EXTRACELLULAR VESICLE-ASSOCIATED C-SRC ACTIVATES LATENT HIV-1

by

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A Thesis
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Science
Biology

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Bachelor of Science
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Summer Semester 2019
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DEDICATION

This is dedicated to my loving husband Derrick and my two siblings, Euginia and Godwin.

ACKNOWLEDGEMENTS

I would like to thank my family, friends, and lovedones who have supported me. My mentor, Dr Kashanchi lent me his expertise and guidance at every step. All the members of the Kashanchi lab, especially Alex who helped me throughout this journey. Drs. Hakami, and Luchini of my committee were of invaluable help. Finally, a big thanks goes out to the Fenwick Library for providing a clean, quiet, and well-equipped repository in which to work.

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LIST OF ABBREVIATIONS

Extracellular Vesicles ..... EVs
Cellular Src Kinase ..... c-Src
Human Immunodeficiency Virus Type-1 ..... HIV-1
Epidermal Growth Factor Receptor ..... EGFR
Phosphoinositide PI3K
Protein Kinase B ..... AKT
Mammalian Target of Rapamycin ..... mTOR
Protein Kinase B ..... AKT
Signal Transducer and Activator of Transcription 3 ..... STAT3
Steroid Receptor Co-activator 1 ..... SRC-1
HIV-associated Neurocognitive Disorders ..... HAND
Phorbol 12-myristate 13-acetate ..... PMA
Receptor Tyrosine Kinase ..... RTK
Whole Cell Extract ..... WCE
Transactivating Response Element ..... TAR
Combined Antiretroviral Therapy ..... cART
Nanoparticle ..... NT

ABSTRACT

EXTRACELLULAR VESICLE-ASSOCIATED C-SRC ACTIVATES LATENT HIV-1

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HIV-1 is a retrovirus that has infected more than 37 million people worldwide. Latent reservoirs throughout the body have proven to be a major hurdle when it comes to completely eradicating the virus. In our previous study, we found that exosomes, small membrane-bound vesicles, from uninfected cells activate the transcription of HIV-1 in latent infected cells regardless of combination antiretroviral therapy (cART) leading to increased mostly short and some long HIV-1 RNA transcripts. This is consistent with the notion that none of the FDA-approved antiretroviral drugs used today in the clinic are transcription inhibitors. These HIV-1 transcripts can then be packaged into extracellular vesicles (EVs), including exosomes, and released from the infected cell potentially eliciting detrimental effects in recipient cells such as increased susceptibility to infection. In this study, we investigated the specific mechanism behind the EV activation of latent HIV-1. We discovered that phosphorylated c-Src (Y-416; active) is present in EVs from various cell lines and has the ability to activate epidermal growth factor receptor (EGFR),

initiating a downstream signaling cascade. We proposed that EGFR is able to activate the PI3K/AKT/mTOR pathway, STAT3, and SRC-1 following activation by c-Src. This was verified by examining the levels of HIV-1 TAR, genomic RNA and HIV-1 Gag p24 protein in the presence of inhibitors of each of the specific proteins involved in our proposed pathway. We discovered that upon inhibiting each of the proteins involved in the aforementioned pathway, HIV-1 transcription, as well as levels of HIV-1 Gag p24 in the cell supernatant, was decreased. Furthermore, we found that EVs containing c-Src were able to rescue HIV-1 despite the presence of the inhibitors, validating the importance of EV-associated c-Src in reversing HIV-1 latency. Collectively, our data suggests that the EV activation of latent HIV-1 is initiated by EV-associated c-Src being delivered to a recipient cell, where it is able to activate the PI3K/AKT/mTOR pathway and STAT3, eventually leading to the activation and translocation of SRC-1 to the nucleus, promoting a pro-transcription state.

INTRODUCTION

Human Immunodeficiency Virus type 1 (HIV-1) is a global health crisis, infecting over 37 million worldwide (1). HIV-1 is an enveloped retrovirus that targets immune cells, mainly T-cells and macrophages, and is the causative agent of AIDS (Acquired Immunodeficiency Syndrome) (2). Upon infection, viral RNA is reverse transcribed and integrated into the host's genome, resulting in the production of viral particles (3). Complete clearance of the virus by the host is unachievable due to latent viral reservoirs throughout the body and the brain, especially in resting memory $CD4^+$ T-cells and macrophages (4). Latency is established upon integration of the viral genome into the host's and is characterized by the inability to detect infectious virions in plasma (2,5). The immune system is incapable of distinguishing these latent cells from healthy cells and therefore is unable to target them for elimination. This makes the issue of latency one of the major hurdles in the discovery of a HIV-1 cure (2).

HIV-1 also affects the central nervous system (CNS), infecting microglia leading to the development of HIV-associated neurocognitive disorders (HAND), such as dementia (6). Close to 50% of HIV-1-infected patients suffer from one of the three sub-classes of HAND – asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND), and HIV-associated dementia (HAD) (7). HIV-1-infected microglia stimulate the immune system through the release of toxic viral proteins, resulting in

inflammation and ultimately causing severe cognitive impairment (8). This diseased state stems from the death of neurons due to HIV-1-mediated toxicity (9,10).

HIV-1 comprises of 9 genes: *gag* (group specific antigen), *pol* (polymerase), *env* (envelope), *tat* (transcription activator), *rev* (regulator of viral gene expression), *vif* (viral infectivity factor), *vpr* (viral protein R), *vpu* (viral protein U), and *nef* (negative factor) (11). These genes are organized into 3 categories – structural, regulatory, and accessory – and encode 15 viral proteins (11). *Gag*, *pol*, and *env* comprise the structural genes while *tat* and *rev* are considered to be the regulatory genes (12). The remaining 4 accessory genes are not required for viral replication; however, studies have shown them to increase both the host's susceptibility to infection as well as the severity of infection (11,12). *Gag* codes for structural proteins, such as matrix (p17), capsid (p24), and nucleocapsid (p7), that protect the internal viral RNA genome(11). *Pol* codes for protease (cleaves the Gag polyprotein), reverse transcriptase (converts viral RNA into DNA), and integrase (integrates the viral DNA with the host's)(13). *Env* encodes envelope glycoproteins 120 and 41 (gp120 and gp41) that sit on the surface of the virion and interact with host surface receptors, mainly CD4, on T helper cells, facilitating viral entry (14). The Tat and Rev proteins are required for the transcription and translation of viral genes. Tat significantly increases the efficiency of transcription by binding to the viral RNA at the transactivating response element (TAR) promoting the elongation of viral transcripts (14,15). Tat has also been shown to have a toxic effect on uninfected bystander T cells causing apoptosis (14). Rev mediates the transport of spliced and unspliced viral mRNAs between the nucleus and cytoplasm (11). Vif, Vpr, Vpu, and Nef proteins function to

increase viral replication, enhance release of virus from the cell, down-regulate host cell CD4 and Class I MHC expression, and increase the survival of infected cells, respectively (12,14). Together, the accessory proteins contribute to the virus' ability to circumvent the host immune system and promote latency.

Combined Antiretroviral Therapy (cART)

There is no cure for HIV-1; however, the introduction of combined antiretroviral therapy (cART) has proven to be quite successful in suppressing the replication of the virus and preventing the onset of AIDS. Patients under cART have an increased life expectancy and improved quality of life(16). In addition, cART has been demonstrated to significantly reduce the rates of maternal-infant transmission and decrease the incidence of opportunistic infections (16,17).

cART lowers HIV-1 viral loads to undetectable levels in plasma but is unable to cure latent infected cells (2). As such, the viral infection reactivates upon discontinuation of cART, leading to viral rebound and an increase in opportunistic infections and the advancement of HIV-1 into AIDS (18,19). cART is a multi-regimen assortment of drugs that works by targeting the HIV-1 replication cycle at several steps: viral entry, reverse transcription, integration, and virion maturation(19,20). Fusion inhibitors and CCR5 antagonists, such as maraviroc, prevent viral entry into immune cells by targeting CD4 and CCR5 receptors used by the virus to gain entry into the cell (21). Nucleoside/nucleotide reverse transcriptase inhibitors (NRTI/NtRTI), such as didanosine and nevarapine, prevent the HIV-1 reverse transcriptase enzyme from converting viral RNA into DNA that can then be inserted into the host genome (21). Another class of

antiretroviral drugs, integrase inhibitors, such as raltegravir, work by targeting the catalytic sites of integrase that are involved in the processing and joining of the viral and cell DNA in the nucleus (21,22). Protease inhibitors, such as ritonavir, target virion maturation by HIV-1 protease by binding to it and preventing its proteolytic activity (21,23). Even though cART works to block the fundamental stages of the HIV-1 growth cycle and has been shown to prolong patient survival, it does not eliminate latent reservoirs.

The inability of cART and the immune system to recognize latent infected cells stems from the integration of the viral genome into the host's DNA. This has led to the development of the "shock and kill" method, in which latency reversing agents (LRAs) reactivate latent infected cells in order for the immune system and/or cART to eliminate the virus (18,24). Recently, a biological LRA, exosomes, has been discovered to activate the transcription of latent HIV-1 (25).

Extracellular Vesicles (EVs) and HIV-1

Exosomes, small extracellular vesicles (30-120 nm diameter) originating from multivesicular bodies and released in most cell types, were originally thought to remove cellular waste (26). However, it has now been shown that exosomes participate in cell-to-cell communication, immune response, and neuronal functions (27–29). Exosomes contain proteins, miRNAs, RNAs, and lipids and can transfer these distinctive molecules to neighboring cells, eliciting functional changes in these recipient cells (30–32).

