Dasatinib Inhibits HIV-1 Replication through the Interference of SAMHD1 Phosphorylation in CD4+ T Cells

Mercedes Bermejo[1,ƒ], María Rosa López-Huertas[1,ƒ], Javier García-Pérez[1], Núria Climent[2], Benjamin Descours[3], Juan Ambrosioni[4], Elena Mateos[1], Sara Rodríguez-Mora[1], Lucía Rus-Bercial[1], Monsef Benkirane[3], José M. Miró[4], Montserrat Plana[2], José Alcamí[1], Mayte Coiras[1*]

[ƒ]Both authors contributed equally to this work

[1]AIDS Immunopathology Unit, National Center of Microbiology, Instituto de Salud Carlos III, Madrid, Spain.

[2]Retrovirology and Viral Immunopathology Laboratory, AIDS Research Group, Institut d´Investigacions Biomèdiques August Pi I Sunyer (IDIBAPS), Hospital Clínic, University of Barcelona, Barcelona, Spain.

[3]Laboratory of Molecular Virology, Institute of Human Genetics, Montpellier, France.

[4]Infectious Diseases Service, AIDS Research Group, Institut d´Investigacions Biomèdiques August Pi I Sunyer (IDIBAPS), Hospital Clínic, University of Barcelona, Barcelona, Spain.

[*]Corresponding author at: AIDS Immunopathology Unit, National Center of Microbiology, Instituto de Salud Carlos III, Ctra. Majadahonda-Pozuelo km2, 28220 Madrid, Spain. Tel: +34-918223782; fax: +34-915097966; E-mail address: mcoiras@isciii.es

ABSTRACT

Massive activation of infected CD4+ T cells during acute HIV-1 infection leads to reservoir seeding and T-cell destruction. During T-cell activation, the antiviral effect of the innate factor SAMHD1 is neutralized through phosphorylation at T592, allowing HIV-1 infection. Dasatinib, a tyrosine kinase inhibitor currently used for treating chronic myeloid leukemia, has been described to control HIV-1 replication through its negative effect on T-cell proliferation and viral entry. We demonstrate that Dasatinib can actually interfere with SAMHD1 phosphorylation in human peripheral blood lymphocytes, preserving its antiviral activity against HIV-1. Dasatinib prevented SAMHD1 phosphorylation in vitro and ex vivo, impairing HIV-1 retrotranscription and proviral integration. This was the major mechanism of action because the presence of Vpx, which degrades SAMHD1, in HIV-1 virions impeded the inhibitory effect of Dasatinib on HIV-1 replication. In fact, infection with VSV-pseudotyped HIV-1 virions and fusion of BlaM-Vpr-containing HIV-1 viruses with activated PBMCs in the presence of Dasatinib suggested that Dasatinib was not acting at fusion level. Finally, PBMCs from patients on chronic treatment with Dasatinib showed lower level of SAMHD1 phosphorylation in response to activating stimuli and low susceptibility to HIV-1 infection ex vivo. Consequently, Dasatinib is a compound currently used in clinic that preserves the antiviral function of SAMHD1. Using Dasatinib as adjuvant of antiretroviral therapy during early primary HIV-1 infection would contribute to reduce viral replication and spread, prevent reservoir seeding, and preserve CD4 counts and CTL responses. These events would create a more favorable virologic and immunologic environment for future interventional studies aiming at HIV-1 eradication.

1. Introduction

Infection by the human immunodeficiency virus (HIV) remains a major public health concern all over the world. The start of combination antiretroviral therapy (cART) accomplished the goal of reducing AIDS mortality [1;2], but it cannot completely eliminate the virus from the organism. HIV infection is now a chronic disease that needs life-long treatment, with the subsequent emergence of co-morbidities related to side effects of cART, drug resistances, and the burden on health-care systems that were initially designed for acute care [3]. As cART is not enough to completely eliminate the virus from the organism, new strategies are being developed to eradicate the infection. This aim can only be achieved if the viral reservoirs, responsible for viral rebound during cART interruption, are completely eradicated [4;5], or a significant reduction achieved [6]. Therefore, a strategy of "kick and kill" has been initiated by several laboratories, testing different compounds to reactivate and destroy latent reservoirs [7], but none have proved to be effective so far [8]. However, early cART in primary infection results in smaller viral reservoirs and the increased possibility of viral control by the immune system [9;10]. As the smaller the size of the reservoir the easier could be its elimination, we proposed a concomitant strategy to reduce the size of the reservoir from the beginning of the infection by interfering with T-cell activation. In particular, we targeted the activity of protein kinase C (PKC) theta (θ) [11;12] that is one of the key regulators of signal transduction during T-cell activation [13]. Blocking PKCθ reduces HIV-1 replication in CD4+ T cells, providing a refractory state to HIV-1 infection [12]. However, none of the selective PKCθ inhibitors currently under development have begun clinical testing yet. Consequently, we searched for other cellular targets upstream of the PKCθ signaling pathway, trying to find other suitable candidates.

PKCθ activation occurs by phosphorylation at different residues and subsequent translocation to the plasma membrane [14], which is mediated by a tripartite interaction with CD28 and the tyrosine-protein kinase $p56^{lck}$ [15]. $p56^{lck}$ is an Src family tyrosine kinase (SFK), predominantly –but not exclusively– expressed in T cells [16], that is essential for transmitting signals from the TCR signaling complex [17]. $p56^{lck}$ is activated after TCR engagement, which leads to the phosphorylation of tyrosine 394 (Y394) in the catalytic domain and induces its kinase activity [18]. $p56^{lck}$ activation causes the phosphorylation of tyrosine residues present within immunoreceptor tyrosine-based activation motifs (ITAMs) [19], followed by downstream calcium flux and expression of T-cell activation markers such as CD69 and CD40 ligand/CD154 [20]. $p56^{lck}$ induces the phosphorylation of PKCθ at threonine 538 (T538) by MAP4K3/GLK [14], which is widely used as a surrogate marker for PKCθ kinase activation [21], and at tyrosine 90 (Y90), which leads to the activation of downstream effectors essential for T-cell function and HIV-1 transcription such as NF-κB, NFAT, and AP-1 [21-26]. As the activity of PKCθ is greatly dependent on $p56^{lck}$, we reasoned that $p56^{lck}$ inhibitors could exert a blocking effect on HIV-1 replication similar to the inhibition of PKCθ. In this regard, the influence on HIV-1 replication of the ATP-competitor Dasatinib has already been analyzed by some groups [27;28]. Dasatinib is an oral small molecule inhibitor of non-receptor tyrosine kinases such as Abl and SFK - including $p56^{lck}$ [29;30], that is currently used in clinic for treating patients with chronic myeloid leukemia (CML) [31-34]. Dasatinib impairs TCR-mediated signal transduction, cellular proliferation, cytokine production, and in vivo T-cell responses [35;36], although chronic treatment with Dasatinib does not increase susceptibility to fungal or viral infections [37].

