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Dual effect of the broad spectrum kinase inhibitor midostaurin in acute and latent HIV-1 infection

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A B S T R A C T

Midostaurin is a multi-kinase inhibitor with antineoplastic activity. We assessed the capacity of midostaurin to affect early and late steps of HIV-1 infection and to reactivate HIV-1 latently infected cells, alone or in combination with histone deacetylase inhibitors (HDACi) known to act as latency-reversing agents (LRA). Acute HIV-1 infection was assessed by flow cytometry in three cell types treated with midostaurin in the presence or absence of SAMHD1. Non-infected cells were treated with midostaurin and harvested for Western blot analysis. Macrophage infections were also measured by quantitative RT-PCR. HIV-1 latency reactivation was assessed in several latency models. Midostaurin induced G2/M arrest and inhibited CDK2, preventing the phosphorylation of SAMHD1 associated to inhibition of its dNTPase activity. In the presence of SAMHD1, midostaurin blocked HIV-1 DNA formation and viral replication. However, following Vpx-mediated SAMHD1 degradation, midostaurin increased viral transcripts and virus replication. In three out of four HIV-1 latency models, including primary $CD4^+$ T cells, midostaurin effectively reversed HIV-1 latency and was synergistic in combination with LRA vorinostat and panobinostat. Our study describes a dual effect for midostaurin in HIV-1 infection, antiviral or proviral depending on SAMHD1 activation, and highlights a role for active SAMHD1 in regulating the activity of potential HIV-1 latency reversal agents.

1. Introduction

Highly active therapy of the human immunodeficiency virus (HIV) has transformed a terminal illness into a manageable disease (Broder, 2010). However, a cure for HIV has yet to be found. A major hurdle to the eradication of HIV infection is the persistent infection through a latent virus reservoir for which current therapy is ineffective. Purging of latent reservoir through latency reversing agents (LRA), followed by selective destruction of infected cells, the so called shock and kill has been thought as a possible strategy. Despite current LRA showing efficacy in reactivating HIV-1, so far all efforts have failed to reduce the latent virus reservoir in infected individuals (Blazkova et al., 2012; Kim et al., 2017). There is a critical need for compounds that not only potently reactivate latently infected cells, but also lead to the death of these reactivated cells (Kim et al., 2017) while preventing further rounds of infection of bystander cells.

Recent studies have shown that agents targeting protein kinase (PK) C are highly potent in inducing latent HIV-1 expression from the viral reservoirs (Spivak et al., 2015, 2018; Vemula et al., 2017) and protecting primary $CD4^+$ T cells from HIV-1 infection through down-modulation of their HIV coreceptor expression (Cary et al., 2016; Guochun and Satya, 2015; Kim et al., 2017; Xing et al., 2012). There are multiple molecular mechanisms that contribute to the establishment of HIV latency, such as the down-regulation of transcription factors required for transcription like nuclear factor κB (NF-κB) (Tietjen et al., 2018; Williams et al., 2007) or the chromatin remodeling by histone deacetylases (HDAC), whose inactivation by HDAC inhibitors, or other types of agents (Vargas et al., 2019), boost HIV-1 reactivation (Barton et al., 2014). Therefore, a combination of compounds targeting different mechanisms may have synergistic effects in activating latent HIV-1 expression.

Midostaurin (PKC142) is a tyrosine kinase inhibitor, including the FMS-like tyrosine kinase 3 (FLT3), and was approved in 2017 by the FDA for the treatment of acute myeloid leukemia (AML) with mutations on FLT3 (Levis, 2017; Stone et al., 2017). However, midostaurin can also inhibit a broad spectrum of serine/threonine kinases, including cyclin-dependent kinases (CDK 1 and 2) (Begemann et al., 1998). Inhibition of CDK modulates the activation of the HIV-1 restriction factor SAMHD1 (Ballana and Este, 2015), controlling the dNTP pool required for HIV-1 reverse transcription (Badia et al., 2016a; Bermejo et al., 2016; Pauls et al., 2014a). CDK inhibitors have been revealed as potent antiviral agents through the activation of SAMHD1-dependent

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degradation of dNTPs (Badia et al., 2017; Pauls et al., 2014a). Inhibition or downregulation of SAMHD1 allows for effective virus replication in non-proliferating and resting $CD4^+$ T cells (Baldauf et al., 2012; Hrecka et al., 2011; Laguette et al., 2011). Additionally, SAMHD1 has been shown to modulate the reactivation of HIV-1 latency in $CD4^+$ T cells (Antonucci et al., 2018). Thus, we hypothesized that midostaurin may effectively exert diverse effect on HIV-1 replication and reactivation from latency.

Here, we show the dual effect of midostaurin in acute and latent HIV-1 infection. These findings provide evidence for the development of a new anti-HIV strategy, aimed at simultaneously reactivating HIV-1 and preventing further rounds of infection through the selective modulation of SAMHD1 function.

2. Materials and methods

2.1. Cells

Peripheral blood mononuclear cells (PBMC) from buffy coats of healthy donors were obtained by Ficoll-Paque density gradient centrifugation and used for fresh purification of $CD4^+$ T lymphocytes, naïve $CD4^+$ T lymphocytes or monocytes by negative selection (StemCell Technologies). Purity of the populations was confirmed by flow cytometry. 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. $CD4^+$ T lymphocytes were kept in complete culture medium (RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 100 U/ml penicillin, 100 µg/mL streptomycin (Gibco, Life Technologies)) and rIL-2 (6.5 IU/mL, Roche). Monocytes were cultured in complete culture medium and differentiated to monocyte-derived macrophages (MDM) for 4 days in the presence of monocyte-colony stimulating factor (M-CSF, Peprotech) at 100 ng/mL. The human cell lines ACH-2 (Clouse et al., 1989), Jurkat (J-Lat) (Jordan et al., 2003) clone 9.2, THP-1, HEK293T and MT-4 cells were obtained from AIDS Reagent Program, National Institutes of Health (Germantown, MD). All cell lines were grown in complete culture medium, as the primary cells, and maintained at 37 °C in a 5% CO_2 incubator.

2.2. Viruses and virus infections

R5-tropic HIV-1 strain BaL was grown in stimulated PBMC and titrated for use in MDMs. The HIV-1 viral strain NL4–3, expressing green fluorescent protein (GFP), was obtained from the MRC Centre for AIDS Reagents (London, UK). The envelope-deficient HIV-1 NL4-3 clone (HIG) 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 (Badia et al., 2016b; Pujantell et al., 2018). For the production of viral-like particles carrying Vpx (VLP-Vpx), HEK293T cells were cotransfected with pSIV3 + (Mangeot et al., 2000) and a VSV-G–expressing plasmid. Three days after transfection, supernatants were harvested, filtered, and stored at −80 °C. The protocol for production of HIV-1 NL4-3 virus modified to carry Vpx (NL4-3*Vpx) was performed as described previously (Baldauf et al., 2012). In some cases, viral stocks were concentrated using a Lenti-X concentrator (Clontech).

