THE EFFECT OF A SPECIFIC HEAT SHOCK PROTEIN 90 INHIBITOR ON HIV-1 INFECTION

by

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A Dissertation
Submitted to the
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Doctor of Philosophy
Biosciences

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Fall Semester 2020
George Mason University
Fairfax, VA

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DEDICATION

This is dedicated to my caring and endlessly supportive family and friends, all of whom have continued to encourage me no matter how far down I fell or how slowly I moved forward. My husband Tony, my daughter Amanda and my mother Janet Learmonth have been my biggest positive influences. My mother has frequently been my partner in a lifetime's worth of adventures and has been the best grandmother to Amanda that I could ever ask for. Tony has made me aspire to persist through tasks as he does with the enthusiasm to get to the other side. He has helped to make me a better Army Officer, partner, mentor and parent. Amanda made me a mother 6 weeks before I began this educational odyssey and is the person I hope to inspire the most for the rest of my life. Her smiles, laughter and love have kept me going when all else failed and she is my inspiration, always. To each and every friend and family member who offered words of encouragement, you made a difference.

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I would like to acknowledge and thank Dr. Yuntao Wu for not giving up on me even when I had. He has shown me, and also made me figure out myself, how to think like a scientist. Dr. Jia Guo was endlessly helpful in the lab as well as keeping me motivated. All members over the years of the Wu lab who answered questions or collected a sample for me so I didn't have to make the 2 hour round-trip drive for 15 minutes of work.

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LIST OF ABBREVIATIONS

Human Immunodeficiency Virus ..... HIV
Acquired Immune Deficiency Syndrome ..... AIDS
Highly Active Anti-Retroviral Therapy ..... HAART
Rev Response Element ..... RRE
Inducible T-cell Kinase ..... ITK
Breakpoint Cluster Region ..... BCR
Chronic Myeloid Leukemia ..... CML
Heat Shock Protein ..... HSP
Fetal Bovine Serum ..... FBS
Fluorescein Amidite ..... FAM
Peripheral Blood Mononuclear Cell ..... PBMC
Time of Addition ..... ToA
Fluorescence Activated Cell Sorting ..... FACS
Nucleotide Binding Domain ..... NBD

ABSTRACT

THE EFFECT OF A SPECIFIC HEAT SHOCK PROTEIN 90 INHIBITOR ON HIV-1 INFECTION

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George Mason University, 2020

Dissertation Director: Dr. Yuntao Wu

This thesis describes the effort to characterize several intracellular signaling and transport molecules during HIV-1 infection via chemical inhibition. Many pharmaceutical molecules developed for one purpose find a second or third life in the treatment of another medical condition. The cellular changes resulting from cancer in some cases resemble changes in signaling which occurs during HIV-1 infection. During the course of this research, several pathways of signaling were variously knocked down, or otherwise inhibited using multiple methods to determine if one or more already-developed chemical compounds could be useful in the treatment or study of HIV-1 disease. During the course of this research and writing, the author conducted numerous literature searches and laboratory investigations to develop the evidence necessary to demonstrate one cell-signaling pathway that is vital to viral infection. The inhibition of the heat shock protein

90 protein leads to the conclusion that tanespimycin impacts viral infection in the described models.

CHAPTER ONE: BACKGROUND

The retrovirus Human Immunodeficiency Virus (HIV) has been responsible for approximately 35 million human fatalities and approximately 70 million total infections since being identified in the mid-1980s as the causative agent of Acquired Immune Deficiency Syndrome (AIDS)(source: WHO Global Health Observatory data, 2016). Even several decades post-discovery there are still approximately 2 million new infections per year, worldwide, and nearly 1 million per year who succumb to the disease. In 1996 the estimated annual cost of treating those with HIV disease was estimated to be between $6.7 and $7.8 billion (Hellinger, 2000). Since then costs have only increased along with the effectiveness of anti-retroviral treatment regimens. A recently infected patient can expect to spend between $326,000 and $435,000 on life-saving HIV treatment over the course of their now near-normal lifespan (Schackman, 2015). Despite years of effort and millions of research dollars invested, the ultimate goal of a successful preventive vaccine remains stubbornly out of reach. As such, novel treatments are also being pursued to add to the arsenal of anti-retroviral medications. Some of these approaches involve re-purposing existing compounds that have the potential to inhibit either the virus itself or the host-response processes that contribute to its pathology.

Like all viruses, HIV must hijack the host cell's replication machinery and other cellular pathways to reproduce and propagate a productive infection cycle. HIV attacks

the immune system's CD4 T cells and has been shown to manipulate key cellular signaling pathways that have been instrumental to its success in establishing productive or latent infections in the human host cell.