Furthermore, exosomes have been implicated in the pathogenesis of viruses, such as HIV-1, Ebola Virus, Rift Valley Fever Virus, and Hepatitis C Virus (33,34). The

content of exosomes differs based on the infection or disease, which makes exosomes potential biomarkers for diseases and infections, including HIV-1, Alzheimer's Disease, Huntington's disease, and cancer (35,36). In the case of HIV-1, exosomes from infected cells have been shown to contain HIV-1 viral TAR RNA and viral proteins such as Nef (37,38). Exosomes produced from HIV-1 infected cells have been shown to increase the susceptibility to HIV-1 infection in recipient cells (38,39). This is partly due to the delivery of HIV-1 products, such as TAR RNA, by exosomes to neighboring cells. Exosomal TAR downregulates apoptosis and increases susceptibility of recipient cells to infection (38). Furthermore, TAR can activate NF-κB, a transcription factor associated with HIV-1 activation from latency, by binding to and activating the toll-like receptor 3 (TLR3) signaling cascade (39). Moreover, exosomal Nef can activate HIV-1 from latency through an ADAM17-associated mechanism (40).

Interestingly, exosomes from uninfected cells have also been shown to activate HIV-1 from latency (25). It was found that exosomes could activate HIV-1 despite the presence of cART in latent T cells (ACH2), monocytes (U1), and primary cells (peripheral blood mononuclear cells) (25). It was discovered that there was a significant increase in TAR RNA copies after the addition of uninfected exosomes in the aforementioned cells (25). This was explained by an increase in RNA Polymerase II loading onto the HIV-1 promoter (phosphorylated in the C-Terminal Domain at Ser 2 and 5) (25). In addition, an increase in the recruitment of Cdk9, a component of p-TEFb (positive transcription elongation factor) was observed, leading to the conclusion that exosomes from uninfected cells were responsible for this observed increase in HIV-1

transcription in latent cells (25). We therefore investigated the mechanism behind this process.

EV activation of latent HIV-1

A subsequent study by our lab of the contents of EVs from uninfected cells showed that EVs from uninfected T-cells and monocytes contained the protein kinase, cellular Src (c-Src), a non-receptor tyrosine kinase and a member of the Src family of kinases (41). c-Src is expressed in many cell types and found in the cytoplasm at the plasma membrane; and involved in signal pathways that result in cellular differentiation, proliferation, cell survival, angiogenesis, and motility (41–43). c-Src has also been linked to a number of major pathways including the RAF/MEK/ERK and PI3K/AKT/mTOR pathways (44). c-Src functions by physically interacting with other cellular proteins such as cell surface receptors, integrins, STATs, and cell-cell adhesion molecules, culminating in the phosphorylation and activation of downstream targets, notably the aforementioned pathways, both of which play a critical role in cell proliferation and cell survival(44–46).

The PI3K/AKT/mTOR pathway is a survival pathway that is activated in response to a number of cellular signals including the activation of growth factor receptors, amplification or mutation of AKT-1 and PI3K, and exposure to carcinogens (47). AKT-1, or Protein Kinase B, is a serine/threonine kinase which regulates a number of signaling pathways that result in changes in cell proliferation, metabolism, apoptosis, and cell cycle(48). Similarly, PI3K (Phosphatidylinositol 3-kinase) is a downstream effector of receptor tyrosine kinases (RTKs) and regulates several cellular processes including proliferation, growth, apoptosis, and cytoskeletal rearrangement (47). mTOR

(mechanistic target of rapamycin) is a serine/threonine kinase that integrates and regulates several signals that play a critical role in cellular growth, autophagy, proliferation, metastasis, and protein translation (47). The activation of the PI3K/AKT/mTOR pathway initiates at the cell membrane by the activation of PI3K by tyrosine kinase growth factor receptors such as epidermal growth factor receptor (EGFR) and insulin-like growth factor-1 receptor (IGF-1R), cell adhesion molecules such as integrins, G-protein-coupled receptors (GPCRs), and oncogenes such as Ras (47,49). This leads to the activation of AKT-1 which directly phosphorylates and activates its main downstream target, mTOR (47).The PI3K/AKT/mTOR pathway is activated in many forms of cancer and has recently been shown to be manipulated by HIV-1 by way of viral proteins such as Nef in order to increase pathogenesis(50).

The PI3K/AKT/mTOR pathway, together with c-Src, have been implicated in HIV-1 replication (51). Activation of Src tyrosine kinases have been observed in HIV-1 infected $CD4^+$ T cells within minutes after infection (52). In addition, McCarthy and colleagues demonstrated that inhibiting c-Src restricts HIV-1 early entry in activated primary $CD4^+$ T cells (52). Tyrosine phosphorylation signaling has been shown to be instrumental for viral entry, actin remodeling, and translocation of the viral preintegration complex (PIC) into the nucleus (53–55). Furthermore, it was discovered that the inhibition of mTOR led to the suppression of viral replication in infected cells in both the presence and absence of Tat (56). Together, these discoveries indicate the involvement of c-Src and PI3K/AKT/mTOR pathway in the activation of HIV-1.

Proposed mechanism of activation

Our preliminary results showed that c-Src is found in EVs and that dasatinib, a c-Src inhibitor, significantly decreases EV-induced HIV-1 transcription as TAR RNA levels dropped by up to three logs. This led to the hypothesis that EV-associated c-Src plays a key role in EV activation of HIV-1. However, the specific mechanism behind this process is not clear. The aim of this study is to elucidate the mechanism by which EVs from uninfected cells activate transcription of HIV-1 in latent HIV-1-infected cells.

We hypothesize that uninfected EVs are taken up by HIV-1 infected cells, leading to the phosphorylation and activation of RTK (receptor tyrosine kinase) (i.e. EGFR) by c-Src. This then leads to activation of PI3K, which activates AKT-1. Following activation, AKT-1 phosphorylates mTOR, which results in the phosphorylation and activation of the transcription inducer, STAT3 (Signal Transducer and Activator of Transcription). STAT3 then recruits the cofactor SRC-1 before translocating to the nucleus and promoting HIV-1 transcription. This model will be tested by examining each of the aforementioned proteins using *in vitro* kinase assays as well as inhibitors of each protein. We expect to see that EVs can activate HIV-1 from latency through c-Src/EGFR//PI3K/AKT/mTOR/STAT3, and SRC-1.

MATERIALS AND METHODS

Cells and Reagents

CEM (uninfected T cells), Jurkat (uninfected T cells), U937 (uninfected promonocytic cell), ACH2 (HIV-1-infected T cells), and U1 cells (HIV-1-infected monocytes) were cultured in RPMI 1640 (Quality Biological) complete medium containing 1% L-glutamine, 1% streptomycin/penicillin, and 10% filtered, exosome-free fetal bovine serum (FBS) at 37^{o}C and 5% CO_2. Exosome-free FBS was obtained through ultracentrifugation at 100,000 X *g* for 90 minutes to remove bovine exosomes. Dasatinib (Sellekchem), Gefitinib (Sellekchem), LY294002 (Sellekchem), MK2206 (Sellekchem), Rapamycin (Sellekchem), WP1066 (Sellekchem), and Bufalin (Cayman Chemicals) will be used to treat cells in various experiments.

EV Isolation

CEM cells were grown in complete media supplemented with 10% exosome-free FBS, and EVs were isolated from 500 mL of cell culture grown in a roller bottle over the course of four weeks. Cells were pelleted by centrifugation at 300 X *g* for 10 minutes, and the cell supernatant was collected. An additional centrifugation at 2000 X *g* for 10 minutes was used to pellet dead cells and cell debris. The supernatant was collected and ultracentrifugation in a Ti70 rotor (Beckman) was performed at 100,000 X *g* for 90 minutes to pellet the EVs. All pellets were then re-suspended in Dulbecco's phosphate-

buffered saline without Calcium and Magnesium (PBS), consolidated into a single tube, and washed with PBS. The resulting large pellet was re-suspended in 1000 µL of PBS. All spins were performed at 4º C.

EV Characterization using *Zetaview*

Characterization of EVs isolated from CEM cells was done using the ZetaView® Z-NTA (Particle Metrix) and its corresponding software (ZetaView 8.04.02). One hundred nanometer polystyrene nanostandard particles (Applied Microspheres) were used to calibrate the instrument prior to sample readings at a sensitivity of 65 and a minimum brightness of 20. For each measurement, the instrument pre-acquisition parameters were set to a temperature of 23°C, a sensitivity of 85, a frame rate of 30 frames per second (fps), and a shutter speed of 250. CEM EVs were diluted in PBS prior to being loaded into the cell. Measurements by ZetaView were taken at 11 different positions throughout the cell, with 3 cycles of readings at each position. The mean, median, mode (indicated as diameter) sizes, and concentration were then calculated by the ZetaView software and analyzed using the same software and Microsoft Excel 2016.

Nanoparticle Capture of EVs

1 mL of cell supernatants was harvested and centrifuged at 25,000 X g for 5 minutes to remove cells. 30 µL of a 30% slurry of NT08/082 particles (Ceres Nanosciences, Inc.) were added to the supernatant and rotated at 4º overnight. This was followed by another centrifugation at 12,000 X g for 10 minutes. The resulting pellet was washed with 1 mL of PBS and resuspended in 15 µL of Laemmli buffer for western blot analysis.

Western Blot Analysis

Samples were loaded onto a 4-20% Tris-glycine gel (Invitrogen) and run at 200V. An overnight transfer of proteins to Immobilon membranes (Millipore) at 50mA was then performed. Membranes were then blocked for 30 minutes with PBS containing 0.1% Tween 20 (PBS-T) and 5% dry milk at 4ºC. Membranes were incubated overnight at 4ºC with the appropriate primary antibody against specified proteins. The next day, membranes were washed twice with PBS-T and incubated with appropriate HRP-conjugated secondary antibody in PBS-T for 2 hours at 4ºC. Membranes were then washed twice with PBS-T and once with PBS. Membranes were developed with Clarity Western ECL Substrate (Bio-Rad) and visualized by the Molecular Imager ChemiDoc Touch system (Bio-Rad).