MDM were infected with HIG in the presence or not of VLP-Vpx and viral replication was measured 48 h later by quantification of GFP + expression by flow cytometry. Alternatively, macrophages were infected with HIV-1 BaL in the presence or not of VLP-Vpx. There is a lag time during which completion of HIV-1 reverse transcription and/or integration is not detectable in MDMs (Bejarano et al., 2018; Surdo et al., 2018). Thus, viral replication was measured 18 h later for total viral DNA, 42 h after infection for integrated viral DNA and 66 h after infection for viral transcripts. Viral replication was quantified by two-step quantitative real-time PCR (qPCR).

THP-1 (SAMHD1 +) and MT-4 (SAMHD1-) cells were infected with HIG and transduced with VLP-Vpx. Viral replication was measured 48 h later for the MT-4 and 72 h later for the THP-1 cells. Viral replication was quantified as percentage of GFP expression by flow cytometry.

2.3. Generation of latently infected cells

Latently infected cells (J-Hig) were generated following a modified protocol (Li et al., 2016). Briefly, cells were generated after acute infection of $CD4^+$ Jurkat cells with HIG and maintained in culture for 10 days to allow for the attrition of productively infected cells.

Latently infected primary $CD4^+$ T cells were generated according to the cytokine-polarized primary T cells model of latency (Badia et al., 2015; Garcia-Vidal et al., 2017) with few modifications. Briefly, naïve $CD4^+$ T cells were activated with αCD3/αCD28 antibodies (1 µg/mL each; BD, Madrid, Spain) and supplemented with TGFβ1 (10 µg/mL, Peprotech), αIL-12 (2 µg/mL) and αIL-4 (1 µg/mL, Peprotech). Medium supplemented with rIL-2 (6.5 IU/mL, Roche) was replaced every 3 days. After 7 days of activation, $CD4^+$ T cells were infected with NL4-3*Vpx by spinoculation (1200 × *g*, 1 h 30 min at 37 °C). Three days later, GFP negative cells containing both latently infected and uninfected cells were sorted using a FACSAria II flow cytometer (BD Biosciences).

2.4. Compounds

Midostaurin was purchased from Sigma-Aldrich, vorinostat (VOR) was purchased from Prochifar srl (Italy) and panobinostat (PNB) was purchased from LC Laboratories. Antiretroviral agent 3-azido-3-deoxythymidine (zidovudine; AZT) was obtained from the NIH AIDS Research and Reference Reagent Program. The integrase inhibitor raltegravir (RAL) was received from Merck Sharp and Dome (MSD, Spain). All compounds were resuspended in DMSO and stored at −20 °C until use.

2.5. Immunoblot

Treated cells were rinsed, lysed, subjected to SDS-PAGE and transferred to a PVDF membrane as previously described (Garcia-Vidal et al., 2017). The following antibodies were used for immunoblotting: anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:5000; Pierce); anti-human Hsp90 (1:1000; 610418, BD Biosciences); anti-SAMHD1 (1:2500; ab67820; Abcam); anti-GAPDH (1:2500; ab9485; Abcam); anti-phospho-pRB (Ser807/811; 9308); anti-pRB (9309); anti–phospho-CDK2 (Thr160; 2561), anti-CDK2 (2546); anti-phospho-SAMHD1 (Thr592; 15038) and anti-p21 (2947) (all 1:1000; Cell Signaling Technologies).

2.6. Flow cytometry

For evaluation of cell death, cells were stained for 30 min with LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (Invitrogen, Thermo Fischer Scientific) in PBS according to manufacturer's instructions. Alternatively, viable cells were identified according to forward and side laser light scatter flow cytometry analysis as described(Pujantell et al., 2016). Cells were washed and fixed in 1% formaldehyde before the analysis.

For cell cycle analysis, cells were treated with 7-aminoactinomycin D (7AAD; Sigma-Aldrich) and pyronin Y (Sigma-Aldrich) as described previously (Badia et al., 2016b; Pujantell et al., 2016). Flow cytometry assays were performed in a FACS LSR II or a FACSCanto II flow

