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Research Article

Dasatinib: Effects on the Macrophage Phospho Proteome with a Focus on SAMHD1 and HIV-1 Infection

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Abstract

Macrophages are one of the main cellular targets of human immunodeficiency virus type 1 (HIV-1). Macrophage infection by HIV-1 is inefficient due to the presence of the viral restriction factor sterile alpha motif and histidine aspartic acid domain containing protein 1 (SAMHD1). Ex vivo, human monocyte- erived macrophages (MDMs) express SAMHD1 in an equilibrium between active (un-phosphorylated) and inactive (phosphorylated) states. Others and we have shown that treatment of MDMs with the FDA approved tyrosine kinase inhibitor, dasatinib, ablates SAMHD1 phosphorylation, thus skewing the balance towards a cellular state that is refractory to HIV-1 infection. We hypothesized that dasatinib inhibits a putative tyrosine kinase that is upstream of SAMHD1. In the search for this tyrosine kinase, we probed several candidates and were unable to identify a single target that, when inhibited, was sufficient to explain the dephosphorylating of SAMHD1 we observe upon treatment with dasatinib. On the other hand, we probed the ability of dasatinib to directly inhibit the serine/threonine cyclin dependent kinases 1, 2, 4 and 6 and confirmed that dasatinib directly inhibits these kinases. Therefore, our results show that inhibition of the proximal CDKs 1, 2, 4 and 6 by dasatinib is clearly detectable, leads to blockade of infection by HIV-1, and may be sufficient to explain the activity of dasatinib against SAMHD1 phosphorylation.

INTRODUCTION

Macrophages and other myeloid cells are long lived and play well-established roles during initial virus infection, viral persistence during antiretroviral treatment, as well as contributing to the spread of HIV-1 to the central nervous system (CNS) [1-3]. Infection of macrophages by HIV-1 is inefficient due to the presence of viral restriction factor sterile alpha motif and histidine aspartic acid domain containing protein 1 (SAMHD1) [4,5]. SAMHD1 is a deoxynucleoside triphosphate (dNTP) triphosphohydrolase that regulates the concentration of dNTPs within cells [6], and when active, maintains dNTP levels below the threshold necessary to support efficient reverse transcription [6-10]. Regulation of SAMHD1 phosphorylation is cell cycle dependent and, in post-mitotic macrophages, SAMHD1 is primarily found in an active (unphosphorylated) state, rendering myeloid lineage cells resistant to HIV-1 infection [11-16]. SAMHD1 phosphorylation/dephosphorylation levels are also responsible for the lower permissivity of female-derived human macrophages compared to their male-derived counterparts [17].

Although many cyclin-dependent kinases (CDK) have been shown to contribute to the phospho-regulation of SAMHD1 [15,18-22], CDKs are serine/threonine kinases whereas dasatinib was identified as a tyrosine kinase inhibitor. Therefore, it is tempting to speculate that a putative upstream tyrosine kinase, which is presumably targeted by dasatinib, ultimately controls the activities of downstream CDKs acting on SAMHD1.

In addition to being cell cycle dependent, SAMHD1 phosphorylation can be modulated in vitro by treatment with γC cytokines [23], interferons (IFNs) [21], and multiple tyrosine kinase inhibitors (TKI) [21,24,25]. One TKI, dasatinib, decreases SAMHD1 phosphorylation levels in macrophages to a much greater degree than others tested [21,24].

Dasatinib is a multi-kinase inhibitor that was designed to target Abl and Src family kinases and is used clinically to treat chronic myelogenous leukemia and acute myeloid leukemia [26-29]. More recently, it has been shown that dasatinib inhibits HIV-1 infection in CD4+ T cells [24], and in macrophages [21], due to its ability to reduce SAMHD1 phosphorylation. However, the specific mechanism through which dasatinib decreases SAMHD1 phosphorylation levels is unknown. Our previous studies demonstrated that the mechanism through which interferons act to induce dephosphorylation of SAMHD1 is different from the pathway inhibited by dasatinib [21]. While types I and II IFN induce dephosphorylation of SAMHD1 in part by diminishing transcription of the CDK1 gene, this change was not observed in dasatinib treated cells [21].