It has been described that the mechanism of action of Dasatinib to thwart HIV-1 replication in CD4+ T cells was to blockade the viral entry [27], as well as by its anti-proliferative effect [38]. However, Pogliaghi et al. [28] observed that Dasatinib was only effective to inhibit HIV-1 replication in peripheral blood lymphocytes (PBMCs) from infected patients when they were activated with phytohemagglutinin (PHA) and interleukin-2 (IL-2) for less than 3 days before treatment with Dasatinib because after this time Dasatinib could not completely shut down viral replication. Accordingly, mitogenic T-cell activation might be triggering some mechanism able to block Dasatinib-mediated inhibition of HIV-1 replication. In this work, we demonstrate by fusion assays of BlaM-Vpr-containing HIV-1 viruses that Dasatinib was ineffective to stop HIV fusion and that its main mechanism of action was actually the interference of the phosphorylation of SAMHD1 (sterile alpha motif domain and HD domain-containing protein 1), which was induced after T-cell activation with PHA and IL-2 for at least 2 days. SAMHD1 is a key regulator of cell cycle progression and a major viral restriction factor that blocks early reverse transcription of HIV-1 genome by depleting the intracellular dNTP pool [39;40] and by degrading viral RNA (vRNA) through its RNase activity [41]. The function of SAMHD1 is regulated through the phosphorylation of threonine 592 (T592) by cyclin A2/Cdk1, an event that is induced by T-cell activation and that renders the cells susceptible to HIV-1 infection [42]. The accessory protein Vpx of HIV-2 and the simian immunodeficiency virus (SIV) targets SAMHD1 for ubiquitination and proteasomal degradation [43]. As HIV-1 does not encode Vpx, it remains sensitive to SAMHD1-mediated restriction until the T cell receives an activation signal. Evidence that the major mechanism of action of Dasatinib for inhibiting HIV-1 replication was through the protection of SAMHD1 activity was that

Dasatinib did not significantly affect HIV-1 infection in CD4+ T cells when the virions carried Vpx.

Accordingly, through the preservation of SAMHD1 antiviral activity, Dasatinib impaired HIV-1 retrotranscription and, consequently, strongly affected proviral integration. The effect of Dasatinib on SAMHD1 phosphorylation in vivo was confirmed by low levels of phosphorylated SAMHD1 observed in CD4+ T lymphocytes from CML patients on chronic treatment with Dasatinib that were activated ex vivo. Moreover, PBMCs from these CML patients were also resistant to HIV-1 infection.

2. Materials and methods

2.1. Cells and patients' samples

Peripheral blood lymphocytes (PBMCs) were isolated from blood of untreated healthy donors by centrifugation through a Ficoll-Hypaque gradient (Pharmacia Corporation, North Peapack, NJ). Human CD4+ T lymphocytes were isolated from PBMCs by using CD4+ T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. Cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM L-glutamine, 100μg/ml streptomycin, 100U/ml penicillin (Biowhittaker, Walkersville, MD). PBMCs were activated by treatment with 5μg/ml PHA (Sigma-Aldrich, St. Louis, MO) and 300 units/ml IL-2 (Chiron, Emeryville, CA) for 48 hours. Then, they were maintained in culture with IL-2. Jurkat E6-1, MT-2 and TZM-bl cells were obtained from the NIH AIDS Reagent Program [44]. Jurkat E6-1 and MT-2 cells were cultured in RPMI 1640 medium supplemented as described above. TZM-bl cells were grown in DMEM medium with 10% fetal bovine serum supplemented with penicillin/streptomycin and 4 mM Lglutamine.

PBMCs from three treated chronic asymptomatic HIV-1 individuals were obtained at the Hospital Clinic (Barcelona, Spain). These patients showed baseline CD4+ T lymphocyte counts > 500 cells/mm^3 and plasma viral loads ranging from 50-10,000 HIV-1 RNA copies/mL. All individuals gave informed written consent and this study was approved by the Institutional Ethical Committee board of Hospital Clinic (Barcelona, Spain).

PBMCs from five HIV-negative, CML Phi Chromosome-positive patients receiving Dasatinib treatment were also obtained at the Hospital Clinic (Barcelona,

Spain). All of them had more than 2 years of follow-up from CML diagnosis and were taking Dasatinib for at least two years. All patients were on hematological remission and none of them presented previous or ongoing serious adverse events related to Dasatinib use, neither infectious complication related to their hematological disease or to the treatment with Dasatinib. All of them had normal routine blood and biochemistry test at sampling. Table 1 summarizes the main clinical characteristics of CML patients. All individuals who participated in this study gave informed written consent.

2.2. Reagents and antibodies

Dasatinib (BMS-354825, Sprycel; Bristol-Meyers Squibb, New York, NY) was kindly provided by Dr. Stephen Mason (Discovery Virology, Bristol-Meyers Squibb) and Dr. Carey Hwang (Discovery Medicine - Virology, Bristol-Myers Squibb). Lck Inhibitor II (Merck Millipore, Darmstadt, Germany) is a cell-permeable, ATP binding site-targeting 3-(2-(1H-Benzo[d]imidazol-1-yl)-6-(2-morpholinoethoxy)pyrimidin-4-ylamino)-4-methylphenol that acts as a highly potent $p56^{lck}$ inhibitor (IC_{50} = 3 nM; [ATP] = 10 μM), blocking IL-2 release in Jurkat E6-1 (IC_{50} = 54 nM) after activation with CD3 cross-linking and PMA [45]. Staphylococcal enterotoxin A from *Staphylococcus aureus* was obtained from Sigma-Aldrich. HIV fusion inhibitor T-20 was used at 10μg/ml and was obtained through the NIH AIDS Reagent Program, (Division of AIDS, NIAID, NIH). Purified Staphylococcal enterotoxin A (SEA) was used at 20ng/ml and it was purchased from Serva Chemicals (Heidelberg, Germany).

Antibody against SAMHD1 phosphorylated at T592 was kindly provided by Dr. Monsef Benkirane and Dr. Benjamin Descours (Institute of Human Genetics, Montpellier, France). Antibody against total SAMHD1 was purchased from Bethyl Laboratories (Montgomery, TX). Monoclonal antibodies against Cdk1/Cdc2 (clone

P0H1) and Cyclin A (clone BF683) were obtained from Cell Signaling Technology. Monoclonal antibody against β-actin (clone AC-15) was obtained from Sigma-Aldrich. Polyclonal antibody against phospho-PKCθ (Thr538) was obtained from Cell Signaling Technology (Danvers, MA). Polyclonal antibody against phospho Lck (Y394) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody against phosphotyrosine (clone 4G10) was obtained from Merck Millipore. Monoclonal antibody against HIV-1 Tat (aa 2–9) was obtained from Advanced Biotechnologies Inc. (Columbia, MD). Secondary antibodies conjugated to Alexa 546 and Alexa 488 were purchased from Molecular Probes (Eugene, OR). Secondary antibodies conjugated to horseradish peroxidase (HRP) were purchased from GE Healthcare (Milwaukee, WI). Antibodies against CD4 conjugated to PerCP, chemokine (CXC motif) receptor 4 (CXCR4) conjugated to phycoerythrin, and chemokine (C-C Motif) receptor 5 (CCR5) conjugated to fluorescein isothiocyanate (FITC) were purchased from BD Biosciences.

2.3. Vectors

Vector pNL4-3 wild-type that contains HIV-1 complete genome and induces an infectious progeny after transfection was kindly provided by Dr M.A. Martin [46]. Vector pNL4.3-Renilla was obtained by replacing *nef* gene of HIV-1 proviral clone pNL4.3 with Renilla luciferase gene [47]. Vector pBx08_Renilla plasmid was generated by cloning in pNL4.3-Renilla the gp160 of the Bx08 strain in the place of NL4-3 *env* [48]. Vector pcDNA-VSV contains DNA for vesicular stomatitis virus (VSV) G glycoprotein cloned in the pcDNA3.1 plasmid (Invitrogen) [49]. Full-length HIV-1 DNA not expressing HIV envelope (pNL4.3-Luc-R_E_) was obtained from AIDS Research and Reference Reagent program (NIAID, National Institutes of Health, MD, USA). Vector pIRES2-EGFP-Vpx was kindly provided by Dr Mario Stevenson (Miami Center for

AIDS Research, Miami, FL) [50]. Vector pCMV-BlaM-Vpr encodes β-lactamase fused to the viral protein Vpr and was kindly provided by Dr Bernard Lagane (Institut Pasteur, Paris, 75015, France) [51].