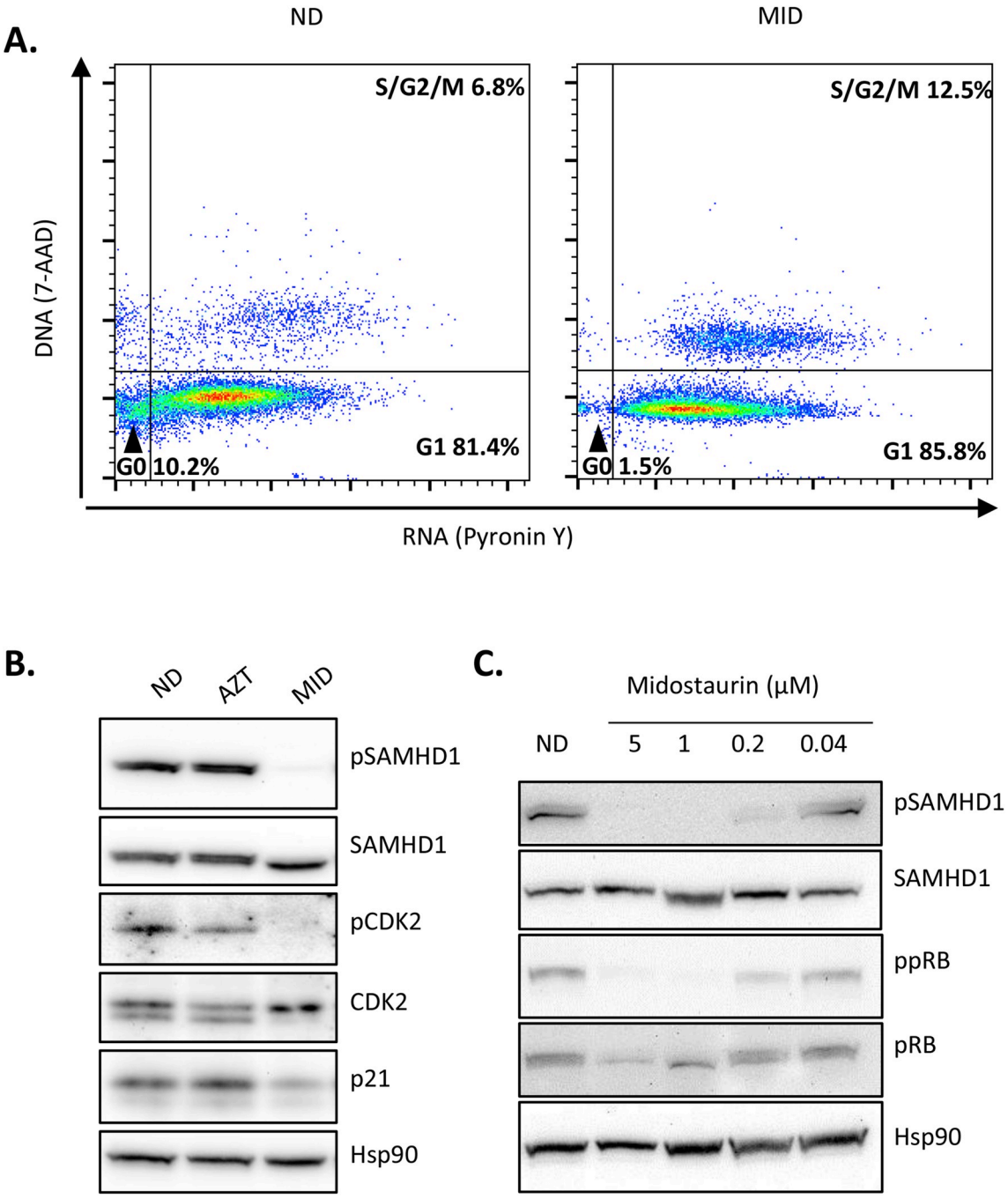

Fig. 1. Effect of midostaurin on cell cycle progression. (A) Cell cycle of treated primary monocyte-derived macrophages (MDM) untreated or treated for 24 h with midostaurin at 5 μM was characterized in a flow cytometer by staining RNA with pyronin Y and DNA with 7-aminoactinomycin D (7AAD). Cells in G0 phase are the ones with low amount of RNA and DNA (lower-left quadrant). In G1 phase the RNA is increased (lower -right quadrant), while in G2-M phase both RNA and DNA are increased (upper-right quadrant). Numbers in each quadrant represent the percentage of the population located in that phase. (B) Expression of cell cycle proteins from 24 h MDM untreated or treated with zidovudine (AZT; 0.2 μM) or midostaurin (MID; 5 μM) was assessed by immunoblot. p21, phospho-CDK2 (pCDK2, active form), CDK2, SAMHD1 and phospho-SAMHD1 (pSAMHD1, inactive form) expression levels were analyzed. Hsp90 was used as a loading control. (C) MDM untreated or treated with midostaurin at different concentrations (5–0.04 μM) for 24 h were harvested for protein analysis by immunoblot. pRB, phospho-pRB (ppRB), SAMHD1 and pSAMHD1 expression levels were analyzed. Hsp90 was used as a loading control. A representative experiment is shown. ND; No drug, AZT; zidovudine, MID; midostaurin.

cytometer (BD Biosciences). The data was analyzed using the FlowJo software (BD Biosciences).

The cell separation of the latently infected primary naïve $CD4^+$ T cells was performed by the Flow Cytometry facility at the Germans Trias i Pujol Research Institute, using a FACSAria II cell separator (BD Biosciences).

2.7. Quantification of total viral DNA, integrated provirus and viral transcripts

MDM were infected with HIV-1 BaL and infections were stopped after 18 h to measure total viral DNA or at 42 h to measure viral integration by two-step quantitative Real-Time PCR as previously described (Pujantell et al., 2017). LTR amplification for total and

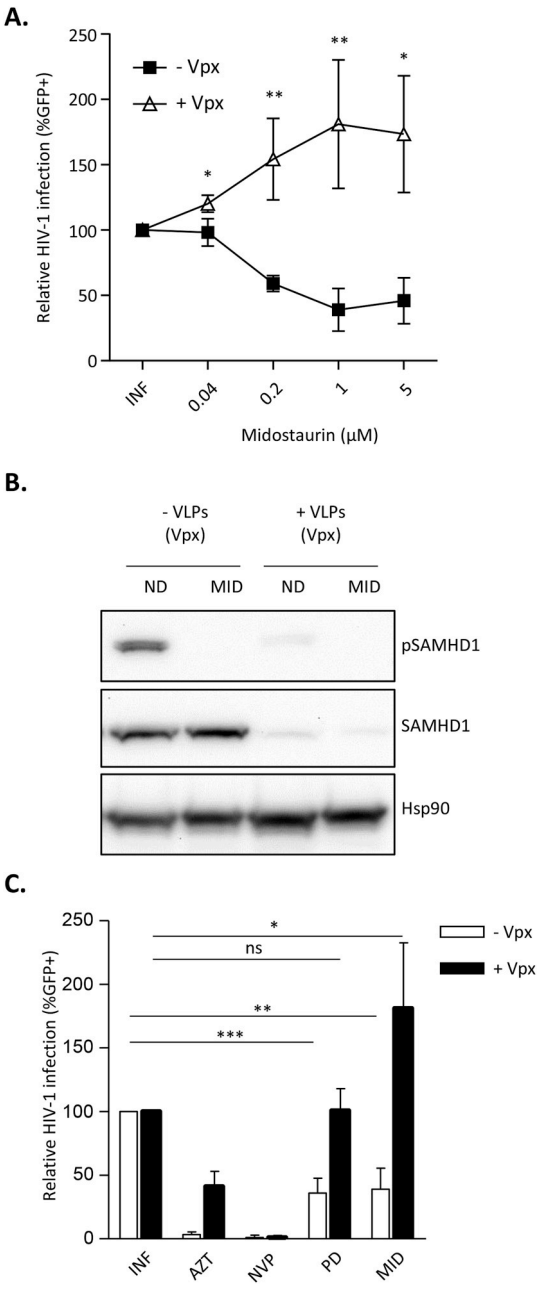

Fig. 2. SAMHD1 expression modulates the effect of midostaurin in acute HIV-1 infection in monocyte-derived macrophages. (A) HIV-1 infection in the presence of midostaurin (5–0.008 μM) in untreated MDM (- Vpx) or Vpx-induced degradation (+Vpx) of SAMHD1 MDM. (B) SAMHD1 and pSAMHD1 expression levels in MDM untreated (ND) or treated with midostaurin (MID; 5 μM) in the presence or not of viral-like particles carrying Vpx (+/− Vpx). Hsp90 was used as a loading control. (C) Effect of zidovudine (AZT; 0.2 μM), nevirapine (NVP; 5 μM), palbociclib (PD; 5 μM) and midostaurin (MID; 1 μM) in the presence (- Vpx) or absence (+Vpx) of SAMHD1. Infection was quantified as percentage of GFP + cells by cell cytometry at 48 h post infection and normalized to the infected non-treated (INF) condition. A representative experiment is shown in (B). Values represent mean ± SD of three independent donors performed in duplicate in (A) and (C). Statistical significance was assessed by comparing the same conditions with and without SAMHD1 in (A) or comparing the treated conditions with the infected non-treated control (INF) in (C). ND; No drug, MID; midostaurin, INF; infected, AZT; zidovudine, NVP; nevirapine, PD; palbociclib. ns (non-significant) $p > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

integrated DNA was performed using the following primers and probe: forward 5′-GACGCAGGACTCGGCTTG-3′, reverse 5′-ACTGACGCTCTC GCACCC-3′, and probe FAM 5′-TTTGGCGTACTCACCAG-3′ TAMRA. A pre-amplification Alu-LTR was performed for the integrated DNA with the following primers: forward 5′-GCCTCCCAAAGTGCTGGGATTA CAG-3′ and reverse 5′-TTGCCCATACTATATGTTTTAA-3. The reverse