We hypothesized that a putative upstream tyrosine kinase

may be a master regulator of several serine/threonine kinases, which collectively can phosphorylate residue threonine-592 of SAMHD1. To begin steps toward identifying a putative upstream tyrosine kinase, we resorted to a previous study [30], that identified multiple protein tyrosine kinases and serine/threonine kinases as being inhibited by dasatinib in addition to Abl and Src. We used specific kinase inhibitors to probe the potential roles of several upstream kinases.

MATERIALS AND METHODS

Isolation of healthy donor PBMC

Healthy volunteer donors aged 18 and above were recruited for this study to undergo peripheral phlebotomy in accordance with the University of Utah institutional review board (IRB) protocol 00067637. Peripheral blood mononuclear cells (PBMCs) were isolated from total blood via Lymphoprep density gradient as previously described [21].

Generation and infection of MDM

Following collection of PBMCs, CD14+ monocytes were isolated through positive selection using magnetic beads (Miltenyi Biotec, US). Cells were plated in untreated 24-well plates at a density of 6x105 in serum free Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 2mM L-glutamine (Gibco, US), and 1% Pen-Strep (Gibco, US) and allowed to adhere for greater than 2 hours. Serum-free medium was then gently removed and replaced with 600µL RPMI 1640 medium supplemented as above, with the addition of 5% pooled human serum (Innovative Research) and 5% fetal bovine serum (FBS) (Gibco, US) (culture medium). The culture medium was changed again on day 5 and experiments began on day 6. MDMs were infected on day 7 with 500ng of p24 of replication competent either HIV-1-NL4-3-BAL-HSA or HIV-1-NL4-3-AD8 viruses diluted in 200-300µL culture medium for 6 hours. Following 6 hours of infection, the total medium volume was brought back up to the culturing volume of 600µL. On day 8, the 600µL of medium containing virus was removed, the cells washed with 1mL of PBS to remove any cell-free virus and replaced with 600µL of fresh culture medium.

Generation and quantification of viruses

Replication competent viruses were generated using calcium phosphate-mediated transfection of HEK293FT cells. Briefly, HEK293FT cells were plated in T175 tissue culture coated flasks (VWR) in 20mL of Dulbecco's Modified Eagle Medium (DMEM) (Gibco, US) supplemented with 10% FBS (Gibco, US), and 2mM L-glutamine (Gibco, US). The following morning, the medium was replaced, and cells were transfected with 25ug of viral plasmid for 6 hours. The HIV-1-NL4-3-BAL-HSA (HIV-1 BAL) virus was generated using the pNL-43-BAL-IRES-HSA plasmid, and the HIV-1-NL4-3-AD8 (HIV-1 AD8) virus was generated using the pNL4-3-AD8 plasmid. After 6 hours, the transfection medium was replaced, and cells were cultured for 2 days. At which time the virus-containing supernatants were removed, filtered at 0.45µm to remove any cellular debris, and stored at - 80°C until further use. All viruses were quantified by p24 ELISA (Zeptometrix).

Inhibitors

Inhibitors were purchased from suppliers (listed in supplementary table X) and re-suspended at either 10mM or 100mM depending on accompanying documentation. MDMs were initially treated with inhibitors on day 6 post isolation. On day 7, MDMs were infected with 500ng of either HIV-1 BAL or HIV-1 AD8, in the presence of inhibitors. On day 8, the infection medium was removed and replaced with fresh culture medium containing the inhibitors. Inhibitors were tested in MDMs from at least two donors, at multiple concentrations. Determination of effect on SAMHD1 phosphorylation was visualized by western blot and flow cytometry.