2.4. Immunofluorescence assay

Cells were adhered on PolyPrep slides (Sigma-Aldrich), fixed with 2% paraformaldehyde in PBS1X and permeabilized as previously described [52]. Cells were then stained with specific antibodies against $p56^{lck}$ phosphorylated at Y394, PKCθ phosphorylated at T538 or phosphotyrosine and a secondary antibody conjugated to Alexa 546. Nuclei were stained with 4′,6-diamidino-2-phenylindole (Dapi) (Sigma-Aldrich). Images were obtained with a Leica DMI 4000B Inverted Microscope (Leica Microsystems, Barcelona, Spain).

2.5. Immunoblotting assays

Whole protein extracts were obtained as described previously [53] and protein concentration was determined by the Bradford method [54]. Forty micrograms of protein extracts were fractionated by sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) and transferred onto Hybond-ECL nitrocellulose paper (GE Healthcare). After blocking and incubation with primary and secondary antibodies, proteins were detected with SuperSignal West Pico/Femto Chemiluminescent Substrate (Pierce, Rockford, IL). Images were acquired in a BioRad Geldoc 2000 (BioRad Laboratories, Madrid, Spain). Representative experiments are shown. Densitometry was performed in a Gel Doc 2000 System (BioRad) by using Quantity One software. Gel bands were quantified and background noise was subtracted from the images. The relative ratio of the optical density units corresponding to each sample was calculated

regarding the internal control (β-actin or total SAMHD1) per each lane. Results are represented as bar diagrams showing the statistical significance regarding the control.

2.6. Cell viability and proliferation

Cell viability was determined with CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) using an Orion Microplate Luminometer and Simplicity software (Berthold Detection Systems, Oak Ridge, TN). Cell proliferation was measured with flow cytometry by staining with carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies, Carlsbad, CA) as described previously [55]. Briefly, cells were labeled with CFSE 2μM, and after extensive washing, cells were resuspended in medium and cultured in 96-well round-bottomed plates in the absence or presence of SEA for 6 days. Samples were acquired with a FACSCalibur flow cytometer (Becton Dickinson, San José, CA) and data analysis was performed with FlowJo software v7.2.5 (TreeStar, Ashland, OR) using non-linear curve-fitting techniques. The number of generations (Gn) was fixed at eight peaks, each including the corresponding number of events for each generation.

2.7. HIV-1 infection

Infectious supernatants were obtained from calcium phosphate transfection of HEK293T cells (provided by the existing collection of the Instituto de Salud Carlos III, Madrid, Spain) with plasmids pNL4-3_wt or pNL4-3_Renilla. VSV-pseudotyped ΔEnv-NL4.3-Luc virus was obtained by co-transfection of pNL4.3-Luc-R_E_ and pcDNA-VSV, that express G protein of vesicular stomatitis virus [56;57]. Infectious supernatants of NL4-3_Renilla strain containing SIVsm Vpx protein were generated by transfecting HEK293T cells with pNL4-3_Renilla and pIRES2-EGFP-Vpx vectors in a ratio 1:2. Classic antiviral assays were performed in 96-well plates with PBMCs

previously activated with PHA/IL-2 for two days before adding 0.5 ng of each HIV-1 strain. Cells were then incubated for 48 hours in the presence of serial dilutions of the inhibitors. Infection by spinoculation was performed in PBMCs pre-treated with the inhibitors for 30 minutes before incubating with PHA/IL-2 for 48 hours. Cells were then infected for 30 minutes at gently rotation, room temperature. After centrifugation at 600xg for 30min at 25°C and extensive washing with PBS1X, each inhibitor was added again and cells were left in culture for 2-7 days. Renilla or luciferase activity, corresponding to HIV-1 replication, was quantified in the cell lysates using Renilla Luciferase Assay (Promega) in Orion Microplate Luminometer (Berthold Detection Systems). IC_{50} was calculated using GraphPad Prism Software (sigmoidal dose–response formula). Cell viability was evaluated in cells treated in parallel as described above. Cytotoxic concentrations 50 (CC_{50}) were calculated using GraphPad Prism Software (sigmoidal dose–response formula). The results were expressed using SI (CC_{50}/IC_{50}).

2.8. Enzyme-linked immunospot (ELISPOT) assay for IFN-γ release

Ex vivo measurement of antigen-specific IFN-γ production from CD8+ T cells was done by ELISPOT as previously described [58;59]. Briefly, 96-well plates were coated overnight at 4°C with 15 μg/ml of anti-IFN-γ mAb 1-D1K (Mabtech, Stockholm, Sweden) in coating buffer (Na_2CO_3 0.1M, pH 9.6). Plates were washed and blocked with RPMI 1640/10% FBS. PBMCs from HIV-infected patients were incubated per well in the coated plates for 18 hours. Different HLA class I-restricted synthetic peptides from Gag, Pol, Env and Nef, as well as peptide pools (15-mer peptides overlapping by 11) spanning the entire HIV-1 consensus B Gag proteins sequences (NIH AIDS Reagent Program), were used. A synthetic HLA*02 restricted-CMV pp56 peptide was also tested. RPMI/10%FCS was used as negative control. A pool of MHC

class I-restricted T cell epitopes from human cytomegalovirus, Epstein-Barr virus and influenza virus (CEF, Mabtech), as well as PHA, were used as positive controls. Cells were lysed, incubated with biotin-labelled, anti-IFN-γ mAb 7-B6-1 (Mabtech) and then with streptavidin-alkaline phosphatase (Mabtech) to visualize the spot forming foci (IFN-γ-secreting cells). After adding a chromogenic alkaline phosphatase conjugated substrate (BioRad), the spot forming cells (SFC) were counted using an AID ELISPOT reader (Autoimmun Diagnostica GmHb, Germany). Results were considered positive if the number of SFC/10^6 PBMCs in stimulated wells was 2-fold higher than that in unstimulated control wells, and if there were at least 50 SFC/10^6 PBMCs after background subtraction.

2.9. Quantification of early and late retrotranscription by TaqMan qPCR

Five hours after infection, DNA was extracted using QIAamp DNA Blood Mini Kit (Qiagen) and quantified using Nanodrop 2000C (Thermo Scientific). Early and late retrotranscription (RT) were assessed by qPCR as described by Konig et al [60]. Briefly, 100ng DNA were mixed with 1μM forward and reverse primers for early RT and late RT, 0.2μM FAM-TAMRA probe, and 1xTaqMan Universal Master Mix II (Applied Biosystems). qPCR was performed in triplicate in a StepOne Real-Time PCR system (Applied Byosistems) using standard cycling conditions. Serial dilutions of genomic DNA from 8E5 cell line, which contains a single integrated copy of HIV-1 [61], were used for the standard curve. *ccr5* gene was used as endogenous control.

2.10. Quantification of proviral integration and 2-LTR circles by TaqMan qPCR

Whole genomic nucleic acid was extracted from purified CD4+ T cells as described above. Analysis of 2-LTR circles was performed as described previously [62]. Proviral integrated DNA was quantified by using a nested Alu-LTR PCR as described

previously [63;64] using a StepOne Real-Time PCR System (Applied Biosystems). In brief, a first conventional PCR was performed using oligonucleotides against Alu sequence and the HIV-1 LTR, with the following conditions: 95ºC, 8 min; 12 cycles: 95ºC, 1 min; 60ºC, 1 min; 72ºC, 10 min; 1 cycle: 72ºC, 15 min. Then, a second qPCR was performed using TaqMan probes with FAM/ZEN/Iowa Black and TaqMan Master Mix (Applied Biosystems). DNA from 8E5 cell line was used for the standard curve. *ccr5* gene was used as housekeeping gene for measuring the input DNA and normalize data.