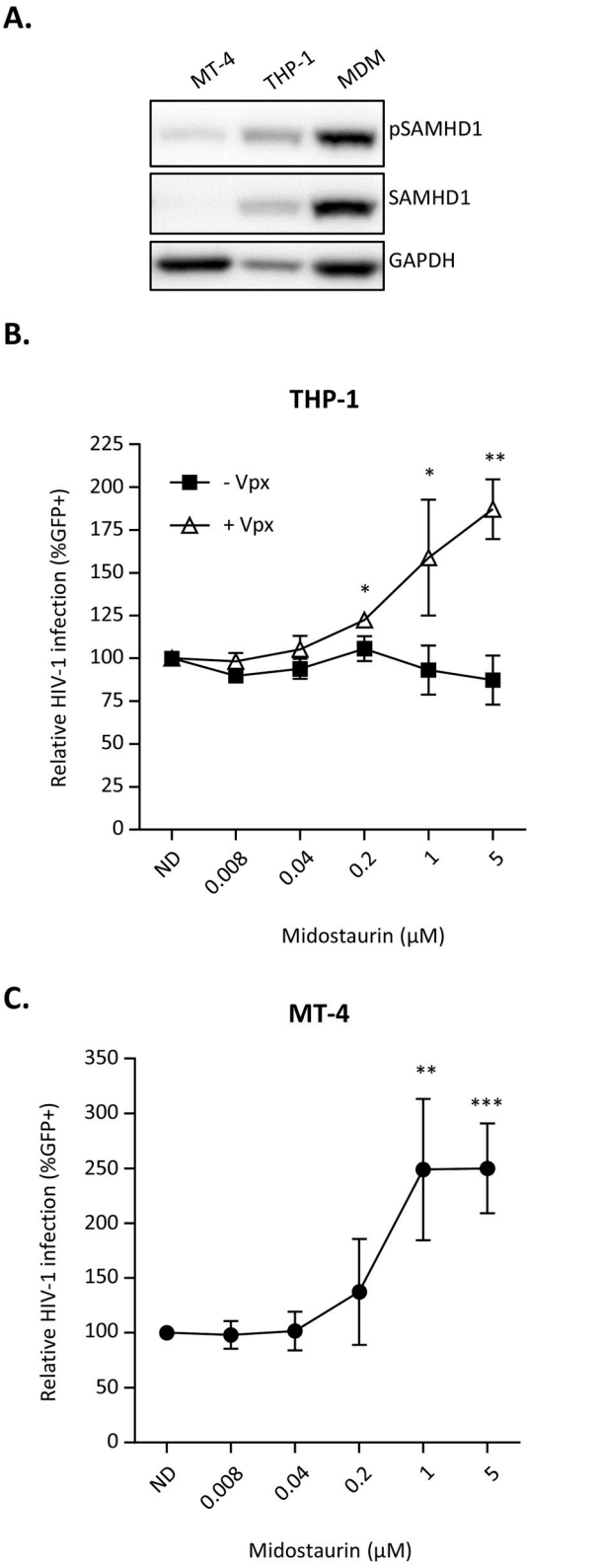

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Fig. 3. Midostaurin enhances HIV-1 infection in the absence of SAMHD1. (A) Protein levels of SAMHD1 and phospho-SAMHD1 in the leukemic cell lines MT-4 and THP-1 and in primary monocyte-derived macrophages (MDM). GAPDH was used as a loading control. (B) HIV-1 infection in THP-1 with (- Vpx) or without (+Vpx) SAMHD1 and (C) MT-4 in the presence of midostaurin (5–0.008 μM). Infection was quantified as percentage of GFP + cells by cell cytometry 72 h post infection (THP-1) or 48 h post infection (MT-4) and normalized to the infected no-drug (ND) condition. A representative experiment is shown in (A). Values represent mean ± SD of at least three independent experiments performed in duplicate in (B and C). Statistical significance in (B) was assessed by comparing the same conditions with and without SAMHD1 in the THP-1 cells or in (C) by comparing the treated conditions with the no drug control (ND) in the MT-4 cells. ND; No drug. *P < 0.05; **P < 0.01; ***P < 0.001.

transcriptase (RT) inhibitor zidovudine (1 μg/mL) and the integrase inhibitor raltegravir (1 μg/mL) were used as negative controls for the total DNA and integrated DNA, respectively.

HIV-1 transcripts were quantified after the reverse transcription using the following primers and probe: forward 5′-GGATCTGTCTCTGTCTCT CTCTCCACC-3′, reverse 5′-ACAGTCAGACTCATCAAGTTTCTCTATCAAA GCA-3′ and the dual-labeled fluorescent probe FAM 5′-TTCCTTCGGGC CTGTCGGGTCCC-3′ TAMRA. All infections were normalized to an untreated control in the presence or absence of VLP-Vpx. All samples were run in duplicate on a 7500 Real-Time PCR System (Applied Biosystems) Real-Time PCR instrument. Cycling conditions were as follows: 50 °C for 2 min followed by 95 °C for 10 min for polymerase activation, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.8. HIV-1 reactivation in vitro in latently infected cells

HIV-1 reactivation was measured as described before (Badia et al., 2015; Garcia-Vidal et al., 2017). Briefly, clonal J-Lat or J-Hig cells were incubated for 24 h with subtoxic concentrations of midostaurin (Table S1). LRAs panobinostat and vorinostat were used as controls for HIV-1 reactivation. Reactivation of HIV-1 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, a T cell latent model with one integrated proviral copy, were cultured for 48 h in the presence or absence of LRA and reactivation was measured by the production of HIV CAp24 antigen using Genscreen HIV-1 Ag ELISA (BioRad) according to manufacturer's instructions.

Sorted latently infected/GFP negative naïve $CD4^+$ T cells were incubated for 24 h with subtoxic concentrations of midostaurin (Table S1). Treatment with anti-CD3 and anti-CD28 was used as reactivation control. Previously, cells were washed with PBS after the sorting and kept in fresh media containing rIL-2 overnight at 37 °C and 5% CO2. Reactivation was measured as the percentage of GFP positive cells by