Kinase Assay

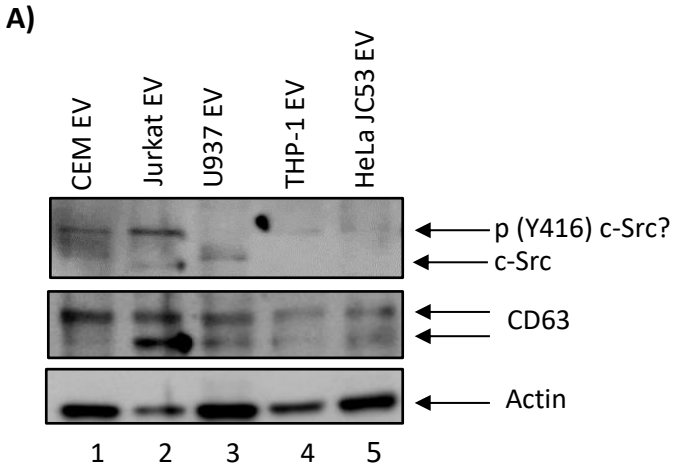
ACH2 whole cell extracts (WCEs) were immunoprecipitated (IP) overnight with antibodies against EGFR. Complexes were then precipitated with A/G beads (Calbiochem) for 2 hours at 4 °C. IPs were then washed twice with TNE buffer (Tris (pH7.5), NaCl, EDTA) and kinase buffer prior to incubation with γ-^{32}P ATP. Reactions were incubated at 37°C for 30 min followed by the addition of Laemmli buffer. The samples were separated by reducing SDS-PAGE on a 4–20% Tris–Glycine gel. Gels were stained with Coomassie blue, destained, and then dried for 2 hours. The gels were exposed to a PhosphorImager cassette and analyzed utilizing Molecular Dynamic's ImageQuant Software.

RESULTS

Phosphorylated c-Src and c-Src are present in EVs derived from multiple cell lines

In our previous 2017 study, we showed that exosomes from uninfected cells cause increased levels of HIV-1 RNA in latent infected cells (25). This was surmised from an amplification in the expression of p24 as well as an increase in the loading of phosphorylated RNA Pol II (Ser2/Ser5) and Cdk9 onto the HIV-1 promoter – all stemming from the addition of uninfected exosomes (25). In this study, our aim was to uncover whether an EV-associated kinase was behind this phenomenon. After a literature search of various kinases linked to HIV-1 transcription (52,57,58), we hypothesized that c-Src could be contributing to the activation of latent HIV-1. To assess the validity of this hypothesis, we isolated EVs from a number of different cell line types including CEM (T- cell), Jurkat (T cell), U937 (monocyte), THP-1 (myeloid), and HeLa JC53 (epithelial cell expressing CD4) via Nanotraps (NT80/82) pull down. This was followed by a western blot to assay for c-Src, CD63, and actin (***Fig. 1***). CD63 served as a control to confirm the successful capture of EVs while actin was used as a loading control. Data revealed that c-Src was present in all cell line EVs with the T-cell EVs (CEM and Jurkat) showing multiple bands while the others had a single band present (***Fig. 1A***). We postulated that the upper band present in T-cell EVs, THP-1 EVs, and HeLa EVs may be a modified form of s-Src, specifically, phosphorylated c-Src.

Next, we sought out to confirm whether the second band we observed in the previous experiment (***Fig. 1A***) was indeed phosphorylated c-Src. We therefore treated CEM, Jurkat, and U937 cells twice with PMA (phorbol 12-myristate 13-acetate), a compound that activates c-Src (59). Cells were allowed to incubate for 72 hours after the first treatment followed by a second treatment for a total of 5 days prior to harvest. EVs were then captured using NT80/82 prior to western blotting for the presence of total c-Src, phosphorylated c-Src, and actin. Here, we used an antibody against phosphorylated c-Src (Y416) because c-Src is active and capable of activating other proteins when it is phosphorylated at Y416 (60,61). Results in ***Fig. 1B*** show that phosphorylated c-Src (Y416) is indeed present in CEM, Jurkat, and U937 EVs, verifying results from ***Fig 1A***. Furthermore, treatment with PMA increased phosphorylation of c-Src (Y416). Taken together, these data indicate that phosphorylated c-Src (Y416) is present in uninfected T-cells and monocytes/myeloids EVs and capable of possibly activating downstream proteins initiating a signal cascade.



B)

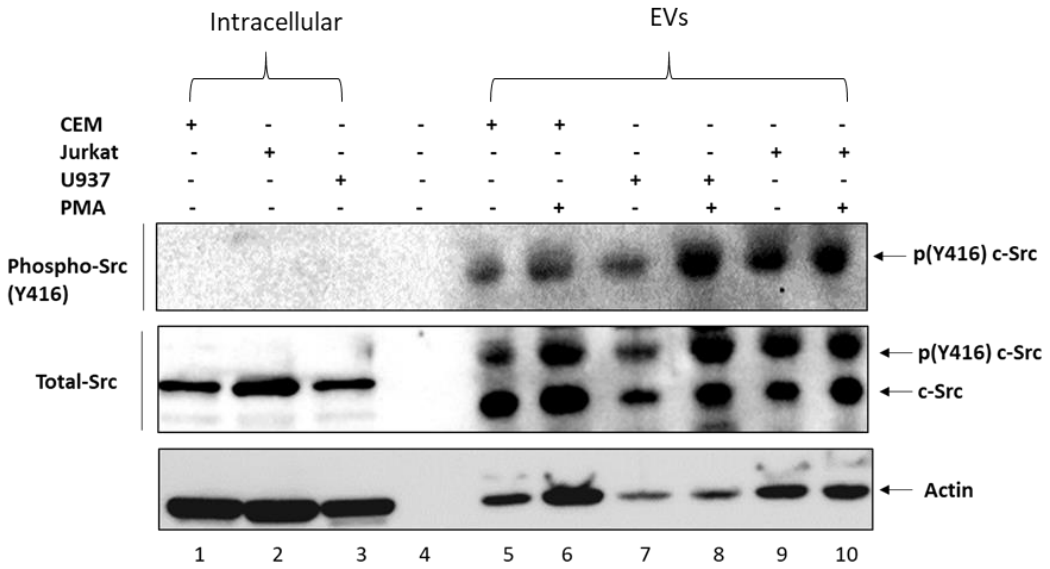


Figure 1: EVs from multiple cell lines contain c-Src and phosphorylated c-Src. A) One milliliter of cell supernatant from CEM, Jurkat, U937, THP-1, and HeLa JC53 cells was collected and treated with nanotraps (NT80/82) prior to rotating for 72 hours at 4° C to concentrate EVs. EVs were then Western blotted for the presence of c-Src and CD63 (EV marker). Actin was used as a loading control. **B)** Cells were treated with 100 nM PMA and allowed to incubate for five days at 37ºC with a second PMA treatment on Day 3. EVs were then concentrated using NT80/82 and rotated overnight at 4 °C. Samples were Western blotted for c-Src and actin. Whole-cell extracts (WCEs) were used as positive control for Western blots. Actin was used as a loading control.

c-Src phosphorylates EGFR

In order to map out the signaling cascade that results from the uptake of activated EV-associated c-Src, we took a look at downstream substrates of c-Src, such as EGFR (62). EGFR is one of the many Receptor Tyrosine Kinases (RTKs) whose stimulation leads to the activation of various downstream pathways that have been shown to regulate HIV-1 latency such as the PI3K/AKT/mTOR pathway (46,56). Hence, we performed an *in vitro* kinase assay with ACH2 (infected T-cells) WCE and EGFR as a substrate. Here, we added 500 ng of purified c-Src to 0.5 mg ACH2 WCE and immunoprecipitated with

anti-c-Src antibody overnight at 4°C. Protein A/G agarose beads were then added to bring down proteins. Data in ***Fig. 2*** revealed that the addition of purified c-Src to ACH2 WCE increased phosphorylation of EGFR. This is consistent with published research stating that the activation of EGFR by c-Src initiates a signal cascade via the activation of downstream proteins (63). Together, this data implies that EV-associated can phosphorylate EGFR following the uptake of EVs by cells.

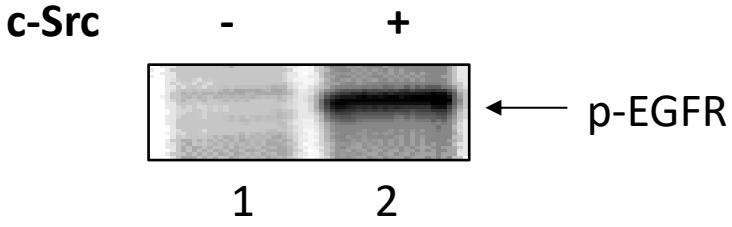
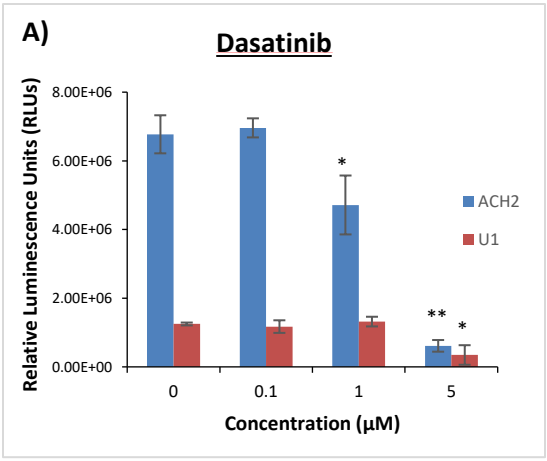


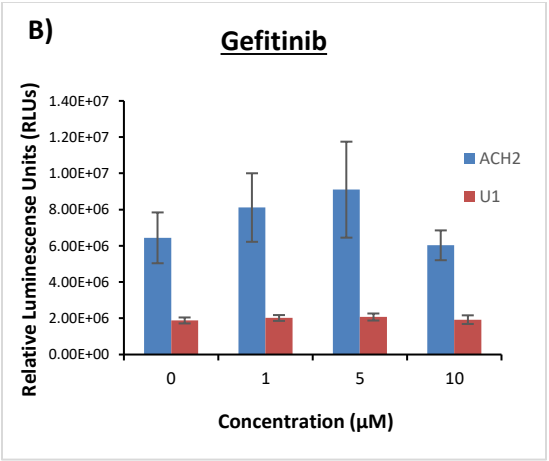
Figure 2: Epidermal Growth Factor Receptor (EGFR) and c-Src *in vitro* kinase assay. ACH2 whole cell extracts (WCEs) were immunoprecipitated (IP) overnight with an antibody against c-Src. Complexes were then precipitated with Protein A/G agarose beads for 1 hour at 4°C. IPs were washed twice with TNE buffer and kinase buffer prior to incubation with γ-^{32}P ATP. The IPs were then used for *in vitro* kinase assays using EGFR as a substrate.