2.11. Flow cytometry analysis

PBMCs from CML patients on treatment with Dasatinib and from untreated healthy donors were activated with PHA/IL-2 for 5 days. Cells were fixed with 1% paraformaldehyde and permeabilized with methanol. After washing, cells were blocked with PBS/BSA 1% and stained with anti-phospho-T592 SAMHD1 and a secondary antibody conjugated to FITC (Dako, Glostrup, Denmark). Cells were then blocked with mouse IgG1 and stained with fluorescent labelled antibodies against CD3 and CD4 (BD Biosciences, San Diego, CA). Flow cytometry acquisition was performed using FACS Calibur cytometer (Becton Dickinsons) and data were analyzed with FlowJo software.

2.12. HIV-1 virion-based fusion assay

HIV-1 particles containing β-lactamase-Vpr chimeric proteins (BlaM-Vpr) were produced by co-transfection of HEK293T cells the different HIV vectors (NL4-3_Renilla, BX08_Renilla, and NL4-3LucR_E_+ pcDNA-VSV) and pCMV-BlaM-Vpr. Following 5 min of incubation with Dasatinib or HIV fusion inhibitor T-20, PBMCs were inoculated with BlaM-Vpr-containing viruses (30 ng p24) by spinoculation at 4ºC, washed and then incubated 2 h at 37 °C in the presence or absence of the drugs. Cells

were washed with CO2-independent medium and then incubated with CCF2/AM dye for 2 h at room temperature in CO2-independent medium supplemented with 10% FBS. After fixation in 2% paraformaldehyde, enzymatic cleavage of CCF2/AM by BlaM was measured by flow cytometry. Data were analyzed with FlowJo software. The percentage of fusion corresponds to the percentage of cells displaying increased cleaved CCF2/AM fluorescence (447 nm).

For drug susceptibility assay, the same PBMCs used for the fusion assay were dispensed in round-bottom 96-well microtiter culture plates in the presence of IL-2. Dasatinib or T-20 was added to the culture medium and cells were infected by spinoculation at 4ºC with equal amounts of viruses as in fusion assay. Cells were washed once and then incubated at 37ºC in the presence or absence of the drugs. At 30 hours post-infection, viral replication was assessed by measuring Renilla luciferase activity as described above.

2.13. Statistical analysis

Statistical analysis was performed using Graph Pad Prism 5.0 (Graph Pad Software Inc., San Diego, CA). Comparisons between groups were made using two-way analysis of variance (ANOVA) with Bonferroni post-test analysis to describe the statistical differences among groups. P values (p) < 0.05 were considered statistically significant in all comparisons and were represented as *, **, ***, or **** for $p<0.05$, $p<0.01$, $p<0.001$, or $p<0.0001$, respectively.

3. Results

3.1. Control of HIV-1 infection by Dasatinib in PBMCs activated with PHA and IL-2

Dasatinib strongly inhibits Abl, Src and other related tirosin kinases [29;30], including $p56^{lck}$. In order to determine whether the antiviral effect of Dasatinib on HIV-1 replication was related to $p56^{lck}$ inhibition, a comparison between Dasatinib and a 2-benzimidazole substituted pyrimidine-based selective inhibitor of $p56^{lck}$ (Lck inhibitor II) was performed. PBMCs previously activated with PHA and IL-2 for 48 hours were then treated for 30 minutes with Dasatinib or Lck Inhibitor II at different concentrations (390 nM to 50 μM) before infection with recombinant HIV-1 NL4-3_Renilla (X4-tropic) or BX08_ Renilla (R5-tropic) strains for 48 hours in the presence of IL-2. Both inhibitors were added to the culture medium only once. Production of Renilla (Relative Light Units, RLUs), corresponding to HIV-1 replication [47], was measured to calculate the half maximal inhibitory concentration (IC_{50}) (Fig. 1A, left line graph). Cell viability was also determined in parallel and the half maximal cytotoxic concentration (CC_{50}) was calculated for each inhibitor (Fig. 1A, right line graph). The selectivity index ($SI = CC_{50}/IC_{50}$) was calculated to determine the therapeutic index, giving maximum antiviral activity with minimal cytotoxicity. Lck Inhibitor II showed similar SI (18.78 for BX08_Renilla and 19.97 for NL4-3_Renilla) to Dasatinib (12.54 for BX08_Renilla and 13.07 for NL4-3_Renilla), as well as similar IC_{50} (2.87 μM for BX08_Renilla and 2.10 μM for NL4-3_Renilla) than Dasatinib (3.67 μM for BX08_Renilla and 3.23 μM for NL4-3_Renilla), in these conditions. There were no significant differences in how each inhibitor affected the infection by R5- or X4- tropic strains.

These results did not correlate with data previously described that reported an IC_{50} of 75 nM for Dasatinib to inhibit HIV-1 infection [27;28]. Intriguingly, Pogliaghi

et al. [28] observed that Dasatinib 75 nM was more efficient when it was added at the same time as PHA, but it was inefficient at this concentration in PBMCs previously treated with PHA for more than 3 days. In order to determine whether PHA-mediated T-cell activation could be triggering some mechanism that interfered with Dasatinib antiviral activity, we calculated IC_{50} in resting PBMCs that were treated with Dasatinib at different concentrations (4.9 nM to 10 μM) for 30 minutes before inducing activation with PHA and IL-2 for the subsequent 48 hours. Cells were then infected with HIV-1 NL4-3_Renilla strain by spinoculation, washed and cultured for 2 days with Dasatinib at the same concentrations, in the presence of IL-2. IC_{50} considerably changed in these conditions and was even lower than previous reports (50.86 nM) (Fig. 1B). SI improved to > 196.62, suggesting that downstream phosphorylation cascade of events induced by PHA/IL-2, such as PKCθ (T538) activation, once in motion could not be easily inhibited by Dasatinib.

Both Dasatinib and Lck inhibitor II interfered with $p56^{lck}$ phosphorylation at Y394, with PKCθ phosphorylation at T538, and decreased the general phosphorylation of tyrosine (pTyr) induced by PHA/IL-2 in PBMCs, at the concentration used for HIV-1 inhibition (Fig. 1C). Intensity per pixel was quantified in every image and results were displayed in a bar diagram showing the statistical significance.

3.2. Effect of Dasatinib on the viral entry

Harmon et al. [27] described that Dasatinib and other related inhibitors of tirosin kinases could be interfering with HIV-1 replication at viral fusion and entry. However, this would not explain the different efficacy of Dasatinib in PBMCs that were activated with PHA/IL-2 before or after adding Dasatinib to the culture medium. Therefore, resting PBMCs were treated with Dasatinib at different concentrations (9.38 nM to 600

nM) for 30 minutes before adding PHA and IL-2 and, after 48 hours in culture, cells were infected with HIV-1 NL4-3_Renilla or pseudotyped VSV-NL4-3 ΔEnv-LUC strains by spinoculation. After washing, cells were cultured for 48 hours with the same concentrations of Dasatinib. HIV-1 replication was monitored by measuring the production of Renilla. Average IC_{50} for pseudotyped VSV-NL4-3 ΔEnv-LUC (59.05 nM) was similar to NL4-3_Renilla (52.71 nM) (Fig. 2A). The absence of a significant difference between IC_{50} for VSV-NL4-3 ΔEnv-LUC and NL4-3_Renilla suggested that the effect of Dasatinib was not on viral entry. Moreover, both Dasatinib and Lck Inhibitor II did not significantly modify the expression of CD4, CCR5 or CXCR4 receptors on the cell surface after 48 hours of treatment (Fig. 2B).