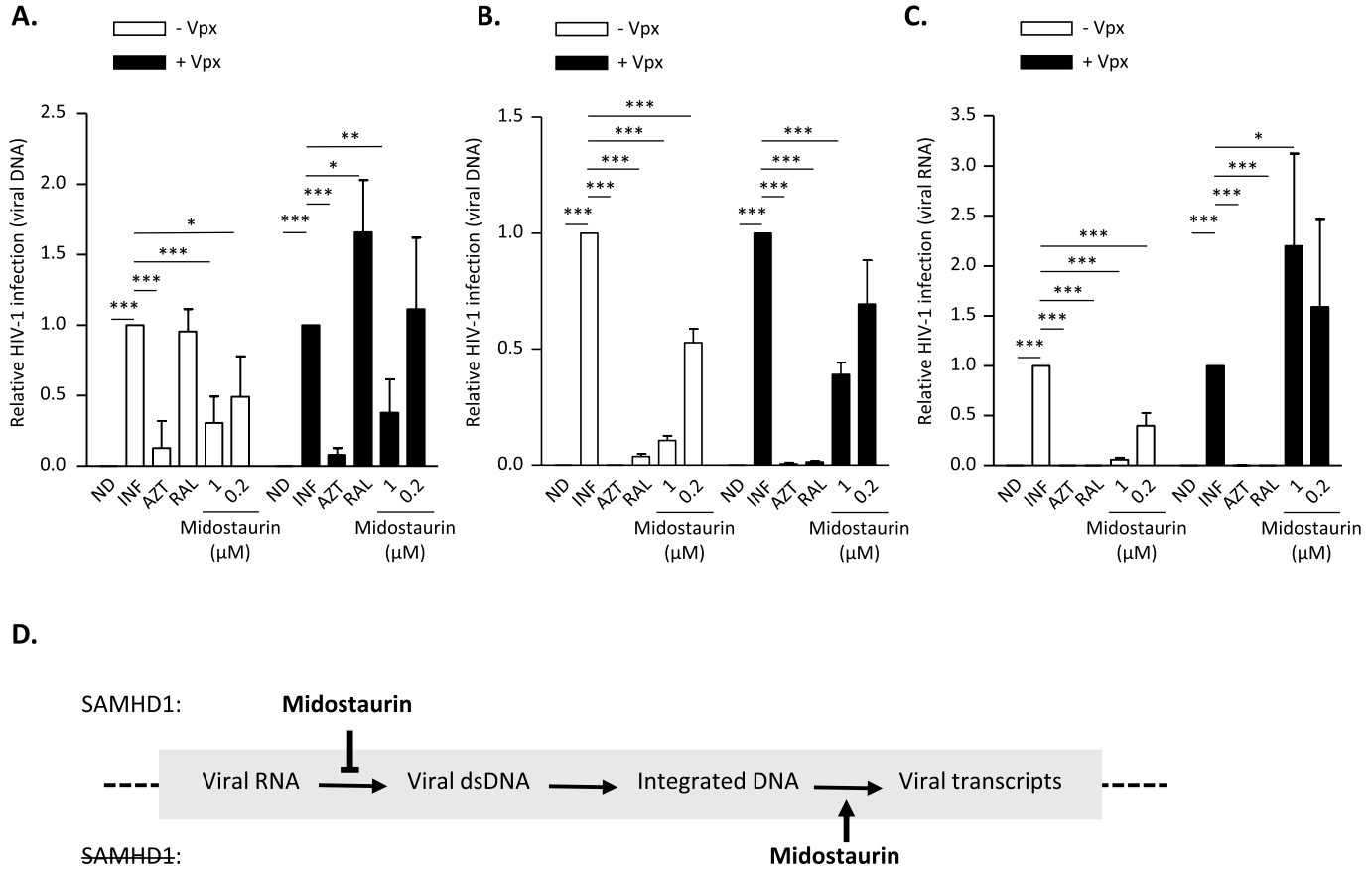

Fig. 4. Effect of midostaurin on HIV-1 infection at different stages of the viral cycle. (A) Total viral DNA, (B) integrated viral DNA and (C) transcribed viral RNA in primary monocyte-derived macrophages (MDM) infected with HIV-1 BaL. Infection was quantified by quantitative RealTime-PCR after DNA (A, B) or RNA (C) extraction at different times: 18 h post-infection for the total viral DNA, 42 h post-infection for the integrated viral DNA and 66 h post-infection for the transcribed viral RNA. Infections were performed in the presence (- Vpx) or Vpx-induced degradation (+Vpx) of SAMHD1. Zidovudine (AZT, 1 μg/mL) was used as a control for assessing HIV-1 reverse transcription step. Raltegravir (RAL, 1 μg/mL) was used as a control for assessing HIV-1 integration step. (D) Schematic representation of the dual-effect of midostaurin in the viral cycle of HIV-1 infected MDM with (SAMHD1) or without SAMHD1. Values represent mean ± SD of at least three independent donors performed in duplicate in (A), (B) and (C). Statistical significance in (A), (B) and (C) was assessed by comparing the conditions with the infected non-treated control (INF). ND; No drug, INF; infected, AZT; zidovudine, RAL; raltegravir. *P < 0.05; **P < 0.01; ***P < 0.001.

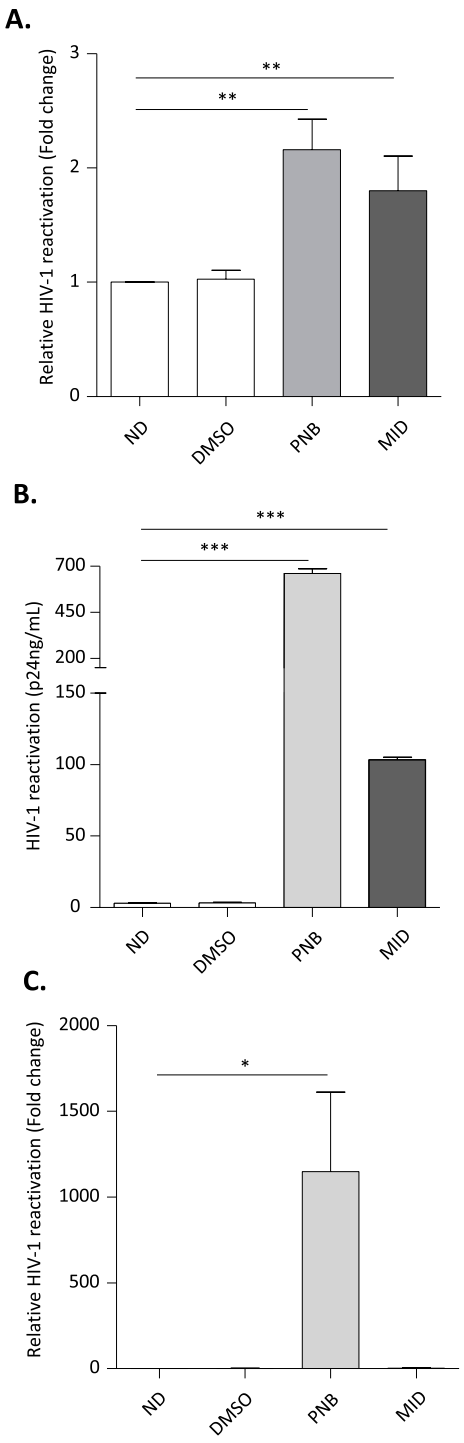

Fig. 5. Midostaurin reactivates HIV-1 latent virus in ACH-2 cells and in a non-clonal Jurkat-derived latent model, but not in J-Lat clone 9.2 cells. (A) HIV-1 reactivation in the latently infected Jurkat cells, J-Hig. HIV-1 reactivation was quantified as the percentage of GFP + cells by flow cytometry 24 h after cell treatment with midostaurin (MID; 5 μM). Panobinostat (PNB; 0.16 μM) was used as a positive control. Dimethyl sulfoxide (DMSO) was used at the same concentration present in the midostaurin condition to exclude nonspecific reactivation effects. (B) HIV-1 reactivation in the HIV-1 latent model ACH-2. Cells were treated as in (A) for 48 h and the supernatant was collected. Reactivation was measured as CAp24 quantification in the supernatant by an ELISA. (C) HIV-1 reactivation in the HIV-1 latent cell model J-Lat clone 9.2. Experiment was performed as in (A). Values represent mean ± SD of three independent experiments performed in triplicate. Data was normalized to the ND condition in (A) and (C). Statistical significance in (A), (B) and (C) was assessed by comparing the treated conditions with the no drug control (ND). ND; No drug, DMSO; dimethyl sulfoxide, PNB; panobinostat, MID; midostaurin. *P < 0.05; **P < 0.01; ***P < 0.001.

flow cytometry.

2.9. Statistical and mathematical analysis

Combination index (CI) was calculated by introducing the reactivation percentages in the CompuSyn software (ComboSyn Inc., Paramus, NJ, USA). Synergistic effect was considered when CI values were below 1. Data are presented as mean ± standard deviation (SD). All p-values were calculated using a t-Student's test calculated with the GraphPad PRISM software (GraphPad Software, San Diego, CA, USA). A p-value of 0.05 was considered to be statistically significant.