Obtaining optimal dosages for kinase inhibitors

Next, we investigated the effects of kinase inhibitors (dasatinib, gefitinib, LY294002, MK2206, rapamycin, WP1066, and bufalin) targeted at the proteins involved in the proposed signal mechanism. In order to determine the optimal dosage for future treatments, ACH2 and U1 cells were treated with different concentrations of the various inhibitors 48 hours prior to a cell titer assay. Results in ***Fig. 3A*** revealed that both cell types (ACH2 and U1) showed less viability at a 5µM concentration of dasatinib (c-Src inhibitor). ***Fig. 3B*** shows no significant loss in cell viability when cells were treated with

gefitinib in both cell type. LY294002 (PI3K inhibitor) treatment saw a loss in cell viability at 10 µM while MK2206-2 HCl (AKT-1 inhibitor) caused a loss in cell viability at 1 µM as shown in ***Fig. 3C-D*** respectively. We did not observe any significant loss in cell viability after treatment with Rapamycin (mTOR) inhibitor (***Fig. 3E***). Treatment with WP1066 (STAT3 inhibitor) caused major loss of cell viability in both ACH2 and U1 cells at concentrations above 1 µM (***Fig. 3F***) while bufalin resulted in loss of cell viability in both cell types starting at 1 nM as shown in ***Fig. 3G***. Furthermore, we investigated cell viability in uninfected cells when a decrease in viability was observed in the infected cells. Results (***Sup. Fig. 1***) from this experiment revealed that treatment with dasatinib, LY294002, MK2206-2 HCl, and bufalin resulted in decreased cell viability at lower dosages than in infected cells, indicating that HIV-1 may confer some resistance to infected cells against these inhibitors. On the other hand, WP1066 only affected infected cells at 1 and 5 µM concentrations. This points to the potential of WP1066 as a possible treatment against HIV-1. Based on these data, we used concentrations of 5 µM, 10 µM, 25 µM, 1 µM, 150 nM, 1 µM, and 5 nM of dasatinib, gefitinib, LY294002, MK2206-2 HCl, rapamycin, WP1066, and bufalin, respectively for cell treatments in future experiments.


A)
Dasatinib
Relative Luminescence Units (RLUs)
8.00E+06
6.00E+06
4.00E+06
2.00E+06
0.00E+00
0
0.1
1
5
Concentration (μM)
ACH2
U1
*
**
*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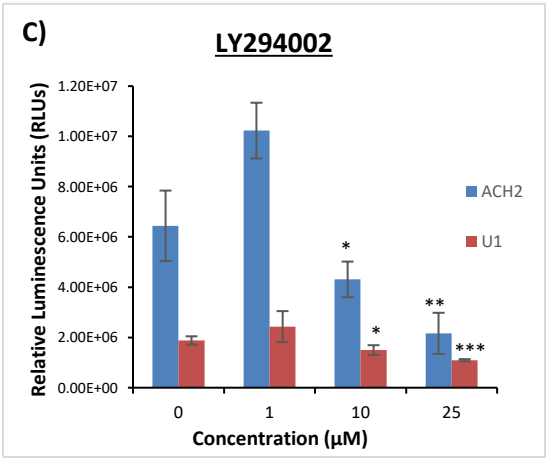
0
1
5
10
Concentration (μM)
ACH2
U1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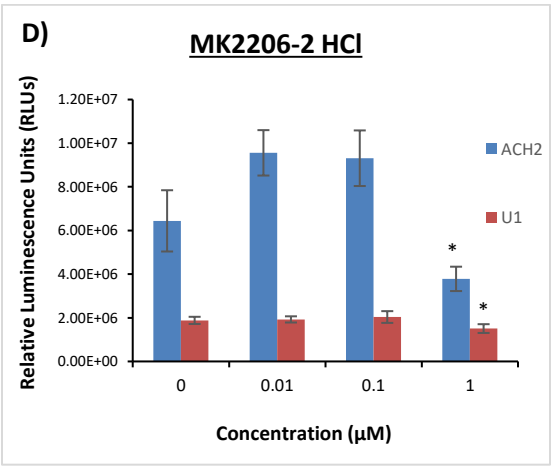
0
1
10
25
Concentration (μM)
ACH2
U1
*
*
**
***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0
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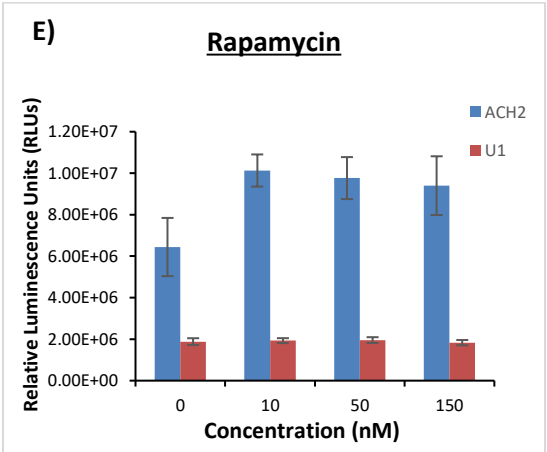
0.1
1
Concentration (μM)
ACH2
U1
*
*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Relative Luminescence Units (RLUs)
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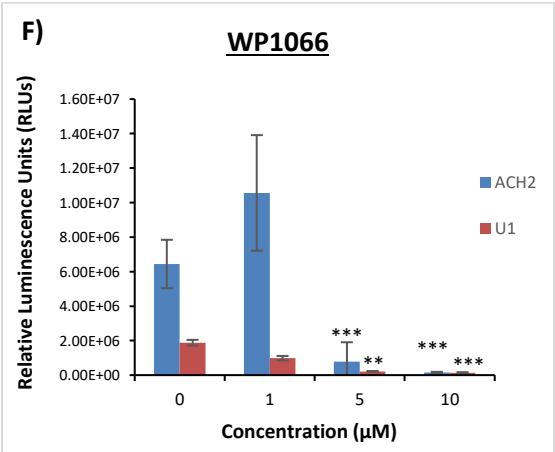
0
10
50
150
Concentration (nM)
ACH2
U1


F)
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6.00E+06
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0.00E+00
0
1
5
10
Concentration (μM)
ACH2
U1
***
**
***
***

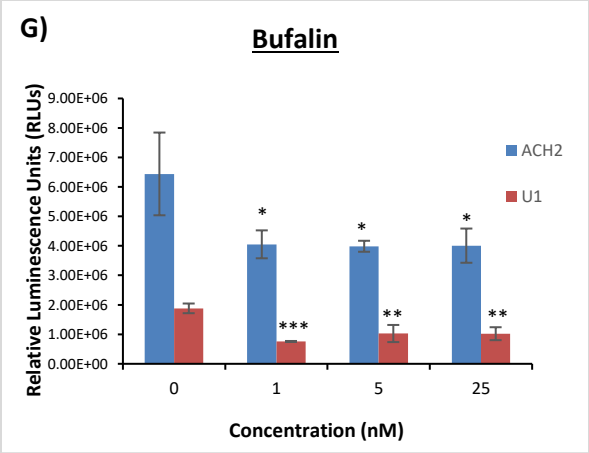


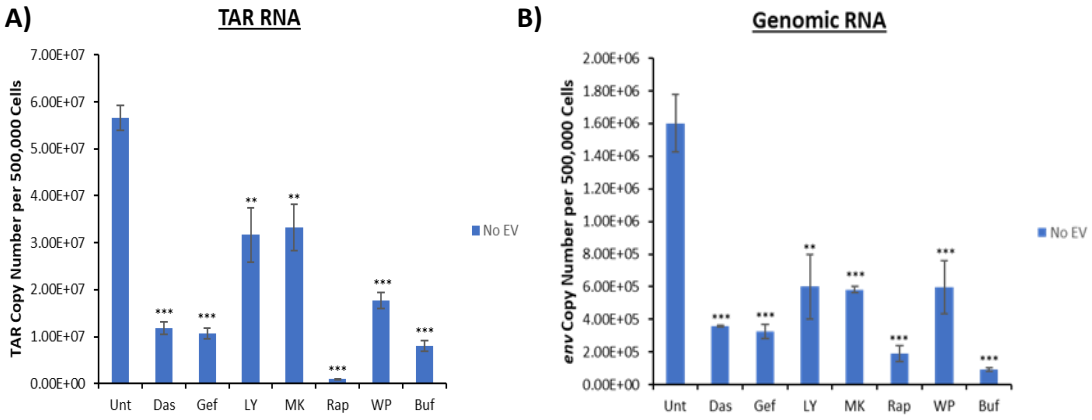
Figure 3: Obtaining optimal dosage for kinase inhibitors. $5X10^4$ cells were plated with different concentrations of kinase inhibitors and allowed to incubate for 48 hours prior to a Cell-Titer Glo assay. ACH2 and U1 cells were treated with **A)** Dasatinib (c-Src inhibitor) at 0, 0.1, 1, or 5 µM. **B)** Gefitinib (EGFR inhibitor) at 0, 1, 5, or 10 µM. **C)** LY294002 (PI3K inhibitor) at 0, 1, 10, or 25 µM. **D)** MK2206-2 HCl (AKT-1 inhibitor) at 0, 0.01, 0.1, or 1 µM. **E)** Rapamycin (mTOR inhibitor) at 0, 10, 50, or 150 nM. **F)** WP1066 (STAT-3 inhibitor) at 0, 1, 5, or 10 µM. **G)** Bufalin (SRC inhibitor) 0, 1, 5, or 25 nM.