In order to discard any possible effect of Dasatinib on the viral entry, fusion assay of BlaM-Vpr-containing HIV-1 viruses was performed in PBMCs activated with PHA/IL-2 before and after adding Dasatinib to the culture medium. HIV fusion inhibitor T-20 was used as control. PBMCs were activated with PHA/IL-2 for 48 hours in the presence or absence of Dasatinib at two different concentrations (75 nM and 5 μM). Cells were then washed, incubated for 5 minutes with Dasatinib or T-20, and infected by spinoculation with BlaM-Vpr-containing NL4-3_Renilla, BX08_Renilla, and pseudotyped VSV-NL4-3 ΔEnv-LUC viruses. After incubation for 2 hours, cells were washed and incubated with the appropriate substrate CCF2/AM. Analysis of changes in CCF2 fluorescence emission spectrum from green to blue was measured by flow cytometry. Dasatinib was ineffective to stop HIV fusion at any concentration assayed, whether it was added before (Fig. 2C, right panel) or after (Fig. 2C, left panel) the induction of T-cell activation with PHA/IL-2. In both conditions, T-20 inhibited HIV fusion of NL4-3_Renilla and BX08_Renilla ($p < 0.001$) but not VSV-NL4-3 ΔEnv-LUC, as expected. Drug susceptibility assays, performed at the same time with

the same amount and types of viruses and with the same cells that the fusion assays, showed that Dasatinib was much more effective when PBMCs were treated before PHA/IL-2-mediated activation ($p < 0.01$) (Fig. 2D, right panel), although Dasatinib also partially interfered with HIV-1 replication when it was added after PHA/IL-2-mediated T-cell activation ($p < 0.001$) (Fig. 2D, left panel). It is important to note that the cells of the positive control (C+) in Fig. 2D, right panel, were also treated with Dasatinib. Therefore, they were not activated despite the presence of PHA/IL-2, SAMHD1 was not phosphorylated and its full antiviral activity prevented HIV-1 infection even in C+. In order to fully appreciate the difference in infection between cells treated with Dasatinib before or after treatment with PHA/IL-2, the percentage of cells with HIV-1 replication from the right panel (treated with PHA/IL-2 and Dasatinib) was referred to C+ (100%) in the left panel (only treated with PHA/IL-2). As Dasatinib inhibited replication of all assayed HIV strains but did not interfere with viral fusion, we concluded it was acting on HIV-1 replication at a post-entry step.

3.3. Dasatinib interfered with the phosphorylation of SAMHD1 induced by T-cell activation

Treatment of resting PBMCs with mitogens such as PHA or the staphylococcal enterotoxin A (SEA) for 6 days induces T-cell proliferation. Both Dasatinib and Lck Inhibitor II abrogated the effect of both PHA/IL-2 and SEA on T-cell proliferation when they were added to the culture medium 30 minutes before the mitogen, being Dasatinib more potent than Lck Inhibitor II at the concentration used for inhibiting cell division (Fig. 3A). As SAMHD1 is a key regulator of cell cycle progression and a major restriction factor for HIV-1 infection [39], we analyzed by immunoblotting the kinetics of SAMHD1 phosphorylation at T592 in purified CD4+ T cells treated with PHA/IL-2 for 5 days. SAMHD1 phosphorylation began on day 2 after PHA/IL-2 activation and it

was maximum on days 3 and 4, correlating with the highest expression of cyclin A2 and Cdk1/cdc2 (Fig. 3B), which have been both described as responsible for SAMHD1 phosphorylation [42]. Dasatinib interfered with PHA/IL-2-mediated phosphorylation of SAMHD1 when it was added at the same time than PHA/IL-2 (t=0) (Fig. 3C, lane 3), 2h after PHA/IL-2 (Fig. 3C, lane 4) or 1 day after PHA/IL-2 (Fig. 3C, lane 5), but it could not impede SAMHD1 phosphorylation when it was added to the culture medium after 3 days of activation with PHA/IL-2 (Fig. 3C, lane 6), proving that the phosphorylated state of SAMHD1 at T592 in response to T-cell activation was quite stable and could not be easily reverted. IC_{50} and SI of Dasatinib to interfere with HIV-1 replication were measured in PBMCs treated with Dasatinib at t=0, 2 hours or 1, 2 or 3 days after adding PHA/IL-2 for 72 hours to the culture medium (Fig. 3C, lower panels). A correlation between the elapsed time of activation with PHA/IL-2 before adding Dasatinib to the culture medium and the increased concentration of Dasatinib that inhibited HIV-1 replication was observed.

Lck Inhibitor II also impeded SAMHD1 phosphorylation when it was added to the culture medium before PHA/IL-2 and incubated together for 3 days (Fig. 3D, lane 2). In CD4+ T cells treated with SEA for 6 days, SAMHD1 phosphorylation at T592 was also induced (Fig. 3E, lane 1), and this event correlated with the expression of cyclin A2 and Cdk1/cdc2. When Dasatinib was added to the culture medium at the same time as SEA (t=0), SAMHD1 was not phosphorylated after 6 days of incubation and neither cyclin A2 nor Cdk1/cdc2 were expressed (Fig. 3E, lane 2). When Dasatinib was added 2 days after incubating with SEA and cells were then incubated for 4 subsequent days, SAMHD1 was not phosphorylated and although Cdk1/cdc2 was expressed, there was no detectable expression of cyclin A2 (Fig. 3E, lane 3). The expression of total SAMHD1 was not modified after treatment with Dasatinib or Lck Inhibitor II or after

T-cell activation. Therefore, in all cases, both total SAMHD1 and β-actin were used as internal loading control. All gel bands were analyzed by densitometry and results were displayed in bar diagrams showing the statistical significance regarding the control.

3.4. Dasatinib inhibited HIV-1 retrotranscription, reducing the integration of the provirus

In order to determine the effect of Dasatinib on HIV-1 replication cycle, PBMCs treated with Dasatinib before inducing T-cell activation with PHA/IL-2 for 2 days were infected with NL4-3_Renilla strain. Dasatinib was only added at the beginning of the experiment, before PHA/IL-2 activation. Analysis by qPCR showed that Dasatinib completely impeded both early ($p < 0.001$) and late retrotranscription 5 hours after the infection (Fig. 4A). As a consequence, 5 days after the infection, the viral DNA did not enter the nucleus, as was determined by the absence of episomal 2LTRs forms ($p < 0.001$), and the integration of the provirus was greatly diminished ($p<0.001$) in comparison to untreated cells. Similar results were obtained with Lck Inhibitor II (data not shown). The progression of infection was followed by quantifying the production of Renilla and it was significantly reduced in cells that were treated with Dasatinib ($p < 0.001$) (Fig. 4B), compared to untreated cells.

3.5. Dasatinib could not inhibit HIV-1 replication in the presence of Vpx

HIV-2 and SIV are able to infect cells expressing active SAMHD1 because they encode Vpx, an accessory protein that targets SAMHD1 for proteosomal degradation [43]. However, HIV-1 does not encode Vpx and consequently, it cannot complete the replication cycle until the T cell is fully activated and SAMHD1 is phosphorylated and neutralized [65]. In order to determine whether the main mechanism of action of Dasatinib to inhibit HIV-1 replication was at the level of retrotranscription by

interfering with SAMHD1 phosphorylation, resting PBMCs were infected with NL4-3_Renilla carrying or not Vpx from SIVsm. Early and late retrotranscription were analyzed by qPCR 5 hours after the infection, in the absence of an activating stimulus to avoid SAMHD1 phosphorylation. In the presence of Vpx, early and late retrotranscription increased 10.6- and 7.0-fold, respectively ($p < 0.001$), regardless of the presence of Dasatinib (Fig. 5A). In the absence of Vpx, treatment with Dasatinib did not significantly affect HIV-1 retrotranscription when cells were in resting conditions, due to the presence of non-phosphorylated, active SAMHD1. These cells were then activated with PHA/IL-2 for 5 days to induce SAMHD1 phosphorylation and full viral replication, which was measured by quantifying the production of Renilla. Dasatinib was now able to reduce 3.0-fold the viral replication ($p < 0.001$), but viral replication did not significantly decrease in cells that were infected with virions carrying Vpx, even in the presence of Dasatinib (Fig. 5B). Degradation of SAMHD1 after infection with HIV-1 virions carrying Vpx was assessed by immunoblotting (Fig. 5C). Gel bands were analyzed by densitometry and relative protein expression was displayed in a bar diagram.

