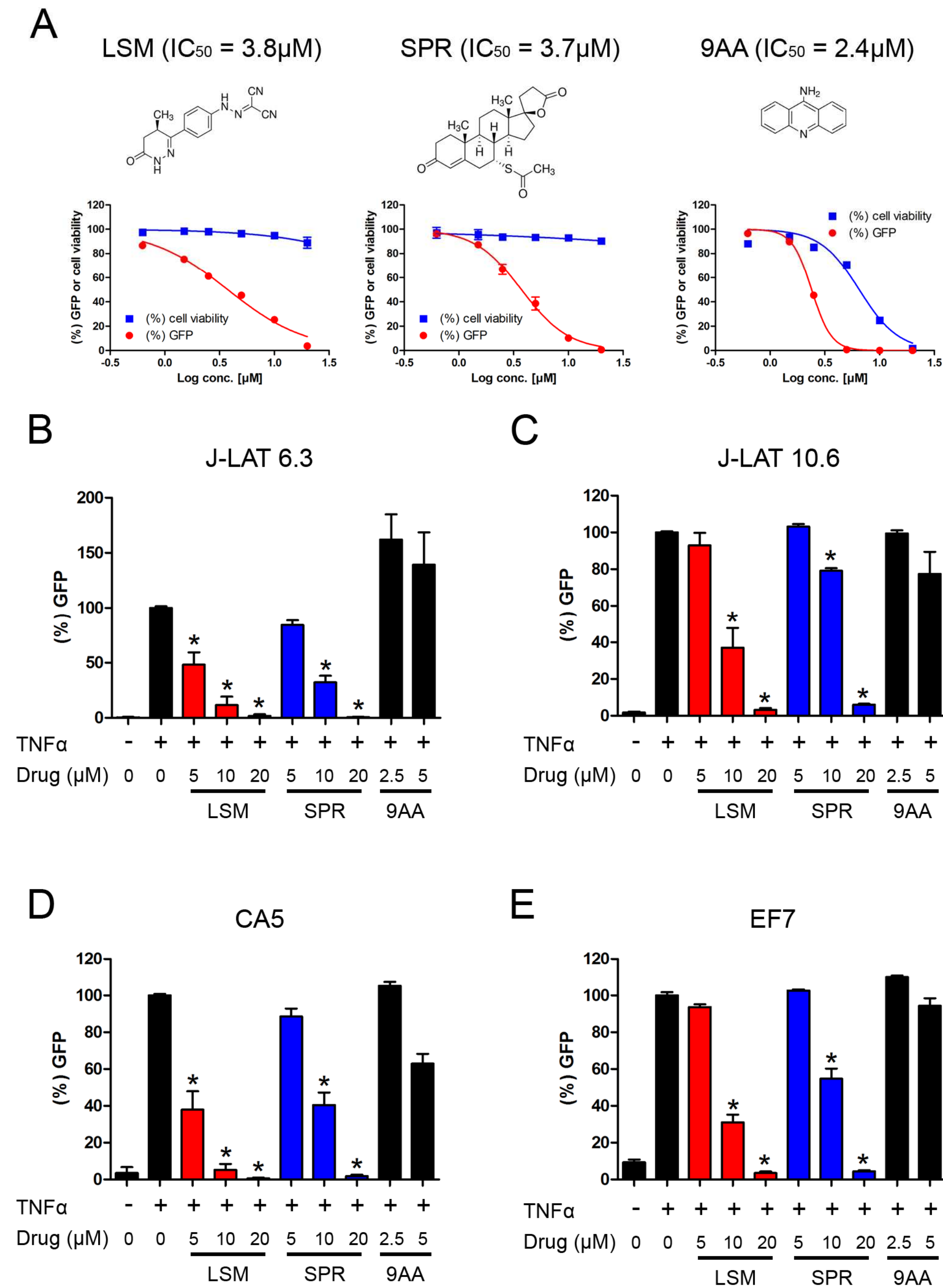

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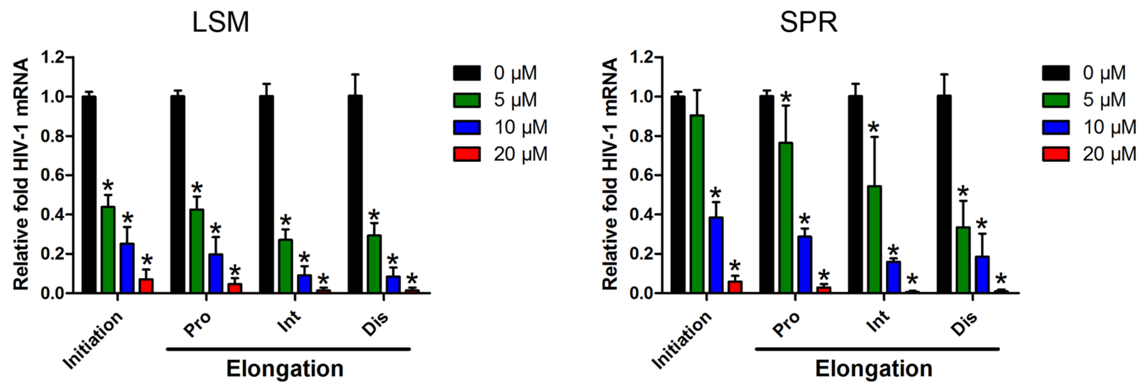
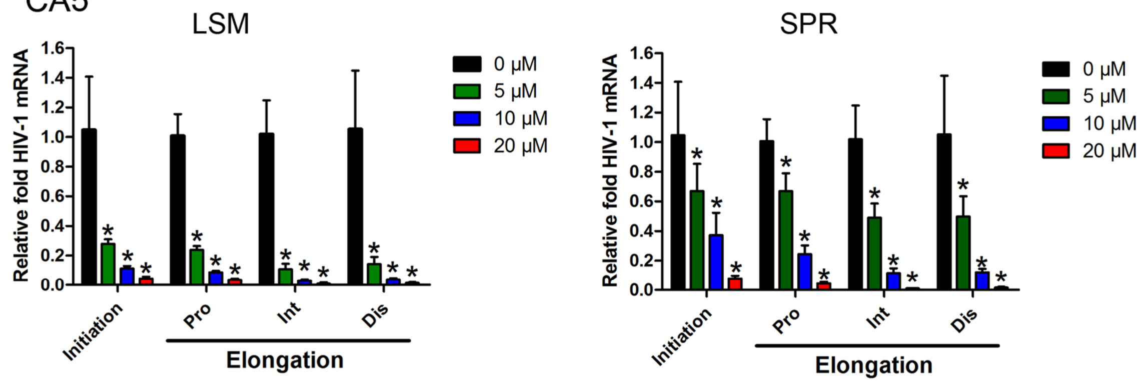
Figure 3