Western blotting

MDMs were lysed for western blotting 8 days post isolation. Culture medium was removed, cells were washed with 1mL of ice-cold PBS, following wash, 125µL of ice-cold NETN lysis buffer containing protease and phosphatase inhibitors (complete ULTRA tablets and Postop, by Roche, respectively). Cells lysed on ice for 10 minutes before protein was collected and transferred to Eppendorf tubes. Lysate remained on ice for a minimum of 1 hour, after which they were sonicated 4 times for 15 seconds, to prevent the water in the solicitor from heating. Following sonication, the lysates were centrifuged at 13000 rpm at 4°C for 10 minutes. After sonication, the lysates were transferred to new Eppendorf tubes, and frozen at -20 °C until needed. The protein concentration of each lysate was determined by BCA assay (Pierce BCA Protein Assay Kit, Thermo Scientific) according to manufacturer's protocol. 10µg of protein was loaded into each well of each gel (Criterion TGX Precast Gels 4-15%, with either 18 or 26 wells, Bio-Rad). Gels were run between 60 and 100 volts and transferred to 0.45 µm PVDF transfer membrane (Thermo Fisher) through semi dry transfer method at 20 volts and 0.4 amps for 30 minutes.

Flow cytometric analysis

MDMs were stained in their 24-well plate to maintain SAMHD1 phosphorylation. Culture medium was removed, and cells were washed with 1mL of PBS. Cells were stained in 200µL PBS at 4°C for 30 minutes with a fixable viability dye (Thermo Fisher) in the presence of an Fc receptor blocking reagent (Miltenyi Biotec) and with or without an anti- mouse heat stable antigen (HSA, CD24) stain (Thermo Fisher) depending on which virus as used to infect cells during each experiment. After 30 minutes, 1mL of PBS was added to each well to dilute the stains and wash the cells. The PBS was removed, and cells were fixed in 200µL of prewarmed BD Cytofix fixation buffer (Becton Dickinson) at 37°C for 15 minutes. Cells were then washed as described in the previous step. Then the PBS was removed, and cells were permeabilized in 200µL of room temperature BD Phosflow perm buffer III (Becton Dickinson) at 4°C for 30 minutes. Cells were then washed with 1mL 1X BD Perm/Wash buffer (Becton Dickinson) as described. After the perm/wash was removed, cells were stained at 4°C for 60 minutes in 200µL perm/wash for phospho-SAMHD1-T592 (Cell Signaling Technologies) with or without a stain for the HIV-1 core antigen, clone KC57 (Beckman Coulter) depending on which

virus was used during each experiment. After 1 hour, 1mL of PBS was added to each well and plates were left at 4 °C overnight. The following morning, cells were scraped from the well surface with mini cell scrapers and transferred to flow tubes (Corning). 2mLs of PBS was added to each tube and cells were centrifuged at 5000 rpm for 5 minutes. Following centrifugation, the majority of PBS was aspirated out of each tube, leaving cells pelleted in approximately 250µL. Samples were run using an Attune NxT Cytometer (Thermo Fisher) and analyzed using FlowJo 10.7.1 (Becton Dickinson).