3.6. PBMCs from CML patients in chronic treatment with Dasatinib showed lower expression of SAMHD1 phosphorylated at T592 after PHA-mediated activation and they were resistant to HIV-1 infection ex vivo

PBMCs isolated from five CML patients in chronic treatment with Dasatinib for more than 2 years and ten untreated controls were treated with PHA/IL-2 for 48 hours and then analyzed by flow cytometry to determine the percentage of expression of phospho-T592 SAMHD1 in the population of CD4+ T cells. After gating CD3+ T cells, all CML patients showed an average 9.8% expression of phospho-T592 SAMHD1 in the CD4+ T cell population, whereas the average expression in untreated controls was

30.5% (Fig. 6A and B). Representative analysis of phospho-T592 SAMHD1 in two CML patients (labeled as CML-1 and CML-2, according to Table 1) and one untreated control is shown (Fig. 6A). CML patients showed an averaged 19% decreased expression of the activation markers CD69 and CD25 on the surface of T cells expressing phospho-T592 SAMHD1 in response to PHA/IL-2, which was nearly 2-fold higher in untreated controls (Fig. 6C). Expression of total SAMHD1 was not significantly modified compared to untreated control (Fig. 6D).

Susceptibility to HIV-1 infection was analyzed in PBMCs from CML patients in comparison to untreated controls. PBMCs were activated ex vivo with PHA/IL2 for 48 hours and then infected with NL4-3_Renilla. Five days after infection, there was a strong decrease in HIV-1 proviral integration ($p < 0.001$) (Fig. 6E, left panel) and in the synthesis of viral proteins (Renilla) ($p < 0.001$) (Fig. 6E, right panel). This experiment was performed without adding Dasatinib to the culture medium, proving that the effects caused by Dasatinib treatment in vivo were quite sustained on CD4+ T cells for at least 7 days after mononuclear cell isolation from the blood of CML patients.

3.7. Effect of Dasatinib on CD8+ T cell activity

A negative effect of Dasatinib on the cytotoxic activity of CD8+ T cells (CTLs) should be discarded. The effect of Dasatinib on the production of IFNγ from PBMCs of treated chronic asymptomatic HIV-1 individuals was then analyzed using ELISPOT assay. The antigen-specific IFN-γ production from CD8+ T cells was analyzed in vitro after treating PBMCs with Dasatinib 75 nM for 30 minutes before incubating with the stimuli for 48 hours (Fig. 7A). Different HLA class I-restricted synthetic peptides from HIV-1 Gag, Pol, Env and Nef proteins, as well as peptide pools spanning the entire HIV-1 consensus B Gag proteins sequences, were used (labeled as HIV). A pool of

MHC class I-restricted T cell epitopes from human cytomegalovirus, Epstein-Barr virus and influenza virus (labeled as CEF) were used to exclude a selective effect of Dasatinib on HIV-1 specific CD8+ T cell clones. RPMI/10%FCS was the negative control (labeled as NEG) and treatment with PHA was the positive control (labeled as PHA). Dasatinib 75 nM totally abrogated the CD8+ T-cell activity in vitro. However, the analysis of IFNγ production by CEF from PBMCs obtained from CML patients on chronic treatment with Dasatinib showed no significant differences with the average data of three untreated controls (Fig. 7B).

4. Discussion

The reservoirs of HIV-1 latently infected cells are formed early during primary infection [66-70]. The size of the reservoir is changeable and is maintained regardless of cART due to low level ongoing replication or homeostatic proliferation of latently infected CD4+ T cells [71;72]. The complete elimination -or at least a significant decrease- of these reservoirs is then compulsory to eradicate the infection or to achieve a `functional cure´. Early cART decreases the size of the reservoir [9;10;73;74] and this has been related to a better prognosis of the disease, as well as a long-term control of viraemia in patients under treatment interruption [75;76]. New strategies aimed at reducing the size of the reservoir from the beginning of the infection will be essential to make HIV `functional cure´ more feasible. In this regard, we reported previously that selectively blocking PKCθ reduces HIV-1 replication in CD4+ T cells [11;12]. We have now evaluated other compounds aimed at kinases upstream PKCθ and involved in its activation such as the tyrosine kinase $p56^{lck}$ [21;77]. The potential positive role of $p56^{lck}$ for the viral cycle was suggested after observing that HIV-1 infection triggered $p56^{lck}$ phosphorylation at Y394 in Jurkat E6-1 cells (Fig. 8).

Inhibitors of tyrosine kinases (TKIs) such as Dasatinib, Imatinib, and Nilotinib are currently used in clinic for the treatment of hematological malignancies such as CML, characterized by a constitutive activation of tyrosine kinases [78]. Dasatinib is also able to inhibit HIV-1 replication in CD4+ T cells from infected patients [28], supposedly through the inhibition of the viral entry [27], as well as through its negative effect on T-cell proliferation [38]. Intriguingly, when we tried to determine the IC_{50} of Dasatinib by classic antiviral assays in PBMCs activated with PHA and IL-2 for 48 hours, IC_{50} was more than 60-fold higher than previously described [27;28]. The observation of Pogliaghi et al. [28] that Dasatinib was less effective in controlling HIV-

1 replication when it was added to the culture medium at day 3 after PHA stimulation suggested that PHA-mediated T-cell activation was triggering some mechanism that Dasatinib could not easily overcome. Accordingly, when IC_{50} was calculated in PBMCs treated with Dasatinib before inducing T-cell activation with PHA, this IC_{50} nicely correlated with other reports [27;28] and was even lower than previously described.

A previous report by Harmon et al. [27] described that Dasatinib and other related TKIs were interfering with HIV-1 fusion and entry. However, we did not find significant differences in Dasatinib IC_{50} to inhibit HIV-1 infection in PBMCs directed by HIV-1 or VSV envelopes. Direct measurement of viral fusion using BlaM-Vpr-containing viruses with HIV-1 envelope (X4 and R5-tropic) or VSV envelope showed that Dasatinib had actually no effect on viral entry in PBMCs. In contrast, the fusion inhibitor T-20 strongly inhibited both X4 and R5-tropic viruses but had no effect on the infection when it was directed by the VSV envelope, as expected. Drug susceptibility assays performed simultaneously proved that Dasatinib strongly inhibited HIV-1 infection when it was added to the culture medium before inducing activation with PHA and IL-2 despite it did not cause a negative effect on viral fusion. Intriguingly, fusion inhibitor T-20 seemed to induce a negative effect on viral infection with VSV-pseudotyped HIV-1 virus in PBMCs previously treated with Dasatinib, but this effect was undoubtedly related to the previous strong inhibition caused by Dasatinib, independently of viral fusion.