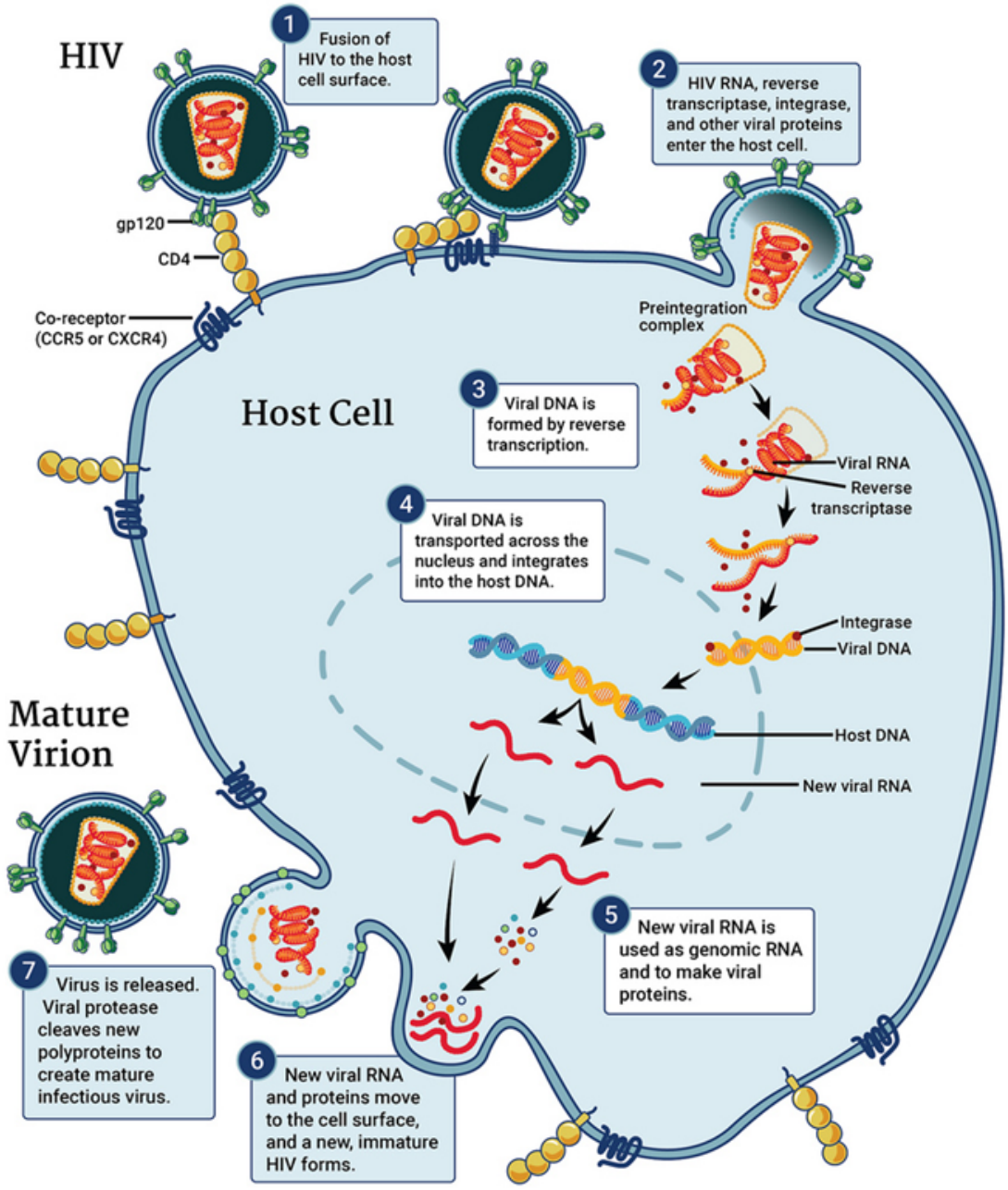


Figure 1 – HIV-1 Infection Cycle:

HIV-1 fuses with the T-cell and the viral capsid containing genomic material and specialized proteins enters the cell. The outer part of the capsid disintegrates and reverse transcriptase

transcribes viral RNA into DNA. Viral DNA is then transported across the nucleus and inserted into host DNA with the aid of viral integrase. Next, host machinery transcribes HIV DNA into HIV RNA, which can either be used as new viral genomic material or become a template for making new HIV proteins. Inactive RNA and protein is releases from the cell surface, and activated by proteases (NIAID, 2020).

Viral Taxonomy

The International Committee on Taxonomy of Viruses (ICTV) classifies HIV 1 and HIV 2 as distinct species within the genus lentivirus (Triangle figure) (Viruses, 2020). Lentiviruses are RNA viruses which contain the transcriptase enzyme necessary to convert RNA into DNA before becoming integrated into the host genome. Lentiviruses are also able to infect non-dividing cells, which allows them to latently infect cells while remaining dormant for years. All lentiviruses have the structural and regulatory genes gag, pol, tat, rev, vif, vpu, vpr and nef. Interestingly, while function is conserved across species, the genes are vastly different (Clements JE, 1996). HIV1/HIV2 share only 35-55% amino acid sequence identity in the key proteins gag, pol, and nef (Motomura K, 2008). Other lentivirus include Visna/maedi virus (VMV) in sheep, the caprine arthritis-encephalitis virus (CAEV) in sheep, the equine infectious anemia virus (EIAV) in horses, the feline immunodeficiency virus (FIV) in cats, and several simian immunodeficiency viruses (SIV) in primates (Durand S, .2011).

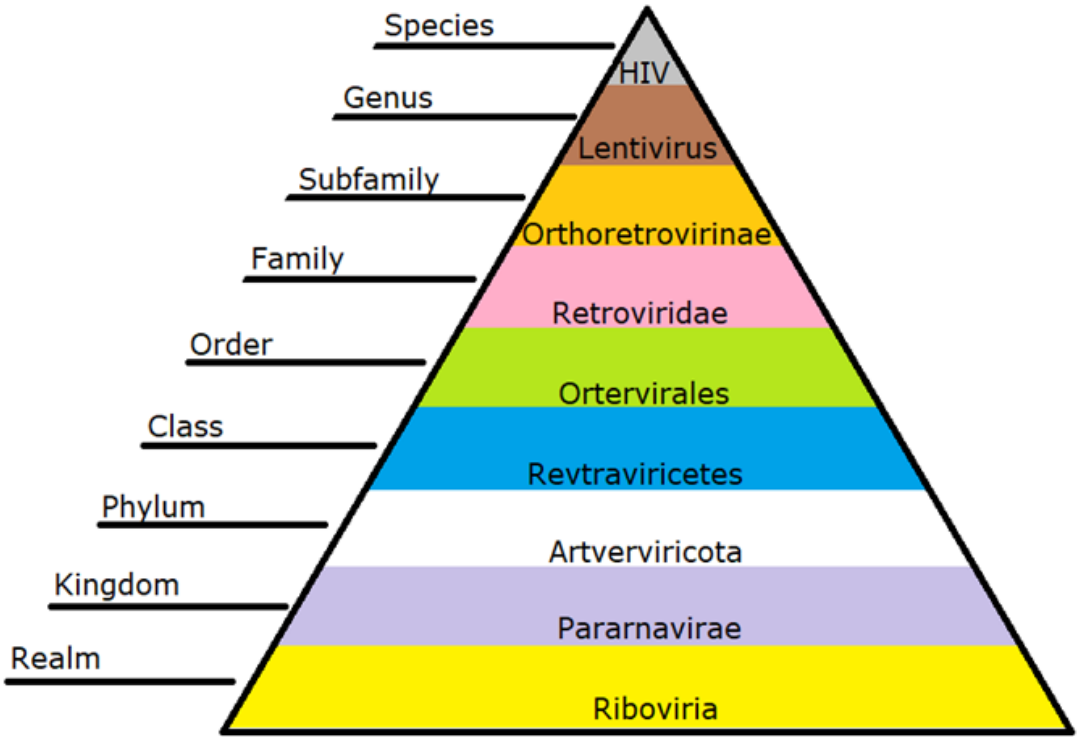


Figure 2 – Taxonomy Triangle

HIV-1 is in a genus of called lentiviruses. Lentiviruses have the unique ability to infect non-dividing cells. Other lentiviruses include several simian immunodeficiency viruses in primates.