3. Results

3.1. Midostaurin-mediated modulation of cell cycle prevents phosphorylation and inhibition of SAMHD1 in primary macrophages

To confirm the effect of midostaurin in cycle progression (Begemann et al., 1998; Fabbro et al., 2000; Kawai et al., 2015), MDM were evaluated for DNA and RNA content as a measure of the distinct cell phases: G0, G1 and G2-M. In comparison to the untreated control (6.8%), a higher percentage of MDM treated with midostaurin (12.5%) were stopped in the G2-M phase (Fig. 1A), as observed by the increase DNA and RNA content. In parallel, protein from 24 h-treated macrophages was harvested for Western-blot analysis. Midostaurin significantly impaired CDK2 activation, as measured by specifically detecting phosphorylation of CDK2 (pCDK2) or by the disappearance of the corresponding band with an anti-CDK2 antibody (Fig. 1B). Additionally, midostaurin decreased the expression of p21, a natural CDK2 inhibitor, known to affect SAMHD1 function (Pauls et al., 2014c; Valle-Casuso et al., 2017). These changes were accompanied by an expected inhibition of the phosphorylated forms of pRB and SAMHD1 (Fig. 1C), both targets of CDK2.

3.2. Midostaurin effect in acute HIV-1 infection is dependent on SAMHD1 expression

We have previously shown that pharmacological inhibition of CDK prevents SAMHD1 phosphorylation and blocks HIV-1 replication (Pauls et al., 2014a, 2014b). In primary MDM, midostaurin showed anti-HIV-1 activity (50% effective concentration, EC_{50}: 0.416 μM) at subtoxic concentrations (Table S1) that was abrogated following Vpx-dependent degradation of SAMHD1 (Fig. 2A and B), similarly to the CDK4/6 inhibitor palbociclib (Pauls et al., 2014a). However, a significant ($p = 0.04$ at 1 μM) stimulatory effect on HIV-1 replication was revealed with midostaurin in the absence of SAMHD1, not observed with palbociclib (Fig. 2C).

The effect of midostaurin was also tested in transformed monocytoid THP-1 cells and proliferating lymphoid MT-4 cells, which showed differential basal levels of SAMHD1 (Fig. 3A). As observed in macrophages, the stimulatory effect in virus replication of midostaurin in the SAMHD1-expressing THP-1 cells was only revealed following SAMHD1 degradation ($p = 0.001$ at 5 μM, comparing −+/− Vpx conditions) (Fig. 3B, left graph). As for the MT-4 cells with an already negligible SAMHD1 expression, midostaurin alone was enough to increase viral replication ($p = 0.03$ at 1 μM and $p = 0.0003$ at 5 μM, compared to untreated conditions) (Fig. 3B, right graph).

3.3. Midostaurin affects different steps of the HIV-1 replication cycle

To understand the role of midostaurin on HIV-1 replication, we evaluated its effect at different steps of HIV-1 replication in the presence or absence of SAMHD1. MDM were treated with VLPs carrying Vpx, incubated with midostaurin (1 and 0.2 μM), infected with the fully replicative R5-tropic HIV-1 strain BaL and collected at different times post-infection. Midostaurin blocked HIV-1 total DNA formation, resembling the effect of the reverse transcriptase

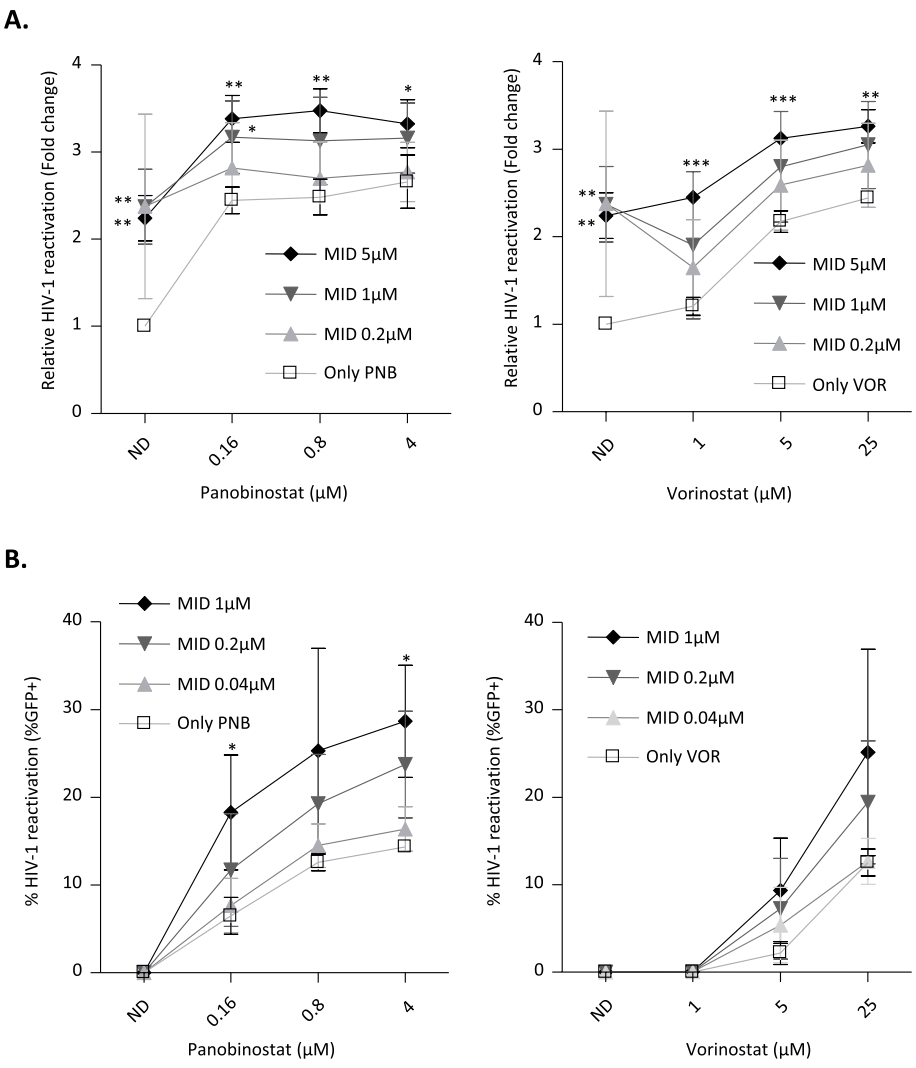

Fig. 6. HIV-1 reactivation by panobinostat and vorinostat is enhanced by midostaurin. (A) HIV-1 reactivation in J-Hig cells treated for 24 h with increasing concentrations of midostaurin (5–0.2 μM) and the HDACi panobinostat (PNB; 4–0.16 μM) (left panel) or vorinostat (VOR; 25-1 μM) (right panel). Reactivation was measured as the percentage of GFP + cells by flow cytometry. (B) HIV-1 reactivation in J-Lat clone 9.2 cells. The experiments and the reactivation quantification were performed as in (A). Values represent mean ± SD of three independent experiments. Data was normalized to the ND condition in (A). Statistical significance in (A), (B), (C) and (D) was assessed by comparing the midostaurin-treated conditions with the conditions treated just with the HDACi (Only PNB or Only VOR). ND; No drug, PNB; panobinostat, VOR; vorinostat. **P < 0.01; ***P < 0.001.