RESULTS AND DISCUSSION

Dasatinib was originally designed to inhibit the tyrosine kinases Abl and Src to treat imatinib- resistant chronic myelogenous leukemia and Philadelphia chromosome-positive acute myelogenous leukemia [26]. Therefore, we first tested whether inhibition of either Abl or Src would result in lower phosphorylation levels of SAMHD1 in macrophages. We confirmed that dasatinib inhibits both Abl and Src (Figure 1A). Next, we inhibited Abl and Src individually to see if their inhibition caused a reduction in SAMHD1 phosphorylation. Figure 1B shows that when we treated cells with the Abl-specific inhibitor, flumatinib [31], we did not observe a change in SAMHD1 phosphorylation. Additionally, when we used the Src inhibitor 1 [32], SAMHD1 phosphorylation was also unchanged (Figure 1C). After ruling Abl and Src out as upstream players in SAMHD1 phosphorylation, we decided to test a broader panel of tyrosine kinases for their potential roles in mediating downstream SAMHD1 phosphorylation. We tested inhibitors of vascular endothelial growth factor receptor VEGFR), platelet-derived growth factor receptor (PDGFR), ephrin receptor (EphR), epidermal growth factor receptor (EGFR), and fibroblast growth factor receptor (FGFR) (Figure 2). Inhibiting each of these tyrosine kinases individually did not reduce SAMHD1 phosphorylation to any degree close to that observed when macrophages were treated with dasatinib. We also tested Janus kinases for their potential roles in regulating SAMHD1 phosphorylation. Figure 3 shows that dasatinib induces dephosphorylation in JAK1, 2 and 3, and tyrosine kinase 2 (TYK2). However, inhibiting any of these four JAKs individually failed to reduce SAMHD1 phosphorylation. Several reports support CDK1 as the sole kinase responsible for SAMHD1 phosphorylation [18,20,21,24,33], although other reports propose that CDK1 acts in combination with cyclin A2 [18,20,24]. Yet, other reports propose that CDK2 alone [20,34], or in conjunction with CDK6 [15], or cyclin D3 [35], can phosphorylate SAMHD1. Still others suggest that CDK6 works with CDK4 to phosphorylate SAMHD1 [36], or that CDK4 under the control of cyclin D2 is responsible [37]. Our inability to identify upstream tyrosine kinases that could modulate SAMHD1 phosphorylation in response to dasatinib treatment could potentially be explained if dasatinib had a direct inhibitory effect on the cyclin dependent kinases CDK 1, 2, 4 and 6.Therefore, we tested this idea in Figure 4, which shows that dasatinib induces dephosphorylation of CDK1 at residue Threo161, which is synonymous with CDK1 inactivation. We then tested whether specific inhibition of CDK1, CDK2, CDK4 and CDK6 with CDK1 inhibitor, CDK1/2 inhibitor III, CDK4 inhibitor, and CDK4/6 inhibitor IV (respectively) would reduce SAMHD1 phosphorylation. Individual inhibition of each of the cyclin dependent kinases 1, 2, 4 and 6 indeed reduced SAMHD1 phosphorylation (Figure 5A). All CDK inhibitors tested uniformly reduced phosphorylation of SAMHD1 (Figure 5A). We therefore predicted that cells treated with CDK inhibitors would be protected from HIV-1 infection. We then evaluated to what extent these dephosphorylation events impacted infections of macrophages by HIV-1 (Figure 5B). Macrophages treated with Dasatinib were strongly protected from infection, as was previously reported [21,24]. The indicated CDK inhibitors showed reduced HIV-1 infection to varying degrees. Comparatively, treatment with the CDK1-specific inhibitor reduced HIV-1 infection by 83.75% (Figure 5B), and with the CDK4-specific inhibitor by 83.13% (Figure 5B), while the dual specificity inhibitors CDK1/2 inhibitor III and CDK4/6 inhibitor IV were less protective, reducing infection by 11.56% and 41.56%, respectively.