Quite a bit of scientific research has been focused on determining the origins of HIV and how it came to be a human pathogen. As a result of exhaustive investigation, it's widely accepted that the original reservoir for HIV-1 is the chimpanzee, while the reservoir for HIV-2 is the sooty mangabey (Sharp PM, 2010). The current belief is that both HIV-1 and HIV-2 share a common viral ancestor, simian immunodeficiency virus (SIV) (Wertheim, 2009). Wertheim et al, used molecular clock dating to establish a common SIV ancestor in sooty mangabeys (1809) and chimpanzees (1492). The

relatively recent introduction of SIV into their population means that they did not co-evolve over thousands or millions of years, which would have explained SIVs relatively low virulence in these primates. HIV on the other hand has killed over 30 million humans. Recently, it has been shown that the difference in pathogenesis is due not to the virus itself, but instead species-specific host factors (Joas, 2018).

P. t. troglodytes (Central chimpanzee) and P. t. schweinfurthii (Eastern chimpanzee) are two subspecies of chimpanzees that harbored a common SIV virus (Sharp PM, 2010) . Once SIV established itself in these chimpanzee, opportunistic encounters with the Central chimpanzee has allowed SIV to infect humans several times (figure pic of monkeys), with each separate cross presents an opportunity to introduce HIV variation into the human population (Sharp PM, 2010). Figure 3 illustrates out how different HIV groups could emerge from a common Central chimpanzee ancestor. HIV1 has 4 main 4 groups; (M, N, O, P) (Tebit, 2016). Group M is the most common HIV variant in humans, accounting for almost 98% of all cases (Tebit, 2016). Group M is distributed worldwide, while Groups N, O, and P are generally found in close proximity to Cameroon (Sharp PM, 2010).

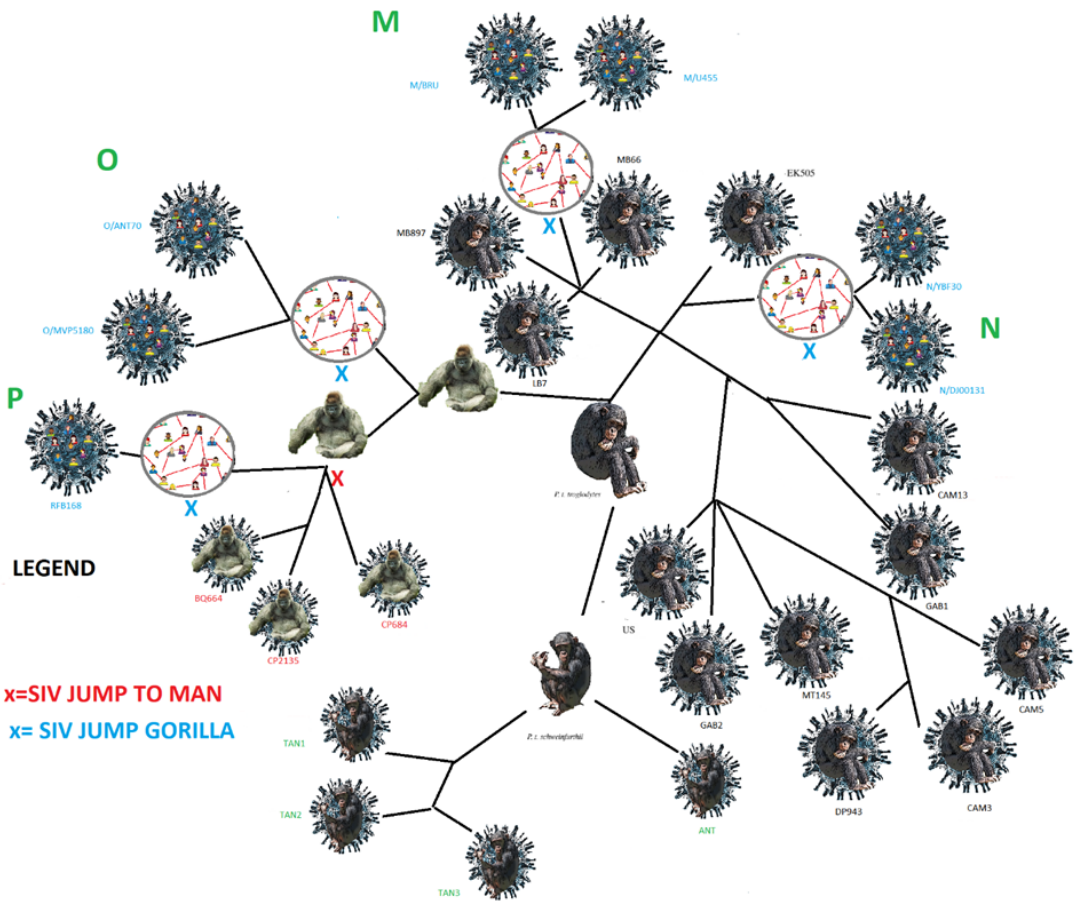


Figure 3 – Origin of HIV-1:

P. t. troglodytes and P. t. schweinfurthii and shared a common SIV infection. Chance encounters with humans and/or gorillas led to cross species infection, giving rise to the 4 HIV1 groups. The terminal branches represent 2 of the many subgroups of each Group. This figure is adapted from Sharp et al (Sharp PM, 2010).

Viral Structure

A single HIV virion ranges in size from 110 nm to146 nm depending on where in the lifecycle the size is measured (Gentile M, 1994). It is spherical in shape and contains distinct cell surface spikes made up of the protein gp41 imbedded in a lipidic membrane and capped with the protein gp120 (see Fig X – HIV virion). The viral matrix is located just inside the lipid membrane and is made up of repeating units of p17 protein. Beyond the matrix layer sits the capsid or viral core, which is made up of repeating units of p24 protein. Within the capsid cavity lies a cluster of several vital proteins: p7, p9, integrase, and reverse transcriptase, as well as the virus genetic material. The HIV genome consists of 2 + sense, single stranded RNA (ssRNA) molecules which encode 9 genes; gag, pol, env, tat, rev, nef, vif, vpr, and vpu (Committee, 2016).