EVs containing c-Src rescue HIV-1 levels in inhibitor-treated cells

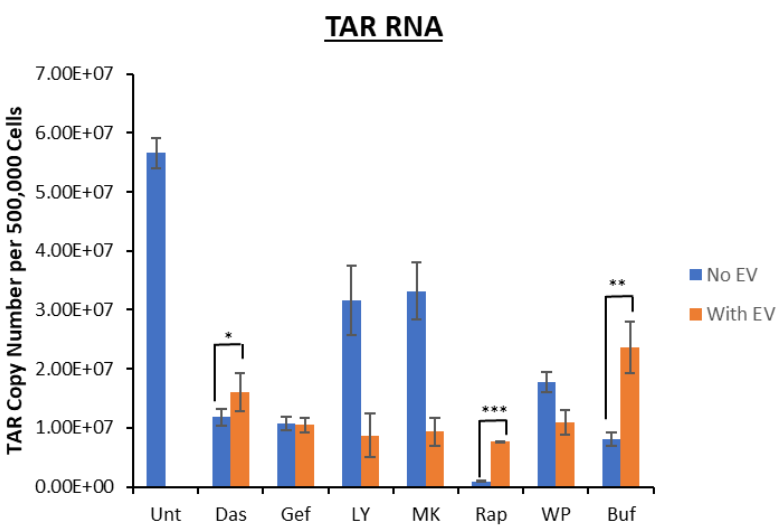
We next asked how EVs would affect the level of HIV-1 transcription in the presence of the kinase inhibitors investigated above. Here, we treated U1 cells with each of the seven kinase inhibitors for 48 hours. A second treatment was performed 2 hours prior to the addition of EVs from uninfected CEM cells. After a 24-hour incubation period, another CEM EV treatment was done. Cell pellets were prepped for RNA isolation and subjected to RT-qPCR. Results from ***Fig. 4A*** show that treatment with inhibitors against the proteins in our proposed signal cascade resulted in a significant decrease in HIV-1 TAR RNA levels. Similar results were observed in ***Fig. 4B*** where genomic RNA levels decreased in inhibitor-treated cells compared to the control. This decrease was especially striking in bufalin-treated cells signifying the importance of SRC-1 in the transcription of full-length, coding HIV-1 RNA. Remarkably, we observed

the opposite effect upon the addition of CEM EVs (containing c-Src) where HIV-1 TAR RNA and genomic RNA levels significantly increased despite the presence of inhibitors suggesting that EVs may be overriding the effects of the inhibitors (***Fig. 4C-D)***.

To confirm these sets of results, we concentrated (NT86) and Western blotted for the presence of HIV-1 virions (Gag p24) in the cell supernatant (as described in Materials and Methods). Data in ***Fig. 4E*** show minimal levels of p24 in inhibitor-treated cells. ***Fig.4F*** demonstrates that upon the addition of EVs, the levels of p24 significantly increased despite the presence of the inhibitors against c-Src, EGFR, PI3K, AKT-1, mTOR, and STAT3 compared to the control. Similarly, the levels of Gag p24 decreased in U1 cells (plated in triplicates) treated with bufalin as shown in **Fig. *4G***. However, treatment with CEM EVs resulted in an increase in Gag p24 expression (***Fig. 4H***). This implies that EV-associated c-Src is able to rescue Gag p24 levels despite presence of inhibitors against key upstream proteins implicated in HIV-1 transcription.



C)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TAR Copy Number per 500,000 Cells
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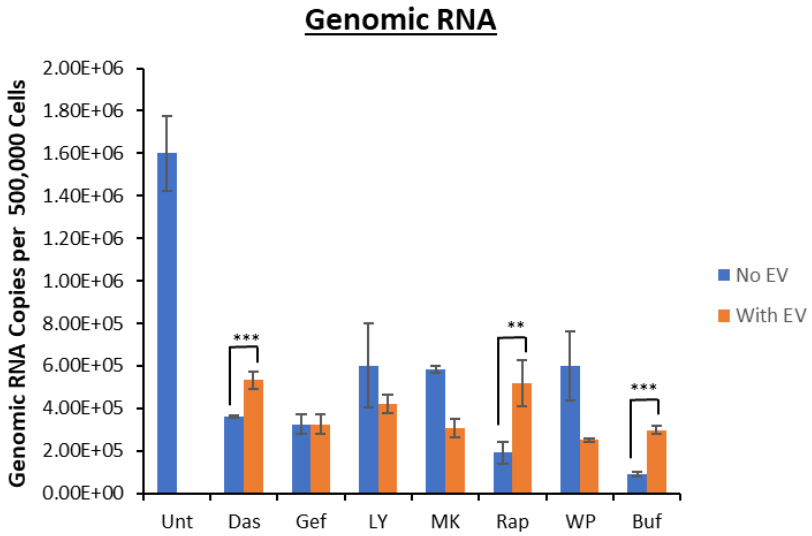
6.00E+07
5.00E+07
4.00E+07
3.00E+07
2.00E+07
1.00E+07
0.00E+00
Unt
Das
Gef
LY
MK
Rap
WP
Buf
*
***
**
No EV
With EV

D)


Genomic RNA
Genomic RNA Copies per 500,000 Cells
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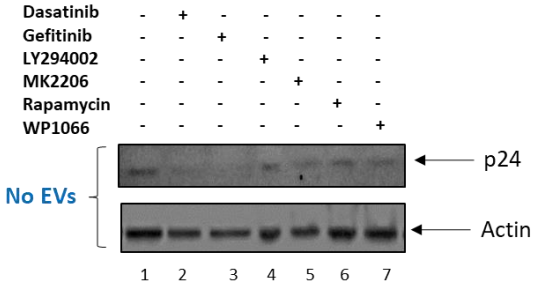
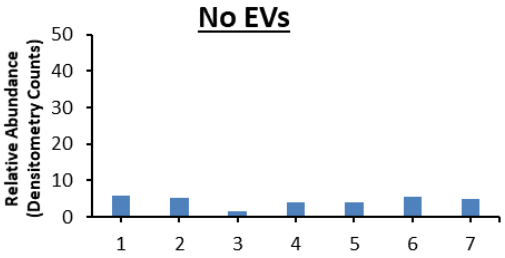
1.60E+06
1.40E+06
1.20E+06
1.00E+06
8.00E+05
6.00E+05
4.00E+05
2.00E+05
0.00E+00
Unt
Das
Gef
LY
MK
Rap
WP
Buf
***
**
***
No EV
With EV

E)


Dasatinib - + - - - - -
Gefitinib - - + - - - -
LY294002 - - - + - - -
MK2206 - - - - + - -
Rapamycin - - - - - + -
WP1066 - - - - - - +
No EVs
p24
Actin
1 2 3 4 5 6 7


No EVs
Relative Abundance (Densitometry Counts)
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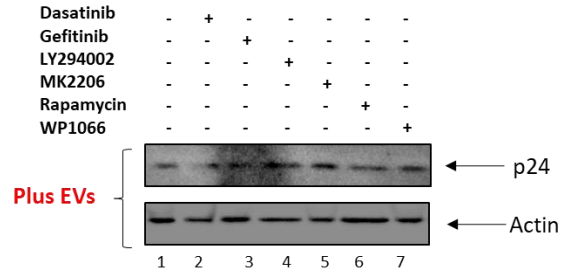
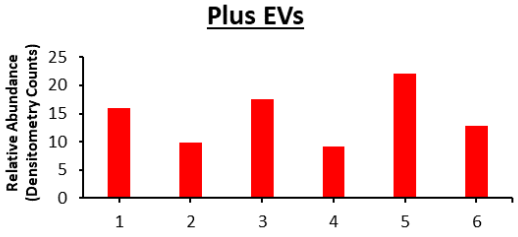
40
30
20
10
0
1 2 3 4 5 6 7

F)


Dasatinib - + - - - - -
Gefitinib - - + - - - -
LY294002 - - - + - - -
MK2206 - - - - + - -
Rapamycin - - - - - + -
WP1066 - - - - - - +
Plus EVs
p24
Actin
1 2 3 4 5 6 7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Relative Abundance (Densitometry Counts)
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15
10
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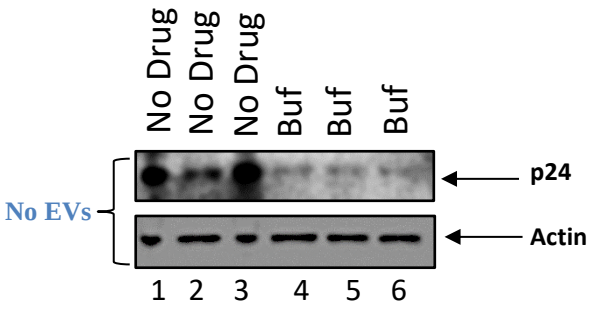
0
1 2 3 4 5 6

G)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No Drug
Buf
Buf
Buf
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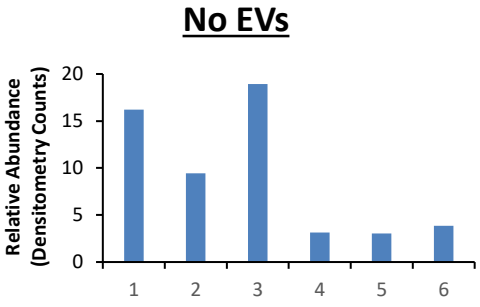
Actin
1 2 3 4 5 6


No EVs
Relative Abundance (Densitometry Counts)
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15
10
5
0
1 2 3 4 5 6

H)