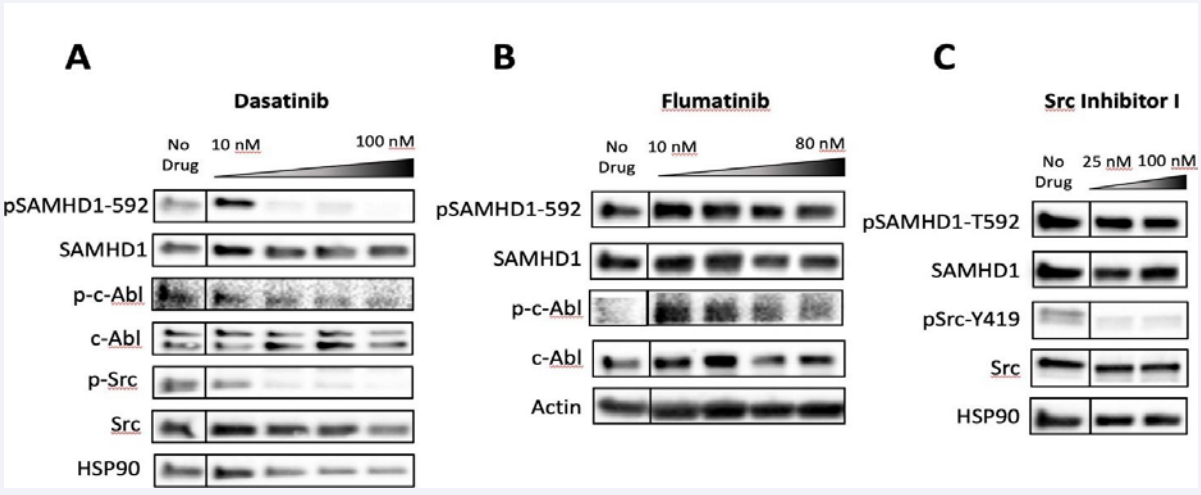

Figure 1 A: Dasatinib inhibits Abl and Src phosphorylation. B: Flumatinib, Abl specifici inhibitor, does not reduce SAMHD1 phosphorylation. C: Inhibiting Src, with Src inhibitor I, does not inhibit SAMHD1 phosphorylation.

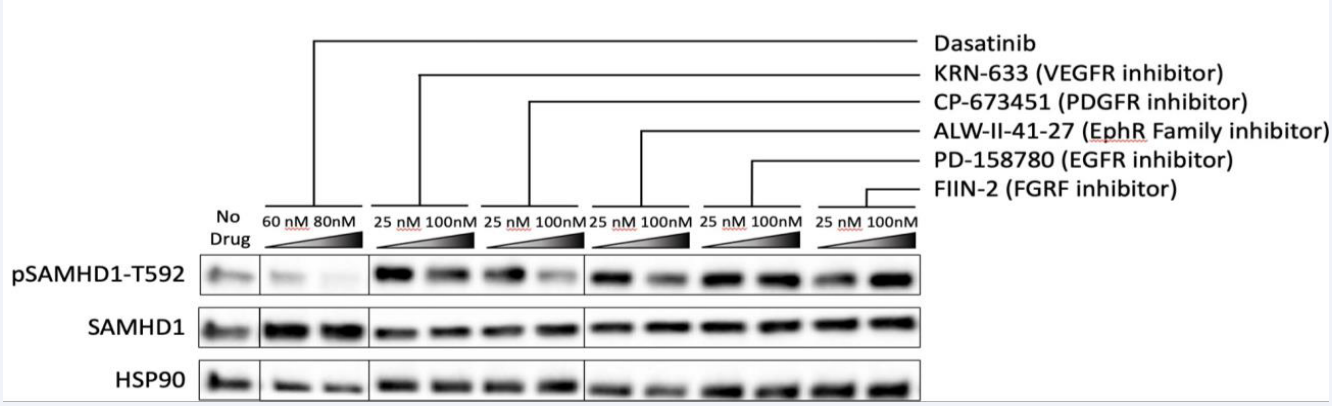

Figure 2 Inhibition of cell surface tyrosine kinase receptors in macrophages does not reduce SAMHD1 phosphorylation to the same degree as dasatinib.