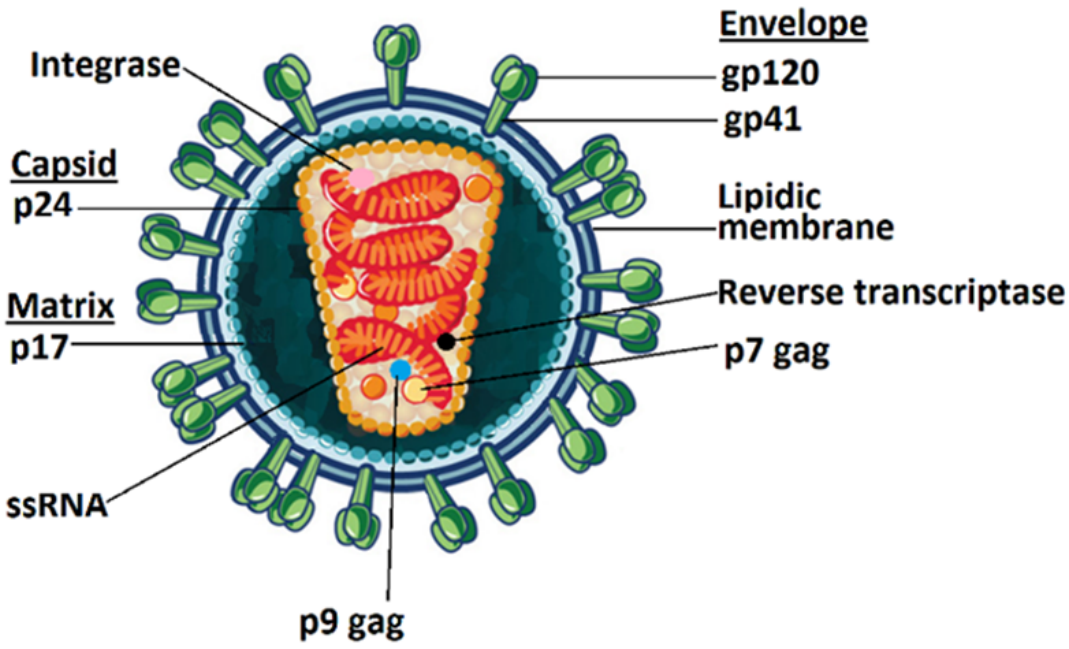


Figure 4 – HIV Virion:

Shows the structural components of the virus after assembly, release and maturation have completed, includes the location of enzymes needed for successful infection and structure of the viral core.

These genes code for proteins that can be divided into 4 groups based on general lifecycle functions; the Gag and Env structural proteins, the Pol enzymes, the gene regulatory proteins, and the accessory proteins. The Gag and Env structural proteins are MA (matrix), CA (capsid), NC (nucleocapsid), p6, SU (surface), and TM (transmembrane). The Pol enzymes are PR (protease), RT (reverse transcriptase), and IN (integrase). The gene regulatory proteins are Tat and Rev. The accessory proteins Nef, Vif, Vpr, and Vpu (Young, 1998).

The final structural proteins are initially translated as one of several polyprotein precursors; Gag, Pol, or Env gp160 (Arrildt KT, 2012). Gag, also known as p55 Group antigen protein, is a single myristoylated precursor which recruits the other essential components and drives HIV virion assembly. After translation, it is subsequently enzymatically cleaved into Matrix (MA, p17), Capsid (CA, p24), Nucleocapsid (NC, p7) and p6 proteins (Table X, figure X (Gottlinger, 2001; Balasubramaniam M, 2011). MA, CA, and NC are found in this order in all lentivirus genomes, and while p6 is not common to all lentiviruses, it is found in all primate lentiviruses. (Li G, 2016). The env gene codes the 160 kD glycoprotein Env, which is also known as gp160. After translation, gp160 is enzymatically cleaved into gp120 and gp41. Although cleaved, Pg120 and gp41 remain non-covalently linked and form one subunit of a trimeric spike on the surface of the HIV virion (Arrildt KT, 2012).

One in 20 Gag gene transcripts is accompanied by a frameshift mutation whereby translation of the Gag polyprotein continues thru Pol to form a single conjoined Gag-Pol polyprotein ($Pr160^{gag\text{-}pol}$) (Jacks T, 1988; Figueiredo A, 2006). The resulting 20:1 ratio of Gag to Gag-Pol ensures that a proper ratio of proteins is produced in order to assemble functional virions (Park J, 1991). $Pr160^{gag\text{-}pol}$ contains the gag-encoded proteins mentioned above as well as the pol-encoded enzymes protease (PR, p10), reverse transcriptase (RT, p66/p51), and integrase (IN, p31).

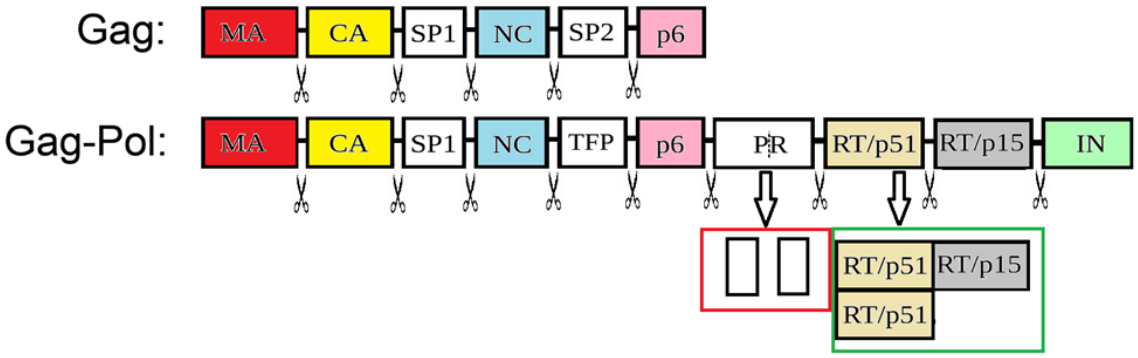


Figure 5 – Gag & Gag-Pol splicing:

Adapted from a model developed by Könnyű shows sites (scissors) where proteases cleave the Gag and Gag-Pol polyproteins, releasing active viral proteins. Reverse transcriptase is activated when one molecule of p51 forms a heterodimer with p66, which is an uncleaved p51 and p15 (green box). Protease activity is self-modulated by PR which inactivates itself by self-cleavage (red box). (Könnyű, 2013)