Table 1
Combinatory index (CI) of midostaurin and HDAC inhibitors in J-Lat clone 9.2 cells.

		Midostaurin					
		CI (1 μM)		CI (0.2 μM)		CI (0.04 μM)	
Vorinostat	25 μM	0.545	Synergy	0.669	Synergy	0.916	Additive
	5 μM	0.226	Synergy	0.268	Synergy	0.327	Synergy
Panobinostat	4 μM	0.011	Synergy	0.039	Synergy	0.366	Synergy
	0.8 μM	0.005	Synergy	0.028	Synergy	0.148	Synergy
	0.16 μM	0.008	Synergy	0.098	Synergy	0.957	Additive

CI values were obtained from the CompuSyn software. Classifications used were: CI > 1 antagonism, CI = 1 additive, CI < 1 synergism. Data represent the mean of at least two independent evaluations.

inhibitor AZT (Fig. 4A), integrated DNA (Fig. 4B) and viral RNA transcripts were decreased in the presence of SAMHD1. However, these effects were lost in SAMHD1-depleted (transduced with VLP-Vpx) cells and an increased number of transcripts ($p = 0.044$ at 1 μM) was detected in VLP-Vpx-treated cells (Fig. 4C). Taken together these results indicate that midostaurin blocks the early formation of viral DNA, prior to integration, in a SAMHD1-dependent manner and has a proviral effect at the later time of transcription of new viral RNA (Fig. 4D).

3.4. Midostaurin reverses HIV-1 latency and enhances HDACi-mediated latency reversal

The enhancing effect of midostaurin in acute viral transcription suggests that midostaurin may help revert HIV-1 latency. HIV-1 reactivation was observed in the non-clonal model J-Hig when cells were incubated with midostaurin ($p = 0.0099$) (Fig. 5A). Midostaurin-mediated HIV-1 reactivation was also observed in the clonal model ACH-2 ($p < 0.0001$), although the effect was less potent compared to that of panobinostat ($p < 0.0001$) (Fig. 5B). In the clonal model J-Lat clone 9.2, midostaurin did not show any effect at 5 μM, in contrast to the reactivation observed with the HDAC inhibitor (HDACi) panobinostat at 0.16 μM, a known HIV-1 LRA (Fig. 5C). In J-Hig cells, midostaurin enhanced the reactivation observed with vorinostat or panobinostat (up to 2.4-fold compared to the HDACi alone) (Fig. 6A and Fig. S1A). However a synergic increase in reactivation was observed when midostaurin was combined with either vorinostat or panobinostat (Fig. 6B and Fig. S1B) (Table 1).

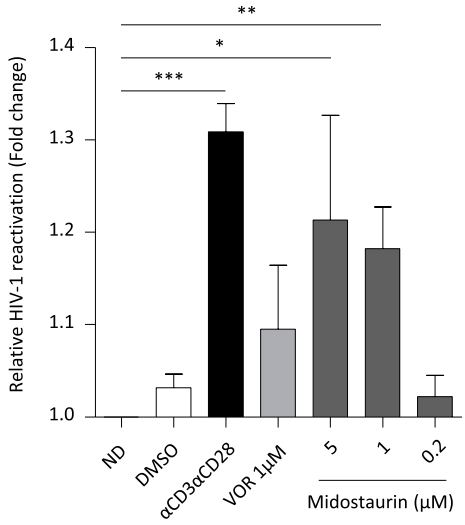

Fig. 7. Midostaurin reactivates latent HIV-1 latent in a model of primary CD4$^+$ T cells. Latently infected naïve CD4$^+$ T cells isolated from peripheral blood mononuclear cells (PBMCs) from healthy donors were incubated for 24 h with different concentrations of midostaurin (5-0.2 μM). HIV-1 reactivation was quantified as percentage of GFP + cells by flow cytometry. The HDACi vorinostat (VOR; 1 μM) was used as a positive control for HIV-1 reactivation. Antibodies αCD3αCD28 were used as a control of cell activation and expansion and HIV-1 reactivation. Dimethyl sulfoxide (DMSO) was used at the same concentration present in the condition with higher DMSO content (VOR 25 μM, data not shown) to exclude nonspecific HIV-1 reactivation. Values represent mean ± SD of three independent donors performed in triplicate. Data was normalized to the ND condition. Statistical significance was assessed by comparing the treated conditions with the no drug control. ND; No drug, DMSO; dimethyl sulfoxide, αCD3αCD28; antibodies αCD3αCD28, VOR; vorinostat. *P < 0.05; **P < 0.01; ***P < 0.001.

The activity of midostaurin to reactivate HIV-1 latency was also assessed in a latent model of primary naïve CD4$^+$ T cells. A significant increase in HIV-1 reactivation was observed for midostaurin ($p = 0.03$) (Fig. 7) in the absence of the acute toxicity observed with known LRA (Fig. S2).

4. Discussion

There is no effective cure for HIV-1 infection yet. Identification of molecules able to induce HIV-1 reactivation and inhibit further infection may help developing new strategies for HIV-1 eradication. In this study, we used several cellular models to assess the effect of the multikinase inhibitor midostaurin in both acute and latent HIV-1 infection. Using a flow cytometry assay we identified a dual effect of midostaurin in HIV-1 acute infection: inhibition of virus DNA formation following reverse transcription and a later proviral effect at increasing viral transcripts (Fig. 8). Midostaurin had already surfaced, in a screen of over 1500 small molecules and kinase inhibitors, as an enhancer of viral transcription through a mechanism involving NF-κB signaling (Ao et al., 2016). Here, we extend this finding to other models of HIV-1 latency, including latently-infected primary resting CD4$^+$ T cells, and provide evidence of the role of the virus restriction factor SAMHD1 in the activity of midostaurin.

SAMHD1 is a major restriction factor of HIV-1 infection in resting CD4$^+$ T cells (Baldauf et al., 2012). Overcoming this restriction by degrading SAMHD1 through expression of HIV-2 Vpx and/or RNA interference has been used in attempts to identify correlates of HIV-1 latency and/or reactivation (Badia et al., 2018; Descours et al., 2017). Moreover, overexpression of wild-type SAMHD1 was shown to suppress HIV-1 LTR-driven gene expression at a transcriptional level (Antonucci et al., 2018), indicating a possible role of SAMHD1 function in regulation viral and cellular transcription. In turn, degradation of SAMHD1, increases HIV-1 replication (Hrecka et al., 2011; Laguette et al., 2011) and negatively affects the antiviral potency of nucleoside analogues commonly used in the treatment of HIV-1 (Amie et al., 2013; Ballana et al., 2014). Pharmacological activation of SAMHD1 (Ballana and Este, 2015) increased expression of host factors that activate it such as p21 (Abdel-Mohsen et al., 2015; Chen et al., 2011; Pauls et al., 2014c; Valle-Casuso et al., 2017) and higher SAMHD1 expression (Fu et al., 2016; Riveira-Munoz et al., 2014) appears also to affect HIV-1 infection.