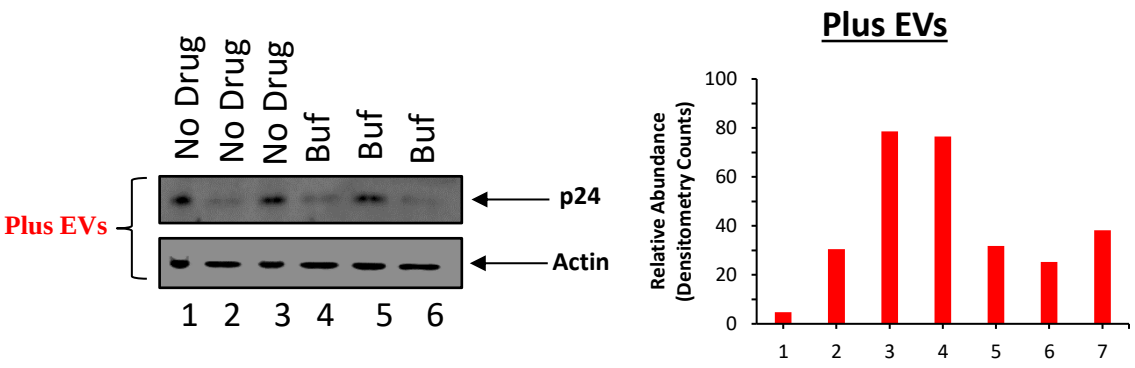


Figure 4: EVs containing c-Src rescue HIV-1 levels in inhibitor-treated cells. U1 cells were treated with each of the various kinase inhibitors and allowed to incubate for 48 hours. A second treatment with inhibitors was performed 2 hours prior to the addiction of CEM EVs. After a 24-hour incubation period, another CEM EV treatment was performed. Cell pellets were prepped for RNA isolation and subjected to RT-qPCR for **A)** HIV-1 TAR RNA levels. **B)** genomic RNA levels. C) Cell supernatants were Western blotted for HIV-1 Gag p24 levels in the absence of EVs (**C, D**) and with treatment of EVs (**E, F**). *Error bars, S.D.*

EV-associated c-Src activates HIV-1 in infected T-cells

Next, we asked whether EV-associated c-Src, rather than intracellular c-Src, activates HIV-1. The rationale for this experiment was that intracellular c-Src, which is present in all cell types (44), could be responsible for the activation of latent HIV-1 and possibly overriding the effects of EV-associated c-Src. To test whether latent HIV-1 activation was due to EV associated c-Src (and not intracellular c-Src), we serum-starved ACH2 (infected T-cells) cells to force them into transcriptional silence. We then knocked out intracellular c-Src using siRNA against 4 different sequences within the c-Src gene. CEM EVs (containing c-Src) were then added to ACH2 cells, which were allowed to incubate for 24 hours. Cells were then harvested and Western blotted for HIV-1 Pr55, p24, and actin. We observed increased levels of Pr55 in EV-treated ACH2 cells

compared to untreated cells (***Fig. 5A)***. Pr55 levels were dependent on EV concentration, highlighting the possible correlation between Pr55 expression and the amount of EV-associated c-Src. Results in ***Fig. 5B*** revealed that siRNA knockdown of intracellular c-Src resulted in minimal levels of p24 in infected cells. However, upon addition of uninfected EVs to infected cells, p24 expression levels dramatically increased. Here, we saw a 4-fold increase in the level of p24 demonstrating the activation of HIV-1 replication. Taken together, these results suggest that the increase in latent HIV-1 activation is due to EV-associated c-Src, and not intracellular c-Src, thereby signifying the importance of c-Src in the EV activation of latent HIV-1.

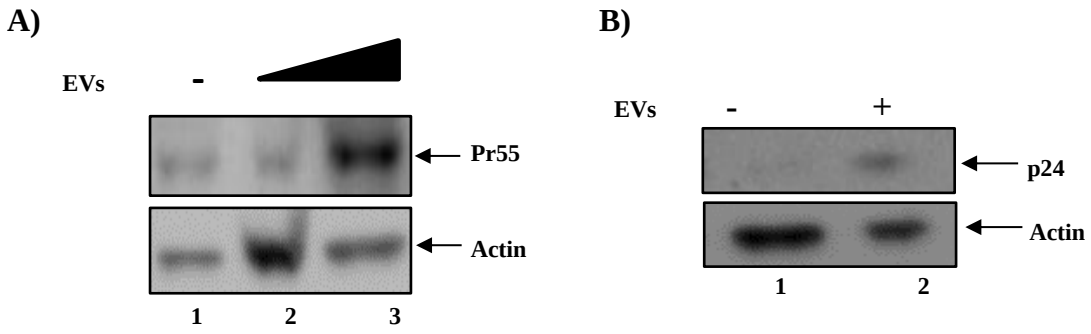


Figure 5: EV-associated c-Src Activates HIV-1 in infected T cells. ACH2 (infected T cells) cells were forced into transcription silence by serum starvation. This was followed by intracellular c-Src knockout in ACH2 cells by transfecting cells using siRNA against c-Src. CEM EVs (containing c-Src) were added to ACH2 cells and allowed to incubate for 72 hours at 37^{o}C. Samples were then analyzed by western blot for **A)** Pr55 and **B)** Gag p24, and Actin. Pr55 levels from untreated infected T-cells was compared to that of infected T-cells treated with a titration of CEM EVs (0, 10^9, and 50^9 EVs respectively).

Effects of Dasatinib and Bufalin on HIV-1 infected PBMCs treated with cART

In order to determine the veracity of our signal pathway, the effects of EV-associated c-Src on the activation of latent HIV-1was investigated in primary cells. Here,

PBMCs from 3 independent donors were activated with PHA/IL-2 and cultured. PHA/IL-2 treatments were performed every other day for a week. Prior to EV isolation, cell supernatants were incubated overnight at 4^{o}C with ExoMAX, a polyethylene glycol-based precipitation reagent, to increase EV yields. PBMCs were then infected with HIV-1 89.6 dual tropic strain (MOI 10). On Day 11, PBMCs were treated with cART and IL-7 every other day for a week to promote latency. This was followed by treatment with dasatinib (c-Src inhibitor) and bufalin (which was selected because it had the most dramatic decrease of HIV-1 genomic RNA in the cell lines – ***Fig. 4D***), at 5 µM and 25 nM respectively. After a 2-hour incubation period, the EVs isolated from each PBMC were added back to their corresponding PBMC. The next day, cART/IL-7 were added to the PBMCs and allowed to incubate for another 24 hours. PBMCs cell pellets were then assayed for HIV-1 TAR RNA and HIV-1 genomic RNA via RT-qPCR while the cell supernatant was collected and treated with NT86 prior to Western blot for the presence of HIV-1 Gag proteins, Pr55 and p24. Results in ***Fig. 6A*** shows a decrease in the HIV-1 TAR RNA levels of two out of the three PBMC donors in the presence of bufalin and dasatinib. A decrease in the genomic RNA levels was observed in all three donors (***Fig 6B***). These results were consistent with those in ***Fig. 6C*** where the treatment of PBMC supernatants with dasatinib and bufalin resulted in decreased levels of HIV-1 Gag Pr55 compared to the controls. Based on these results, our proposed signal pathway applies to primary cells and not just cancer cell lines suggesting that this mechanism could potentially occur *in vivo*.

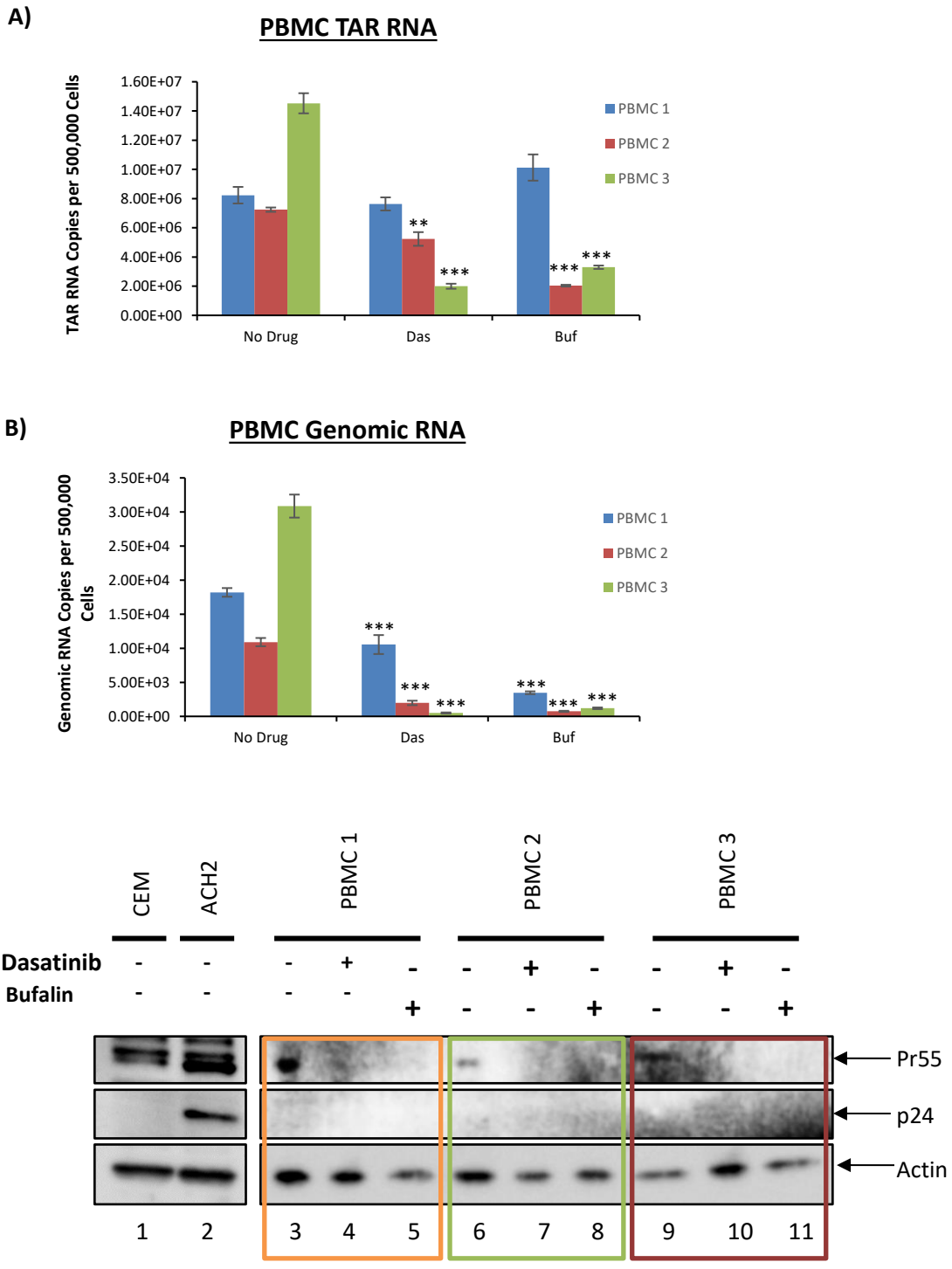


Figure 6: Effects of Dasatinib and Bufalin on HIV-1 infected PBMCs treated with cART. EVs were isolated from PBMCs from 3 donors followed by infection with HIV-1. Uninfected EVs were then added to

their corresponding PBMC at a 1 cell:10,000 EV ratio 24 hours prior to cART and IL7 treatment. PBMCs were then treated with 5nM and 5□M bufalin and dasatinib respectively. Cells were then harvested after 24 hours of incubation at 37oC. Total RNA was isolated and subjected to RT-qPCR from cell pellets. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Number of stars indicate the level of significance of the decrease in TAR RNA levels between no drug treatment and drug treatments.