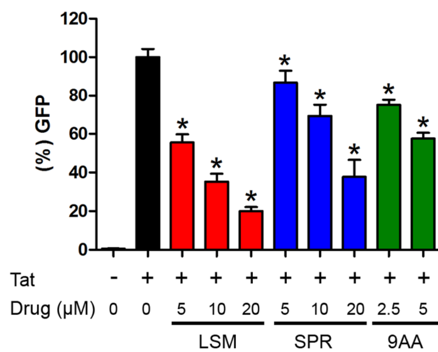
A J-LAT 10.6



B CA5



C JLTRG



D Jurkat

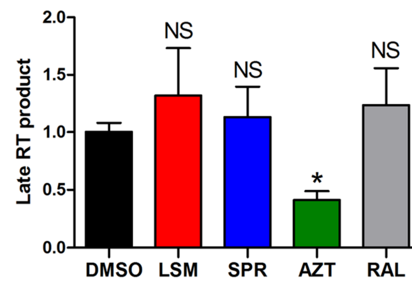


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Figure 4

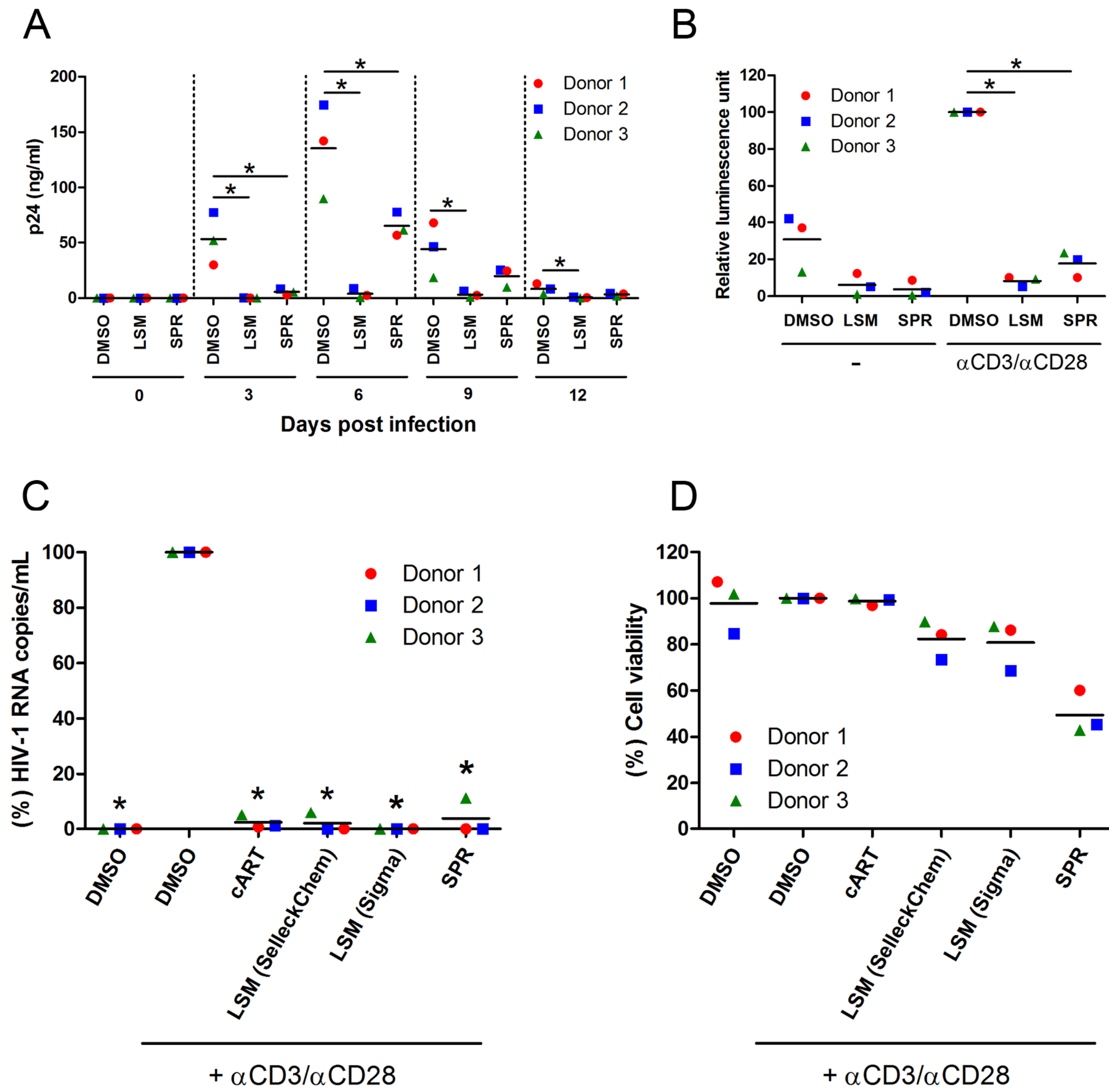

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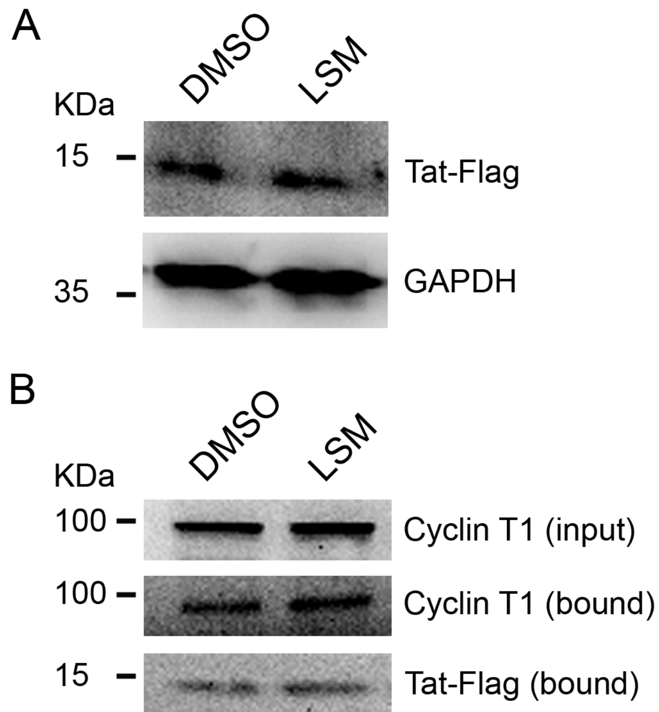
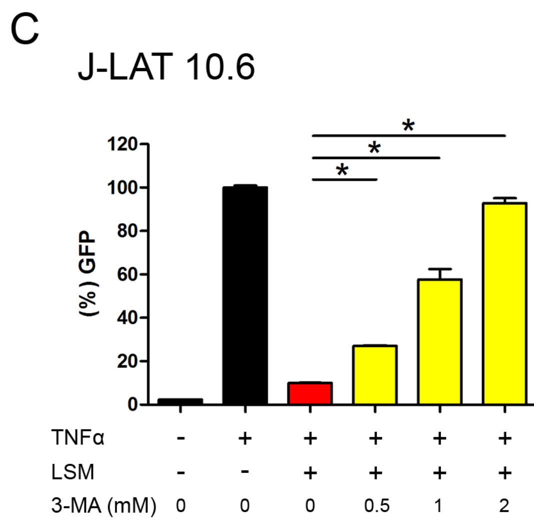
Figure 5





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- 978 FDA-approved compounds were screened for their ability to block TNFα-induced HIV-1 reactivation.
- Levosimendan was identified as a novel and leading anti-HIV-1 inhibitor from the screenings.
- Levosimendan blocked HIV-1 reactivation in $CD4^+$ T cells isolated from cART-treated, HIV-infected aviremic patients.
- Levosimendan suppressed HIV-1 Tat-LTR mediated transcription, which was rescued by inhibition of the PI3K pathway.

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