Here, we show that midostaurin blocked CDK2 activation, a kinase known to phosphorylate SAMHD1 (Pauls et al., 2014c; Valle-Casuso et al., 2017), providing evidence of the mode of action of the anti-HIV-1 activity of midostaurin, i.e. activation of SAMHD1 and restriction of HIV-1 through inhibition of HIV DNA formation. This inhibition was completely lost, and even reversed to a proviral effect, upon SAMHD1 degradation, indicating that the antiviral effect of midostaurin depends on SAMHD1 function. Alternative mechanisms of action, although not formally excluded, were not apparent in our cell culture conditions. Our analysis showed that midostaurin also increased HIV-1 transcription and reversed HIV-1 latency in a non-clonal model of virus latency. This increase in HIV-1 transcription may be due to a NFκB-dependent mechanism, as suggested by other authors before (Ao et al., 2016). However, we did not observe any reactivation in the J-Lat cell clone 9.2. An explanation for this apparently contradictory result may lie in the insertion site of the virus in those cells. LRA can differentially target and reactivate HIV depending on the viral insertion site (Chen et al., 2017), indicating that the specific integration site in J-Lat clone 9.2 cels may not be accessible to the activity of midostaurin, and partly explaining the combined effect of midostaurin with the LRAs panobinostat and vorinostat. Effective reactivation of latent HIV-1 may come from combinations of different but possibly overlapping LRAs able to target HIV-1 provirus integrated at different genomic locations. Future studies involving the sequencing of the midostaurin-reactivated provirus and their insertion sites may prove helpful to corroborate our assumptions on the differential specificity of midostaurin on HIV-1 insertion sites.

Thus, agents conveyed of a SAMHD1-dependent antiviral activity that at the same time promote virus reactivation from latently infected cells could provide an alternative to effectively purge the virus reservoir. Curiously, histone deacetylase inhibitors (HDACi) such as vorinostat, have been shown to induce a SAMHD1-dependent block to HIV-1 (Mlcochova et al., 2017), revealing an intricate mechanism associated to cell cycle control and eventual cell death that could be exploited for the selective killing of latently infected cells.

Resting CD4$^+$ T cells with an active (unphosphorylated) form of SAMHD1 are the major reservoir driving HIV-1 persistence. Nevertheless, acute infection of monocyte-derived macrophage and their susceptibility to antiviral drugs as well as to subtle variation in SAMHD1 function provide an excellent model for the evaluation of new strategies to purge and eliminate the HIV-1 reservoir that can be later translated to T-cell models of virus persistence.

In conclusion, we identified and characterized the effect of midostaurin in both acute and latent HIV-1 infection. This compound showed dual but opposing effects by inhibiting HIV-1 reverse transcription in acute HIV-1 infection and enhancing viral transcription in SAMHD1-depleted cells or in latently infected cells, reversing HIV-1 latency and enhancing the potency of known HDAC inhibitors. Our results suggest that the use of agents with similar properties to that of

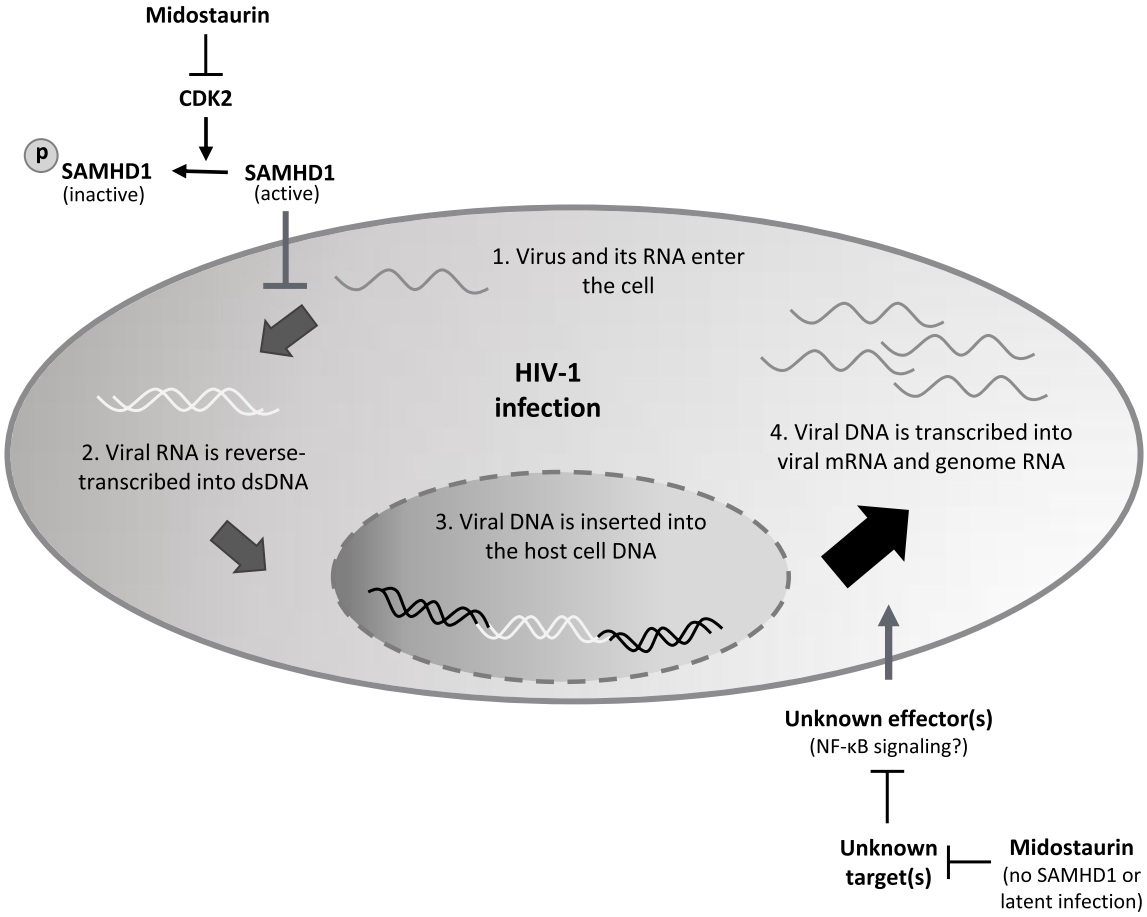

Fig. 8. Proposed model for the effect of midostaurin in HIV-1 infection. In the presence of the HIV-1 restriction factor SAMHD1, midostaurin prevents its phosphorylation and inactivation by inactivating CDK2. Thus, leading to a SAMHD1-restricted pool of dNTPs that prevents HIV-1 reverse transcription. Aside from the effect on SAMHD1, the multi-kinase inhibitor midostaurin is also able to inhibit other proteins. The inhibition of one or more of those targets leads to a direct or indirect increase in HIV-1 transcription observed in both HIV-1 acute and latent infection. However, this effect is only noticeable when SAMHD1 is not performing its function due to inactivation, degradation or in latent infection, where the virus is already integrated in the host genome. As proposed in previous studies, midostaurin-mediated increase in HIV-1 transcripts could be the response to an increase in the NF-κB signaling.

midostaurin could be useful to develop new anti-HIV-1 strategies, by reactivating the HIV-1 latent reservoir while preventing subsequent rounds of infection.

Conflicts of interest

We declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.05.003.

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