Consequently, Dasatinib was interfering with HIV-1 infection at a post-entry step. We then analyzed the effect of Dasatinib on viral retrotranscription. In this regard, SAMHD1 is a major antiviral factor that blocks early reverse transcription of the HIV-1 genome [39;41]. It is also a key regulator of cell cycle progression and remains active in resting CD4+ T cells as long as it is not phosphorylated at T592 by cell cycle complexes

such as cyclin A2/Cdk1, rendering T cells susceptible to HIV-1 infection [42]. Activation of PBMCs with mitogens such as PHA or SEA triggered cyclin 2/Cdk1 expression and, consequently, SAMHD1 phosphorylation. This phosphorylation was inhibited by Dasatinib especially when it was added to the culture medium before the activating stimuli. When Dasatinib was added to cells treated with PHA for more than 2 days, the level of SAMHD1 phosphorylation was then be so high that higher concentrations of Dasatinib were needed to preserve SAMHD1 antiviral function and other proteins could be affected as well. As SAMHD1 blocks HIV-1 reverse transcription, we determined that the protection of SAMHD1 against phosphorylation by Dasatinib had in fact a complete effect on HIV-1 early and late retrotranscription and, as a consequence, on the entry of viral DNA to the nucleus and proviral integration. This effect of Dasatinib on preserving SAMHD1 antiviral function in non-activated CD4+ T cells was essential for its inhibitory activity, as Dasatinib could not inhibit the replication of HIV-1 virions carrying SIVsm Vpx, which targets SAMHD1 for proteasomal degradation [43;65]. Moreover, the activity of Dasatinib to inhibit HIV-1 replication was observed only when PBMCs were subsequently activated and not when they were in a resting state, where SAMHD1 would not be phosphorylated. These data provided solid evidence that the preservation of SAMHD1 activity was the major mechanism for Dasatinib antiviral effect, as well as a feasible explanation for the contradictory results shown by Harmon et al [27]. In their work, TZM-bl cells were used to analyze the effect of Dasatinib on viral entry, but these cells do not express SAMHD1, as occurs with many other cell lines such as Jurkat E6-1 and MT-2 (Fig. 9). The activity of Dasatinib to inhibit HIV-1 replication at retrotranscription by preserving SAMHD1 antiviral function would then be lost in these cell lines.

The effect of Dasatinib to avoid SAMHD1 phosphorylation in vivo was confirmed in PBMCs obtained from five CML patients treated chronically with Dasatinib. All patients had more than 2 years of follow-up from CML diagnosis and all were taking Dasatinib for at least two years. CD4+ T cells from these patients showed lower percentage of SAMHD1 phosphorylation compared to untreated controls when activated with PHA/IL-2 ex vivo, proving that Dasatinib could be used to preserve SAMHD1 antiviral function in vivo. Moreover, PBMCs from CML patients were less susceptible to HIV-1 infection than PBMCs from untreated controls and proviral integration was greatly diminished. A possible negative effect of Dasatinib on CTL-mediated cytotoxicity should be discarded before even considering a potential use of Dasatinib in HIV-infected patients. Although a great inhibitory effect of Dasatinib on CD8+ T cell function was observed in vitro, it was not so clear in vivo, as IFNγ production ex vivo from PBMCs of CML patients was quite similar to untreated controls. As only five patients could be recruited for this study, these results need to be confirmed in a larger number of patients. However, these data are consistent with previous report that chronic treatment with Dasatinib does not increase susceptibility to fungal or viral infections or that lymphopenia and severe opportunistic infections defined as AIDS-related events are rare in Dasatinib-treated patients with CML [37]. In fact, no CML patient recruited for this study presented previous or ongoing serious adverse events related to Dasatinib use, nor infectious complication related to their hematological disease or to the treatment with Dasatinib. However, as an increased risk of opportunistic infections cannot be discarded in HIV-infected patients, only patients with CD4 counts above 350-500 cells/μL should be included in clinical studies using Dasatinib even if it is administered in combination with ART.

On the other hand, it has been described that HIV-1 impaired reverse transcription and subsequent accumulation of incomplete reverse transcripts could activate the inflammasome, causing caspase-1-mediated cell death of abortively infected CD4+ T cells [79-81]. Although we used for this study PBMCs instead of CD4+ T cells from lymphoid tissues, we did not observe accumulation of retrotranscripts in HIV-infected T cells treated with Dasatinib. Moreover, SAMHD1 may also act as a restriction factor for other ubiquitous viruses such as herpes simplex virus 1 (HSV-1) [82] and no related effect has been described for CML patients on treatment with Dasatinib. Nevertheless, although Dasatinib-mediated inhibition of proliferation does not induce apoptosis and signal transduction and proliferative responses via IL-2 remain unperturbed [36], the possibility that HIV-1 abortive infection caused by Dasatinib might produce CD4+ T cell depletion should be excluded before starting clinical assays.

In conclusion, to our knowledge, this is the first time that a compound currently used in clinic has been described to preserve in vivo the function of the innate antiviral factor SAMHD1. The use of Dasatinib in combination with cART during primary HIV-1 infection could control the massive activation and destruction of CD4+ T cells characteristic of early stages of the infection [83;84], making the cells refractory to infection by interfering mostly with retrotranscription. However, although the administration of Dasatinib would be mostly recommendable during the immediate first days after infection, as both processes of CD4+ T cell destruction and reinfection occur during the first months after infection, Dasatinib would supposedly reduce the size of the reservoir and the destruction of CD4+ T cells even when administered few months after primary infection, as it has been demonstrated for early cART [74;85]. Among the immunologic and virologic benefits of a rapid shutdown of immune activation during primary HIV-1 infection would be the reduction of viral replication and spread, the

preservation of $CD4^+$ T cell counts and CTL response, and a low plasma viral load. These events would reduce the reservoir size and preserve the HIV-specific immune responses, creating a more favorable virologic and immunologic environment for future interventional studies aiming at HIV-1 eradication and cure and therefore merits the evaluation of the efficacy and safety of Dasatinib in a pilot clinical trial.

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Figure Legends

Fig. 1. Measurement of IC_{50} and CC_{50} of Dasatinib in PBMCs activated with PHA/IL-2 and infected with HIV-1 strains. (A) PBMCs activated with PHA and IL-2 for 48 hours were incubated in 96-well plates with increasing concentrations (390 nM to 50 μM) of Dasatinib or Lck Inhibitor and then infected with NL4-3_Renilla or BX08_Renilla strains. IC_{50} and CC_{50} were calculated by measuring the production of Renilla (RLUs). (B) Resting PBMCs treated with increasing concentrations (4.9 nM to 10 μM) of Dasatinib for 30 minutes were then activated with PHA/IL-2 for 48 hours and then infected with NL4-3_Renilla by spinoculation. After extensive washing, cells were incubated for 48 hours with the same concentrations of Dasatinib. In all experiments, the production of Renilla was quantified 48 hours post-infection. SI was calculated with the formula CC_{50}/IC_{50}. All measurements were done in triplicate and mean is represented with the standard error of the mean (SEM). R^2 is a measure of goodness-of-fit of linear regression. (C) PBMCs treated with Dasatinib (75 nM) or Lck Inhibitor II (2.5 μM) 30 minutes before incubating with PHA/IL-2 for 48 hours were analyzed by immunofluorescence using specific antibodies against $p56^{lck}$ phosphorylated at Y394, PKCθ phosphorylated at T538 and phosphorylated tyrosine and a secondary antibody conjugated with Alexa 546. Nuclei were stained with Dapi. Intensity mean per pixel was calculated and values were represented in bar diagrams showing statistical significance. Representative images are shown.