Table 1 - HIV Genes, proteins, and function:

The table shows the gene, its product and the results of splicing events in assembly of the functional virion as well as the function of each

HIV Genes, Proteins, and Function				
Group	**Gene**	**Primary protein product**	**Modified proteins**	**Function**
structural	gag	Gag polyprotein	Matrix, MA, p17	Assembles into trimers to form a coat on the inner surface of the viral membrane. Role in infection: important in viral budding from the surface of infected cells.
	gag	Gag polyprotein	Capsid, CA	Assembles stable hexamers which form a cone-shaped coat around the viral RNA. Role in infectioin: protect/deliver viral RNA into the cell during infection.
	gag	Gag polyprotein	Nucleocapsid, NC	Forms a stable and protective complex with the viral RNA.
	env	Pol polyprotein	gp120, SU and gp41, TM	gp120 and gp41: HIV surface receptors that bind to CD4+ cells. Antigenicity is masked by abundant carbohydrates.
	gag	Gag polyprotein	p6	Involved in the incorporation of Vpr into new viruses.
Pol enzymes	pol	Pol polyprotein	Protease	HIV protease cleaves long polyproteins into the proper functional pieces. This protein is susceptible to protease inhibitors.
	pol	Pol polyprotein	Reverse Transcriptase, RT	Builds a DNA copy of the viral RNA genome in order to assemble new viruses. RT is a common target for anti-HIV drugs.
	pol	Pol polyprotein	Integrase	Integrates DNA copy of the viral genome into the infected cellular genome. Another treatment strategy is the prevention of integration.
gene regulatory proteins	tat	tat	Tat	(trans-activator of transcription) protein binds to a hairpin in the viral RNA and greatly enhances the amount of protein that is made.
	rev	rev	Rev	Binds to a hairpin in the viral RNA and regulates the splicing and transport of viral RNA.
Accessory proteins	vif	vif	Vif (viral infectivity factor)	Targets human cell's defense proteins, which forces the cell to destroy it.
	vpu	vpu	Vpu (viral protein u)	Weakens the interaction of the new envelope proteins with cell receptors.It also forms an ion channel in the viral membrane
	vpr	vpr	Vpr (viral protein r)	Guides the viral genome into the nucleus following infection.
	nef	nef	Nef (negative regulatory factor)	Induces infected host cells to stop making proteins required for cellular defense as part of the progression of HIV infection to Acquired Immune Deficiency Syndrome (AIDS).

Matrix protein p17 is a 17 kD protein which has critical roles in most stages of the HIV lifecycle. P17 also binds to preactivated human T cells, which causes the release of proinflammatory cytokines, cell proliferation, and viral replication (Fiorentini S, 2006). CA is the protein that makes up the HIV viral capsid core. As previously mentioned, viral proteases cleave multiple copies of Gag into the proteins MA, CA, NC and p17. Approximately 1500 CA monomers that have an 80 amino acid C terminal domain

(CTD) and a 150 amino acid N terminal domain (NTD). These monomers self-assemble into hexamers and pentamers to form the HIV capsid core. When the capsid core is completed, the NTD faces outward and the CTD faces inward, which stabilizes the capsid core (Campbell EM, 2015).

The HIV-1 nucleocapsid protein (NC) is derived from cleaving part of the 55KDa Gag precursor protein ($Pr55^{gag}$) (Thomas JA, 2008). NC is a nucleic acid chaperone which facilitates the formation of stable nucleic acid structures that are instrumental in viral replication and reverse transcription fidelity (Levin JG, 2010). NC ma also play a role in integrating viral DNA into the host chromosome (Carteau S, 1999).

The Pol enzymes are PR (protease), RT (reverse transcriptase), and IN (integrase). Protease (PR) is an enzyme that cleaves the HIV polyproteins Gag and Gag-Pol into individual proteins (Soares, 2016). This is an essential step for HIV maturation and infectivity (N E Kohl, 1988). The class of HIV medications called protease inhibitors takes advantage of this critical function of PR to prevent virus maturation and spread to uninfected cells (Emamzadeh-Fard, 2013). RT (Hu WS, 2012).

RT is a heterodimer which contains a 560 amino acid subunit called p66 and a truncated p66 subunit called p51, which contains only the first 440 amino acids of p66 (Lightfoote MM, 1986). p66 contains a highly conserved polymerase domain that has been described as looking like a right hand, and an RNase H domain (Esposito, 2012). p66 facilitates the enzymatic activity of RT, which includes RNA- and DNA-dependent DNA synthesis and RNase H activity (Liu, 2008). p51 is similar but the absent amino

acids cause it to fold differently and lose enzymatic activity. Its role is that of an anchor to ensure proper p66 folding (Kohlstaedt LA, 1992).

IN is an essential viral protein that is responsible for integrating newly formed viral double-stranded DNA into the host cell. IN achieves viral DNA insertion in 3 general steps; first, it catalyzes the removal of two nucleotides from each 3' end of the viral DNA as part of the pre-integration complex (PIC). Then IN binds the ends of the viral DNA, forming a stable synaptic complex (SSC) in order to enter the nucleus. Once inside the host nucleus, the 3' end of the viral insert is joined at the host insertion site. The 5' end is then processed by cellular nucleases and ligases (Craigie, 2012; Engelman A, 2013).

The gene regulatory proteins are Tat and Rev. Trans-activator of transcription (Tat) is an amino acid encoded by the tat gene. Tat consists of 86–101 amino acids depending on the subtype (Jiang Y, 2018). During HIV replication, Tat serves as a trans-activator that enhances the efficiency of viral transcription (Frankel AD, 1998). Tat functions in transcription promotion and membrane transduction (Jiang Y, 2018). Rev is an approximately 13 kD accessory/regulatory protein which combines with other Rev proteins to attach to the 350 nucleotide Rev Response Element (RRE) in the nucleus and facilitate export of mRNAs (Rausch JW, 2015) . Rev is essential for viral replication and the Rev-RRE complex, a possible target for future therapeutic efforts (Pond SJ, 2009).