DISCUSSION

Extracellular vesicles have been shown to be more than cellular waste carriers and are present in virtually all body fluids (35) . They have been found to mediate cell-to-cell communication, immune function, and disease pathogenesis through the transport of materials between cells, eliciting changes in recipient cells (27,35). In previous studies, EVs from uninfected cells were found to activate the transcription of latent HIV-1 in infected cells (25). Upon the addition of uninfected EVs, there was an increase in the levels of HIV-1 Gag p24 as well as an increase in the loading of phosphorylated RNA Pol II (Ser2/Ser5) and Cdk9 onto the HIV-1 promoter (25). This current study supports the above findings and aims to elucidate the mechanism underlying this phenomenon. Our findings indicate that c-Src, a non-receptor tyrosine kinase, initiates a signal cascade involving EGFR/PI3K/AKT/mTOR/STAT3/SRC-1 that culminates in the activation of latent HIV-1.

We found that c-Src is present in EVs derived from multiple cell lines, including T-cells, monocytes, and HeLa cells (***Fig. 1A***). We observed that T-cell EVs (CEM and Jurkat) had the most amount of c-Src present and this c-Src was modified, possibly phosphorylated at Y-416, which would make the c-Src catalytically active and therefore capable of activating downstream proteins (60,61). To confirm this, we Western blotted for the presence of p-c-Src (Y-416) using a specific antibody against c-Src

phosphorylated at Y-416. Results in ***Fig. 1B*** confirmed that EV-associated c-Src was indeed phosphorylated at Y-416, indicating that EV-associated c-Src could be catalytically active and able to activate downstream targets. One such target of c-Src is the receptor tyrosine kinase, EGFR (45). An *in vitro* kinase assay revealed that c-Src phosphorylates EGFR in ACH2 cells (***Fig. 2***). This finding is similar to studies that have established the activation of c-Src to be a prerequisite of the activation of EGFR in initiating a signal cascade (45,64). The ensuing signal cascade stemming from the activation of EGFR has been shown to result in a plethora of downstream pathways, including Ras/MAPK, PLCγ1/PKC, PI3K/AKT/mTOR and STAT pathways (65). Collectively, this data suggests that EV-associated c-Src has the potential to initiate a signal cascade starting with EGFR and bring about functional changes within the cell.

We then investigated downstream proteins of c-Src and EGFR that could possibly be linked to HIV-1 activation. In a preliminary experiment, we observed that treatment of infected U1 cells with dasatinib, an inhibitor of c-Src, led to decreases in AKT-1 and SRC-1 protein levels (data not shown). Therefore, we hypothesized that c-Src could be linked with AKT-1 and SRC-1 via EGFR, the PI3K/AKT/mTOR pathway, and STAT3 (66–70). We then inhibited each protein in our hypothesized signal cascade and examined the levels of HIV-1 RNA and protein in response to the inhibitors using the optimal dosages obtained from experiments in ***Fig. 3***. When inhibitors against each protein were added to infected cells, we saw significant decreased levels of HIV-1 TAR RNA and genomic RNA (***Fig. 4A-4B***). This was also true with Gag p24 levels found outside the cell. Data in ***Fig. 4E*** and ***4H*** demonstrated that inhibiting each protein in our

hypothesized cascade led to decreased amounts of p24 released into the cell supernatant regardless of the inhibitor used. Taken together, this suggests that each of these proteins are involved in activation of latent HIV-1.

We then added EVs from uninfected cells, containing c-Src, to HIV-1-infected latent cells in order to determine whether the EV-associated c-Src could rescue HIV-1 levels in the inhibitor-treated cells. We observed that intracellular HIV-1 TAR and genomic RNA levels were rescued despite the presence of dasatinib, rapamycin, and bufalin (***Fig. 4C-4D***). Corresponding western blots of the cell supernatants showed that uninfected EVs resulted in the increased levels of HIV-Gag p24 despite the inhibitor used (***Fig. 4F*** and ***Fig. 4H***). This data suggests that EV-associated c-Src is able to activate HIV-1 despite the presence of the c-Src inhibitor, dasatinib. Furthermore, the increased HIV-1 RNA and p24 levels observed with uninfected EV addition to latent, infected cells in other inhibitor-treated cells (i.e. MK2206-2 HCl) could mean that there may be other proteins present in EVs that can bypass the effects of some of the inhibitors, implying that there may be other pathways in addition to our proposed pathway that result in the EV activation of latent HIV-1. Collectively, this data implies that EVs are able to override the effects of various kinase inhibitors and activate latent HIV-1.

In this study, we observed increased levels of latent HIV-1 RNAs, and associated increases in Gag p24 levels in the cell supernatant, in cells treated with EVs compared to the untreated control. To confirm this was due to EV-associated c-Src rather than intracellular c-Src, we serum-starved ACH2 cells to force them into transcriptional silence and knocked out intracellular c-Src using siRNA. Upon addition of EVs

containing c-Src to the siRNA-treated cells, we observed increased levels of Pr55 Gag and p24 in EV-treated cells compared to the untreated control (***Fig. 5A, B***). Interestingly, we observed a direct relationship between the concentration of EVs and the levels of Gag proteins. This confirmed our hypothesis that EV-associated c-Src rather than intracellular c-Src was responsible for the observed activation of latent HIV-1.

Importantly, we reproduced our results in human PBMCs from three different donors. EVs were isolated from each PBMC. This was followed by HIV-1 infection, and once latency was established, the PBMCs were treated with dasatinib and bufalin. Uninfected EVs were then added back to their corresponding PBMC. Our data show that upon the addition of uninfected PBMC EVs to the latent HIV-1 infected cells, 3 out of the 3 donors saw a significant decrease in genomic RNA levels while HIV TAR RNA levels decreased in 2 out of the 3 donors (***Fig. 6A-B***). The lack of decrease in TAR RNA levels observed in Donor 1 may be due to high background levels of TAR as EVs saturated the system. This would potentially have allowed for recruitment of RNA Pol II to the HIV-1 promoter, thereby overriding the effects of inhibitors. However, in the case of the decreased levels of HIV-1 genomic RNA in the same donor, dasatinib or bufalin could potentially have inhibited the phosphorylation of RNA Pol II, subsequently preventing it from moving down the HIV-1 promoter (i.e. completing elongation). Western blot analysis of the cell supernatants of the 3 PBMCs showed that Gag Pr55 was expressed only in untreated cells and absent in cells treated with dasatinib and bufalin. Collectively, these data show the importance of c-Src and SRC-1 in the proposed signal cascade as well as the cascade's relevance in normal cells.

Our proposed signal cascade could shed light on the persistence of low levels of viral RNA in certain parts of the body despite cART treatment and how patients undergoing cART could still produce defective and/or mutant viruses (71,72). This is because current cART treatments consist of fusion, integrase, reverse transcriptase, and protease inhibitors but not a transcription inhibitor (21,74). Based on our results, WP1066 (STAT3 inhibitor) could be a viable candidate for further investigations as a supplement to cART. This is because our results showed that of all the inhibitors tested, WP1066 had selective killing of both infected T-cells and monocytes at 5 μM (***Sup. Fig. 1***). Also, of note is that dasatinib had selective killing of HIV-1 infected T-cells at 1 μM but not selective killing of infected monocytes. This is very important as T-cells and monocytes are latent reservoirs of HIV-1 (2).

Based on our collective results, we have proposed a model of the specific mechanism behind the EV activation of latent HIV-1 **(*Fig. 7*)**. We hypothesize that uninfected EVs from healthy cells are taken up by HIV-1 infected cells, leading to the phosphorylation and activation of EGFR by EV-associated c-Src. EGFR then activates PI3K, which in turn activates AKT-1. Following activation, AKT-1 phosphorylates mTOR resulting in the phosphorylation and activation of the transcription inducer, STAT3. STAT3 then recruits the cofactor SRC-1, which translocates to the nucleus and promotes HIV-1 transcription. Inhibition of the aforementioned proteins led to decreased levels of HIV-1 RNA inside the cell as well as decreased HIV-1 Gag p24 in the cell supernatant, demonstrating the importance of these proteins in HIV-1 activation. This leads us to conclude that EV-associated c-Src is indeed able to initiate our proposed

signal cascade. Nonetheless, there could be alternate pathways for EV activation of latent HIV-1 as evidenced by the activation of HIV-1 in the presence of EVs despite the inhibition of downstream proteins in the cascade. Lastly, WP1066 (STAT3 inhibitor) could be a potential candidate to supplement antiretroviral therapy, especially since the current cART regimen lacks a transcription inhibitor. Future studies should be conducted to investigate possible alternate pathways for EV activation of HIV-1 as well as the viability of WP1066 as a cART supplement *in vivo*.