Fig. 2. Effect of Dasatinib on HIV-1 fusion step in PBMCs treated with Dasatinib and PHA/IL-2 and infected with HIV-1 or VSV-pseudotyped HIV strains. (A) Measurement of Renilla production in PBMCs pre-treated with increasing concentrations of Dasatinib and then activated with PHA/IL-2. Cells were infected with NL4-3_Renilla or pseudotyped VSV-NL4-3 ΔEnv-LUC strains for 48 hours. SI was calculated with the

formula CC_{50}/IC_{50}. All measurements were done in triplicate and mean is represented with SEM. R^2 is a measure of goodness-of-fit of linear regression. The rate between IC_{50} of VSV-NL4-3 ΔEnv-LUC and NL4-3_Renilla was calculated to determine the inhibitory effect of Dasatinib on the viral entry. (B) Analysis by flow cytometry of the expression of receptors CD4, CCR5 and CXCR4 in PBMCs after treatment with Dasatinib or Lck inhibitor II for 48 hours, regarding cells in basal conditions. (C) Analysis by flow cytometry of fusion of BlaM-Vpr-containing NL4-3_Renilla, BX08_Renilla and VSV-NL4-3 ΔEnv-LUC viruses with PHA/IL-2-activated PBMCs is shown in the presence of Dasatinib (75nM or 5 μM) and T-20 or in the absence of drugs (positive control, C+). Results are expressed as percentage of BlaM-Vpr positive cells displaying cleaved CCF2/AM fluorescence at 447 nm. (D) The same PBMCs were infected with NL4-3_Renilla, BX08_Renilla and VSV-NL4-3 ΔEnv-LUC viruses in the same conditions described above for the fusion assays, and cultured for 30 hours at 37ºC. Results are expressed as percentage of RLUs. Cells were activated in the absence of the inhibitors that were added only during the fusion assay (C and D, left panels) or cells were treated with Dasatinib before adding PHA/IL-2 and were then maintained with Dasatinib during all the experiment (C and D, right panels). Representative experiments out of 3 independent experiments are shown.

Fig. 3. Dasatinib interfered with SAMHD1 phosphorylation at T592 in response to T-cell activation. (A) Analysis by CFSE of PBMCs proliferation in response to PHA/IL-2 or SEA, in the presence or absence of Dasatinib or Lck inhibitor II. The number of events per generation is indicated as G#, as well as the percentage of proliferating cells compared to cells in basal conditions (untreated). (B) Analysis by immunoblotting of the kinetics of SAMHD1 phosphorylation in CD4+ T cells in the presence of PHA/IL-2 for 5 days. (C) Analysis by immunoblotting of SAMHD1 phosphorylation in CD4+ T

cells treated with Dasatinib at different times after the activation with PHA/IL-2 regarding markers of cell cycle Cyclin A2 and Cdk1/cdc2. In order to establish a correlation between SAMHD1 phosphorylation and the ability of Dasatinib to interfere with HIV-1 replication, both IC_{50} and SI were measured in PBMCs treated with Dasatinib at t=0, 2 hours (h) or 1, 2 or 3 days (d) after adding PHA/IL-2 for 72 hours to the culture medium. Virus was added directly to the wells and cells were incubated for additional 48 hours before reading Renilla. Results from two independent experiments are shown. (D) Analysis by immunoblotting of the inhibition of SAMHD1 phosphorylation in CD4+ T cells treated with Dasatinib or Lck Inhibitor II before PHA/IL-2 activation for 3 days (E) or treated with Dasatinib at different times after activation with SEA for 6 days. In all cases, total SAMHD1 and β-actin were used as loading control. Gel bands were quantified by densitometry and values were represented in bar diagrams showing the statistical significance. Representative experiments are shown.

Fig. 4. Dasatinib interfered with HIV-1 replication cycle at retrotranscription and, consequently, at proviral integration and viral transcription. (A) Analysis by qPCR of HIV-1 early and late retrotranscription (RT), 2LTRs circles, and integrated DNA in PBMCs treated with Dasatinib before activation with PHA/IL-2 for 48 hours and then infected with NL4-3_Renilla for 5 hours (for RT) and 5 days (for 2LTRs and integrated provirus) (p.i., post-infection). (B) Synthesis of Renilla was analyzed in these cells 2, 3 and 4 days after the infection. All measurements were done in triplicate and mean is represented with SEM.

Fig. 5. Dasatinib did not inhibit the infection by HIV-1 virions carrying Vpx. (A) Analysis of early and late retrotranscription (RT) in resting PBMCs infected for 5 hours with NL4-3_Renilla with or without SIVsm Vpx, in the presence or absence of

Dasatinib 75 nM. (B) These cells were then treated with PHA/IL-2 for 5 days and the production of Renilla was then analyzed. All measurements were done in triplicate and mean is represented with SEM. (C) SAMHD1 degradation after infection with HIV-1 virions carrying Vpx was assessed by immunoblotting in PBMCs activated with PHA/IL-2 for 48 hours and then infected for 5 days with NL4-3_Renilla carrying or not Vpx. Representative experiment is shown. The relative ratio of the optical density units corresponding to each sample was calculated by densitometry regarding the internal loading control (β-actin) per each lane and displayed in a bar diagram as relative protein expression.

Fig. 6. Analysis of SAMHD1 phosphorylation in PBMCs from CML patients on chronic treatment with Dasatinib. (A) A representative experiment of the analysis of PBMCs from two CML patients (CML-1 and CML-2) and one untreated control is shown. Cells were gated by CD3+ CD4+ and then SAMHD1 phosphorylation was quantified in CD4+ T cells by flow cytometry. (B) Average percentage of SAMHD1 phosphorylation in CD4+ T cells from five CML patients and ten untreated controls. Mean is represented as a bar diagram and SEM is shown on top of the bars. (C and D) Representative experiment of the surface expression of activation markers CD69/CD25 regarding SAMHD1 phosphorylated at T592 (C) or total SAMHD1 (D) in PBMCs from patients CML-1 and CML-2 and one untreated control after gating by SSC (side scatter) and FSC (forward scatter) and CD3+ CD4+ staining. (E) Analysis of proviral integration (left panel) and synthesis of Renilla (right panel) in PBMCs from CML patients activated ex vivo for 48 hours before infecting with NL4-3_Renilla for 5 days. Mean is represented as a bar diagram and SEM is shown on top of the bars.

Fig. 7. Effect of Dasatinib on CD8+ T cell activity. (A) IFNγ release from PBMCs of HIV-1 infected patients was analyzed by ELISPOT. Cells were treated in vitro with

Dasatinib 75 nM before adding PHA/IL-2 to the culture medium and incubate for 48 hours. Different HLA class I-restricted synthetic peptides from Gag, Pol, Env and Nef proteins and peptide pools spanning the entire HIV-1 consensus B Gag proteins sequences were used (labeled as HIV). A positive control formed by a pool of MHC class I-restricted T-cell epitopes from human cytomegalovirus, Epstein-Barr virus and influenza virus (labeled as CEF). PHA was used as additional positive control. RPMI/10%FCS was used as negative control (labeled as NEG). (B) The effect of Dasatinib in vivo on IFNγ release was analyzed in PBMCs from four CML patients and three untreated controls, represented as a bar diagram of mean and SEM.

Fig. 8. HIV-1 infection induced the phosphorylation of $p56^{lck}$ at Y394 in Jurkat E6-1 cell line. Jurkat E6-1cells infected with NL4-3_wt for 48 hours or 5 days were analyzed by immunofluorescence in order to evaluate the phosphorylation of $p56^{lck}$ at Y394 as the infection progressed. Cells were stained with specific antibodies directed against $p56^{lck}$ phosphorylated at Y394 and the viral protein Tat, conjugated with Alexa 546 and Alexa 488, respectively. Nuclei were stained with Dapi. Intensity per pixel was quantified in every image stained with anti-phospho $p56^{lck}$ Y394 and results were displayed in a bar diagram showing the statistical significance. Representative images are shown.

Fig. 9. Comparison of SAMHD1 expression in several cell lines regarding purified CD4+ T cells. Analysis by immunoblotting of the expression of SAMHD1 phosphorylated at T592 or total SAMHD1 in TZM-bl, MT-2, and Jurkat E6-1 cell lines, as well as in CD4+ T cells isolated from PBMCs of healthy donors, resting or activated with PHA/IL-2 for 3 days. β-actin was used as internal loading control. Representative experiment is shown. The relative ratio of the optical density units corresponding to

each sample was calculated by densitometry regarding β-actin as internal loading control per each lane and displayed in a bar diagram as relative protein expression.