The viral accessory proteins Nef, Vif, Vpr, and Vpu (Young, 1998). Nef (Negative factor) is an accessory protein that downregulates CD4 and other molecules from the surface of infected cells (Sugden, 2016). Vif (Virion infectivity factor) is

essential for infection in primary cells (Baraz, 2004). It induces the cellular ubiquitin ligase complex in order to counteract the host's antiviral defenses, including APOBEC3 enzymes and PP2A phosphatase regulators. Vpr (Viral protein r) is packaged with the virion core along with IN and bridges viral DNA complexes in a way that favors nuclear import (Lyonnais, 2012). Vpr (Viral protein r) enters the cell and shuttles between cytoplasm and the nucleus to help facilitate viral replication and promote early T cell activation (Sugden, 2016). Vpu (viral protein u) along with HIV-1 Nef alter the composition of the infected cell's plasma membrane surface in such a way that contributes to evasion of the host immune response and an increase in viral fitness - (Sugden, 2016)

Biology of Infection and HIV Transmission

HIV-1 cannot be contracted by casual contact. It requires sexual contact across a mucosal surface, a needle stick/blood splash to a compromised surface, or transmission from mother to child (Shaw GM, 2012). The risk of sexual transmission is zero among two non-infected individuals. The estimated risk of transmission from penile-vaginal sex is 4 per 10,000 exposures, and rises to 138 per 10,000 for penile-anal intercourse (Patel P, 2014). These numbers vary depending on the present viral load, and if the infected partner is on antiviral therapy (Albert J, 2014). The risk of penile-oral transmission is low, and is considered minimal if the infected party is on antiviral therapy. Risk of transmission can be further reduced if the uninfected partner has been prescribed pre-exposure prophylaxis (PrEP) (CDC, 2020). Regardless of the method of entering the

body, HIV-1 Env will bind to the T-cell CD4 receptor once encountered, causing a conformational change in ENV, resulting in binding of one of the host cell's co-receptors. This process leads to the fusion of the viral and host cell membranes, initiating the infection (Wilen CB, 2012).

Vulnerable Cells: As the name suggests HIV targets cells that comprise the human immune system. B cells, granulocytes, Langerhans cells, and even dendritic cells are all susceptible to HIV infection (Woodham AW, 2016). The 2 main types of T lymphocytes (T-Cells) are CD4 and CD8 cells, which are characterized by the antigen specific pathogen-recognizing receptors displayed on their surface. CD4 cells (also known as T helper cells) can be further divided into Th1 and Th2. Th1 are instrumental for killing intracellular parasites and its pro-inflammatory cytokines are, in effect, counterbalanced by Th2 cells, which generally produce more anti-inflammatory cytokines. The balance must be maintained for a normal, properly functioning immune response (Berger, 2000).

Binding and entry: Manipulation of cell signaling by HIV-1 begins upon cell surface receptor binding, which triggers a signaling cascade to eliminate one of the cell's first defenses against entry: cortical actin. After binding to the T cell specific CD4 surface receptor, one of two available co-receptors is engaged, either CCR5 or CXCR4 (Huang, 2007). Which co-receptor a virus preferentially uses is known as its tropism. In general most early-circulating virus tends to be CCR5-tropic, however, much research has shown populations of dual-tropic and demonstrated the phenomenon of tropism switching as the circulating virus of a patient transitions from R5- to X4-preferring

(Regoes, 2005) (Mosier, 2009). Once this co-receptor binding succeeds, fusion to the membrane occurs with deposition of the viral core within the cell. At this point the core encounters one of the cell's first defenses, a mesh of cortical actin fibers that can catch it almost like a net. The Wu lab has shown that the initial act of binding to the CD4 receptor, be it by virion-associated or purified gp120, initiates a cascade of signaling that favors infection (Vorster, 2011). By altering the actin activity of the cell, HIV is able to overcome this restriction to entry (Yoder, 2008). The virus manipulates intracellular signals that begin tread-milling the actin, which aids the core on its migration to the cell nucleus.

Reverse transcription and nuclear import. The viral ssRNA genome is reverse transcribed into dsDNA. This process occurs by the activity of virion-associated Reverse Transcriptase enzyme during the journey to the nucleus. The host protein cleavage-and-polyadenylation-specificity-factor-6 (CPSF6) is believed to play a role in nuclear entry of the virus (Rasheedi S, 2016).

Integration, transcription, translation. Another virion-associated enzyme, integrase, may then proceed to splice the transcribed viral DNA into the host-cell genome. This allows the virus to either integrate into the genome of the host cell or productively reproduce without being targeted by the body's immune system.

Assembly, budding and maturation. The newly transcribed viral RNA strand exits the nucleus and is translated into each of the new structural and virion-associated proteins needed to assemble the next generation of viral particles. The enzyme protease cleaves the proteins into their final forms for inclusion. Other strands of viral RNA are

assembled as the genetic material of the new virus at the cell membrane as budding occurs. Protease also plays a role in cleaving the final, new particle off from the cell surface after budding. Full maturation actually occurs after release from the host cell.

Clinical HIV-1 Disease and Immunology

As the early infection proceeds, newly infected patients develop a mild flu-like illness, usually 7-10 days post-infection, followed by apparent recovery. Immunologically these patients typically experience a drop in CD4 count on a complete blood count (CBC) and then no other apparent sequelae as the virus integrates into the genome establishing viral latency. During this period, which can be months or even years long, the patient will experience little in the way of outward clinical symptoms that would raise suspicion under routine medical care. HIV modulates the cell's membrane trafficking pathway to alter the expression of MHC Class I on the cell surface, preventing identification and elimination of infected cells by the immune system (Jia, 2012). Unless the patient's gradually declining CD4 T cell count is detected by a CBC sooner, once the CD4 count drops below the clinical threshold for Acquired Immune Deficiency Syndrome (AIDS) of 200 cells/mL, and the patient develops one or more of the hallmark infectious diseases that can ravage the immunocompromised, this latent period may persist for quite some time. As it does, low level viral replication continues in the body as more and more CD4 T cells become infected and gradually their population shrinks. During this time, the patient's immune system is struggling to maintain homeostasis by regenerating CD4 cells (Cooper A, 2013). There is a correlation to strong CD4+ T cell

response and IL-21 production which are important to maintain CD8+ response (Duvall, 2006).