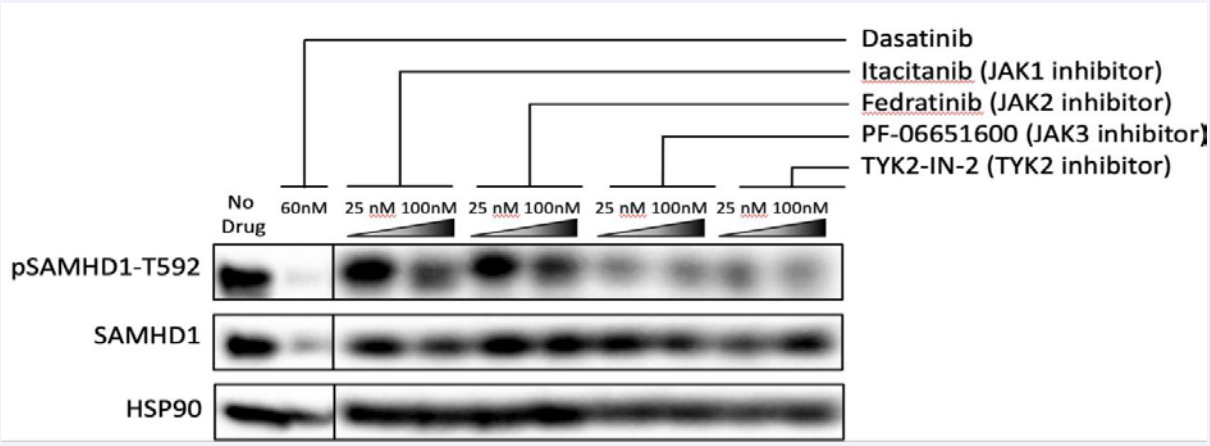

Figure 3 Inhibiting the JAK proteins does not inhibit SAMHD1 phosphorylation to the same degree as dasatinib.

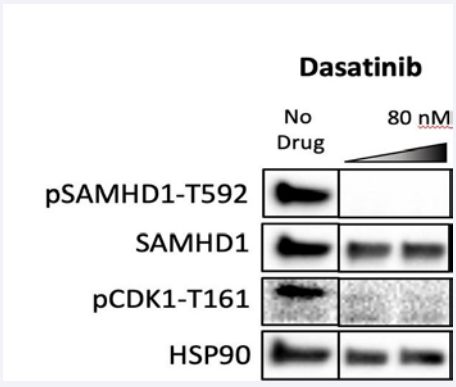

Figure 4 Dasatinib inhibits the phosphorylation of CDK1 at threonine 161, in the kinase loop, of monocyte-derived macrophages.

CONCLUSIONS

Taking our data together with previously available literature [15,18,20,21,38], we propose that dasatinib directly inhibits the serine/threonine kinases CDK1, CDK2, CDK4, and CKD6, thus resulting in potent dephosphorylation of SAMHD1 via redundant pathways, conferring protection of MDMs from infection by HIV-1.

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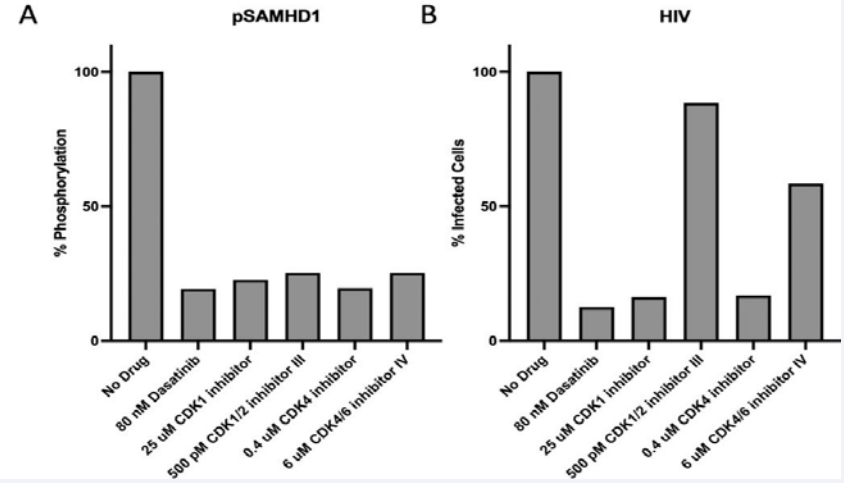

Figure 5 A: SAMHD1 phosphorylation in macrophages is reduced by dasatinib and by specific inhibitors of CDK1, 2, 4 and 6. B: Pre-treatment of macrophages with dasatinib and specific inhibitors of CDK1, 2, 4, and 6 reduces HIV-1 infection.

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