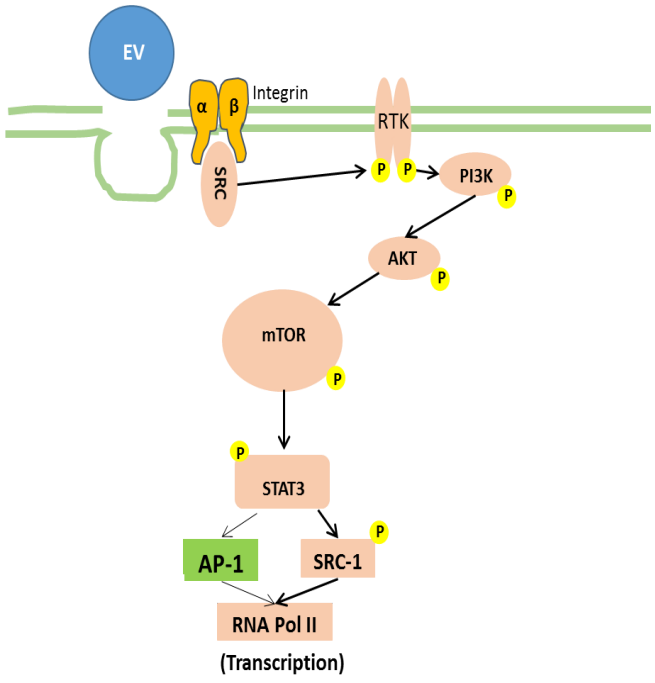
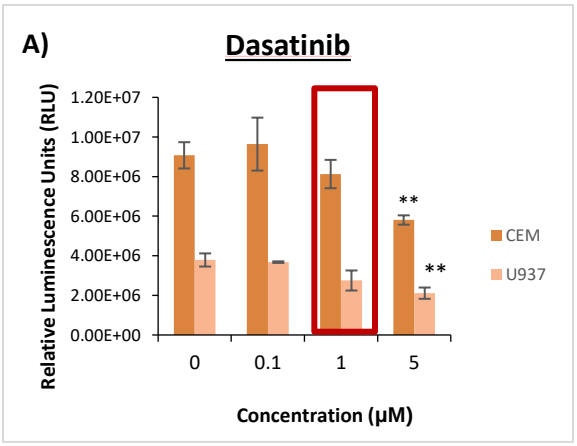
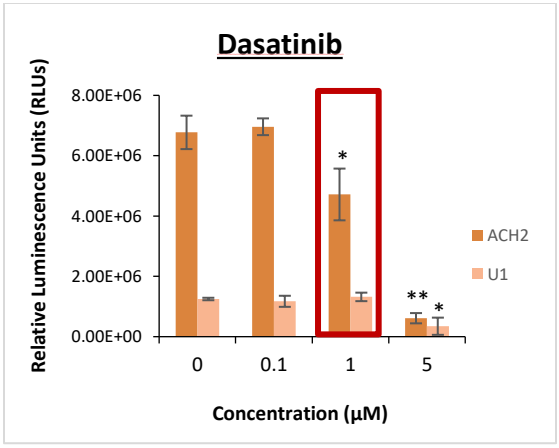
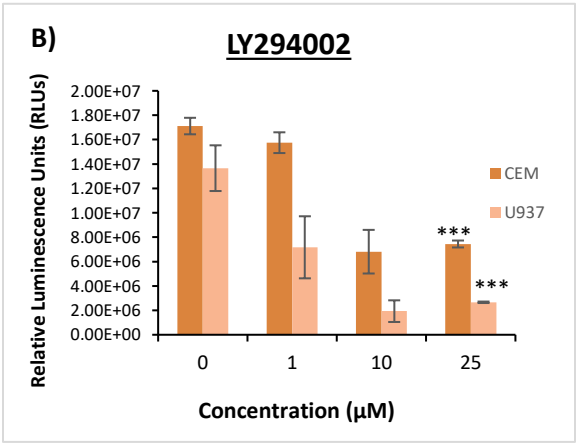


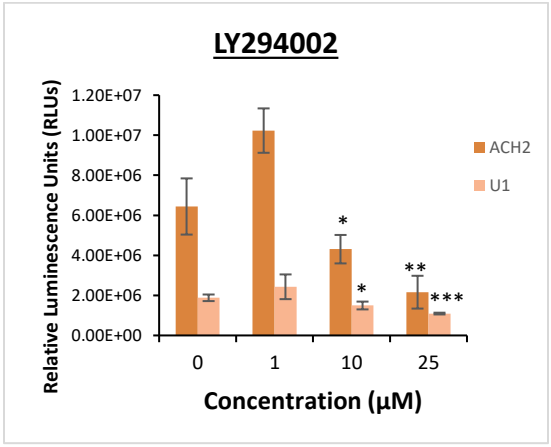
Figure 7: Proposed model for the signaling cascade of EV activation of latent HIV-1. Uninfected EVs from healthy cells are taken up by HIV-1 infected cells, leading to the phosphorylation and activation of RTK by c-Src. RTK then activates PI3K, which in turn activates AKT-1. Following activation, AKT-1 phosphorylates mTOR resulting in the phosphorylation and activation of the transcription inducer, STAT3. STAT3 then recruits the cofactor SRC-1 before translocating to the nucleus and promoting HIV-1 transcription.

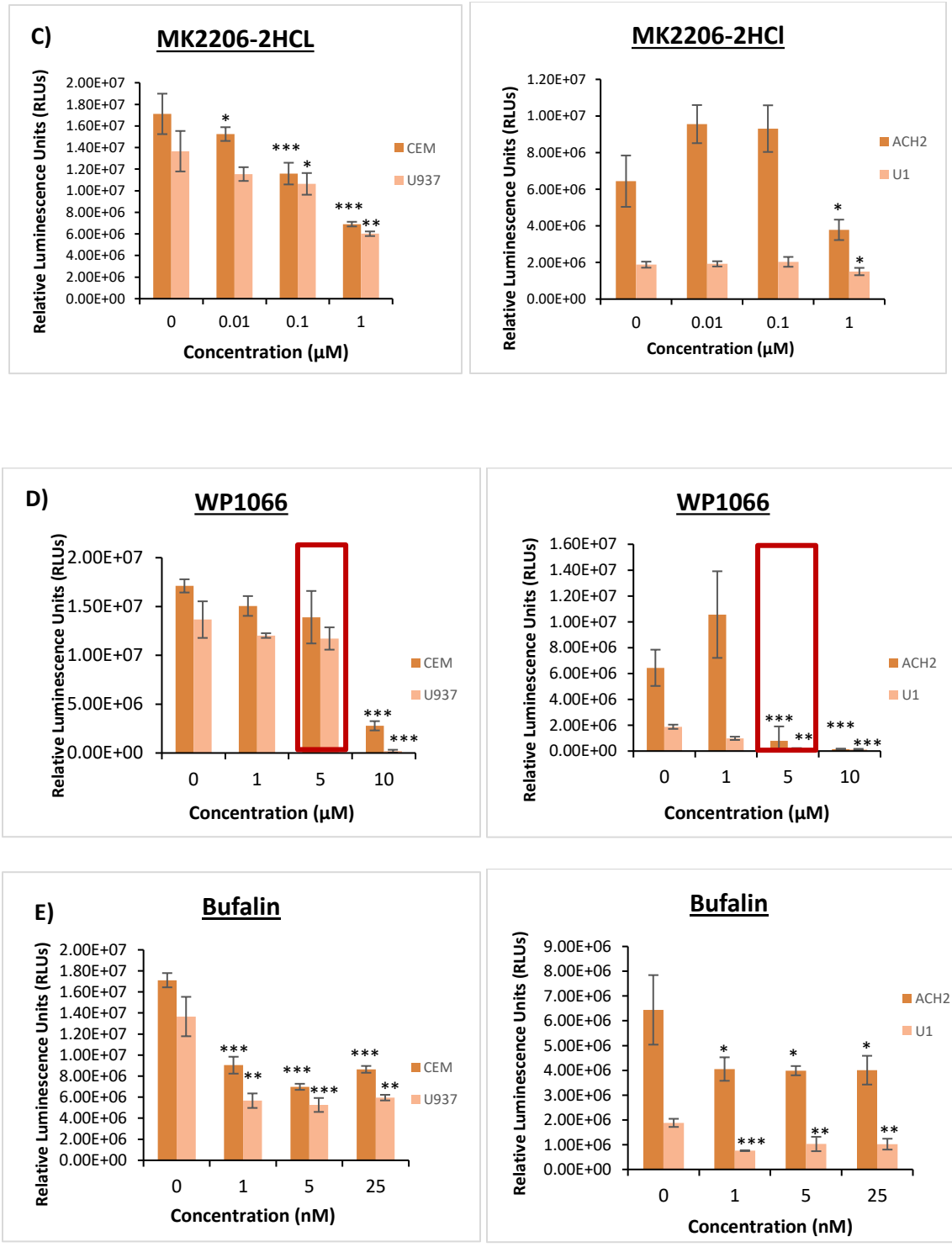
SUPPLEMENTAL FIGURES











Sup. Figure 1: Determining Potential cART Supplements. $5X10^4$ cells were plated with different concentrations of kinase inhibitors and allowed to incubate for 48 hours prior to a Cell-Titer Glo assay.

ACH2 and U1 cells were treated with **A)** Dasatinib (c-Src inhibitor) at 0, 0.1, 1, or 5 μM. **B)** LY294002 (PI3K inhibitor) at 0, 1, 10, or 25 μM. **C)** MK2206-2 HCl (AKT-1 inhibitor) at 0, 0.01, 0.1, or 1 μM. **D)** WP1066 (STAT-3 inhibitor) at 0, 1, 5, or 10 μM. **E)** Bufalin (SRC inhibitor) 0, 1, 5, or 25 nM.

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