Despite the success of HAART, HIV remains a treatable but not curable condition and the development of resistance to current drugs is always a concern (Taylor BS, 2019), resulting in a need to find more effective treatment options, with the ultimate goal of finding a cure. One approach is to determine if any current compounds can be repurposed to eliminate HIV infected cells. As early as 2004 research suggested that cytotoxic chemotherapy could be used to eliminate the HIV reservoir in the body (Yang, 2004). CXCR4 is overexpressed in at least 24 different human cancers and is often associated with a poor prognosis (Chatterjee, 2014). CXCR4 antagonists are important weapons in the fight against cancer and tumor progression and present an interesting target for potential HIV treatment. The possibility of a cure for HIV was bolstered by the report of a stem cell transplant from a CCR5 donor was cured (Mitsuyasu, 2013). In addition to CXCR4 and CCR5, another potential target for drug repurposing are drugs that target inducible T cell kinase and the Bcr/Abl oncogene product.

In simplistic terms, a dominant Th1 response leads to production of interferon γ (IFN-γ) and interleukin 2 (IL-2), which drives the cell–based immune response. In contrast, Th2 cell activation is ITK dependent, which drives an antibody mediated response. The balance between the Th1 and Th2 response is crucial for an effective overall immune response. ITK production is upregulated in some disease states, including cancers and parasitic infections, leading to over activation of the Th2 response. This disrupts the Th1/Th2 balance, effectively lessening the effectiveness of direct effector

cell cytotoxicity (Dubovsky JA, 2013). The result is predictable, intracellular pathogens and aberrant cells cannot be effectively eliminated.

For this reason ITK is an attractive therapeutic target. Blocking it could lessen the Th2 response, which would correct the Th1/Th2 imbalance, thereby restoring the body's ability to fight its own infections. ITK also plays a critical role in HIV pathogenesis (Readinger JA, 2008). Inhibiting ITK disrupts F-actin capping and reduces HIV replication in primary human CD4+ T cells. This suggests that targeting ITK is a promising strategy for controlling HIV infection (Schiralli, 2013).

Similarly, the Bcr/Abl oncogene product is a potential therapeutic target. It is elevated in CD34+ cells found in chronic myelogenous leukemia (CML) patients. Blocking Bcr/Abl helps eliminate CML associated cells (Modi H, 2007). In the crisis stage of CML, CD34+ cells express high levels of Bcr/Abl and are less sensitive to the anti-CML drug Imatinib and are observed to rapidly develop resistance (Barnes, 2005).

Some HIV treatments can have unintended negative effects in individuals with concurrent chronic myeloid leukemia (CML). The commonly used HAART drugs ritonavir (induces) and efavirenz (inhibits) Cytochrome P450 3A4 (CYP3A4), which is an important molecule that oxidizes drugs and toxins for removal from the liver (Beumer JH, 2015). CML patients many times are treated with the drug imatinib. However, altered CY3A4 levels increase or decrease imatinib metabolism which may lead to either treatment failure or toxicity (Silvestri L, 2003). Saquinavir is another protease inhibitor/proteasome blocker used as part HAART. It has also been shown to reverse

imatinib-resistance in CML cell lines in a dose and time dependent manner (Timeus F, 2006).

Repurposing pharmaceuticals previously developed or approved to treat other illnesses is an attractive source of potential anti-HIV medications as they presumably already have demonstrated safety profiles and other data from human use. This research employs this tenet to investigate the performance of several classes of small molecules in order to evaluate the potential to repurpose them as anti-HIV medications.

CHAPTER TWO: INDUCIBLE T-CELL KINASE INHIBITION

Introduction

There are 5 members of the Tec (tyrosine kinase expressed in hepatocellular carcinoma) family of non-receptor tyrosine kinases. The three that are expressed in T-cells are Tec, Itk (inducible T-cell kinase), and Rlk/Txk (resting lymphocyte kinase). They are important in antigen receptor signaling as well as many downstream pathways (Andreotti, 2010). Hain et al. demonstrated that HIV was unable to bind to Itk deficient cells. Readinger et al. demonstrated that blocking ITK blocks several steps of HIV replication. Altered ITK function leads to reduced intracellular p24 levels upon infection and decreased virus spread in culture (Readinger, 2009). Itk is especially important in T-cell receptor signaling where it coordinates PLC-1 activation, calcium ion release, and actin rearrangement (Hain, 2018). ITK recruits kinases and adapter proteins, coordinates actin polymerization/polarization and is required for virion assembly and release (Schiralli, 2013).

These observations led to the conclusion that further ITK pathway characterization in terms of HIV infection coupled with an inhibitor would be valuable in our laboratory. Another goal would be to develop an ITK knockdown or knockout cell line as this would be a valuable research tool. An ITK knockdown model in a mammalian cell line could be used to elucidate the impact of ITK on contributing factors of HIV

infectivity such as actin remodeling and T-cell surface receptor availability. The human T4-lymphoblastoid cell line cloned by P.L. Nara et al. were selected because of its utility in HIV research (Foley, 1965). These CEM-SS cells can be induced to adhere to microtiter plates for generating virus and conducting infectivity assays or used in suspension. These assays can be used for testing novel antivirals and assessing neutralizing antibodies (Nara P. e., 1988) (Nara P. , 1987).

The specific aim of this research was to establish a stable ITK knockout phenotype in CEM-SS cells by using shRNA in a lentiviral vector to silence the production of Itk. Our approach was to use ITK-specific shRNA, pCMVΔR8.2 and pHCMV-G DNA transfected into 293T cells. The expected result was the production of a lentivirus containing the desired ITK shRNA. This virus could then be harvested and used to infect CEM-SS cells. In CEM-SS cells the lentivirus was expected to integrate into the genome at the shRNA targeted site and disrupt transcription and subsequent translation of the Itk protein. Further, our goal was to introduce a sequence of protein labeling at the site of gene transcription. The resulting his- or flag- tagged Itk protein would be non-functional and these cells could be used for high-throughput screening of potential drug targets.

Materials and Methods

Virus preparation and cell infections

Infectious HIV-1_{NL4-3} was prepared by Lipofectamine 2000 (Life Technologies, Cat #L3000008, lot #1345608) transfection of HEK293T cells with purified proviral DNA, pNL4-3 as described in (Wu Y. , 2001). The supernatant was harvested at 48 hours

post-transfection, filtered using a 0.45µm syringe-driven nitrocellulose membrane and stored at -80°C until use. The concentration of p24 in the resulting supernatant was determined by ELISA performed in triplicate. Virus titers used in all experiments unless specified otherwise were $10^{3.5}$ to $10^{4.5}$ $TCID_{50}$ of HIV-1 in 1 x 10^6 cells.

Negative control and shITK knockdown viruses: to build the knockdown virus structure, plasmids pCMVΔ8.2, coding for the HIV packaging components Gag, Gag-Pol, Tat, Rev, Nef, Vif and Vpu but without Env, and pHCMV-G, coding for the Vesicular Stomatitis Virus envelope Glycoprotein G, were used, as otherwise described by Yee et al. (Yee, 1994). These lentiviral packages contain the knockdown plasmids pNTC and pshITK. pCMVΔ8.2, pHCMV-G and either pshITK or pNTC are transfected into HEK293T cells in a 10cm petri dish using Lipofectamine 2000 (Life Technologies), incubated for 48h and the supernatant harvested. Supernatants were cleared of gross debris by centrifugation and then filtered through a Whatman 0.45 µM filter aliquoted and stored at -80°C until use.

Cell lines used

Two mammalian cell culture lines were utilized for this series of experiments. Unless otherwise indicated, environmentally cells are maintained at 37°C in an incubator supplemented with 5% CO_2.

HEK293T cells: Adherent HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen) added, to confluence, trypsinized and plated in sterile, round 50mm petri

dishes. Lipofectamine transfection, harvesting and storage of viral particles as described was performed.

CEM-SS cells: The CEM CD4 T lymphoblastoid cell line, CEM-SS, provided by the NIH AIDS Reagent Program were used for transduction, staining and actin imaging experiments. These cells were maintained at a cell density of 1 x 10^6 cells per milliliter or less in RPMI 1640 (Life Technologies) with 10% FBS and penicillin/streptomycin supplementation.

shITK-containing Lentiviral transduction

CEM-SS were passaged with dilution to a cell density of XXXX and transduced with XXng or uL of ITK specific shRNA-containing virus for XX h, washed and diluted to single-cell (0.5 cells/200μL) in filtered CEM-SS conditioned media (RPMI with 10% FBS 200μL of cells were then plated to

Actin staining and cell imaging

Cells were harvested 48h after transduction by low-speed centrifugation, washed then stained with fluorescein isothiocyanate (FITC)-labeled phalloidin. They were then mounted to microscope slides carefully in ProLong Gold anti-fade with DAPI. Images were generated on Nikon confocal microscope.

Results

Knockdown of ITK reduces cell viability and growth

Knockdown cells were successfully obtained using the described methods but unfortunately the knockdown appears to be eventually lethal to the cells. In all attempts

the cell numbers, as compared to control CEM-SS cells, lagged well behind when held in culture under otherwise optimal conditions.

Knockdown of ITK reduces actin activity

Following knockdown of ITK in these cells, actin staining was performed for examination by fluorescence microscopy. Although equal volumes of cells were applied to the slides, the appearance is of few cells with the actin layer confined to an uneven, localized area of the cell.

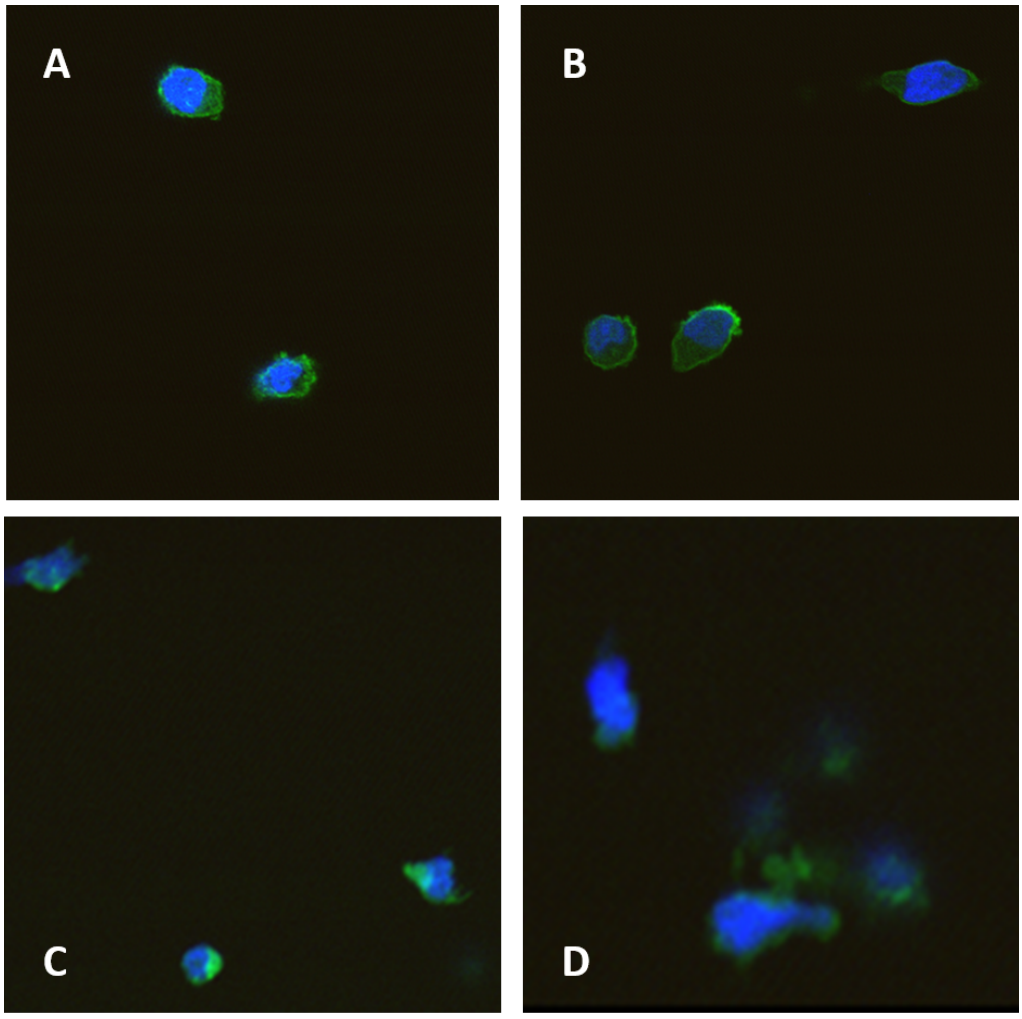


Figure 6 – Inhibition of ITK reduces actin activity:

CEM-SS cells were infected with the lentiviral knockdown virus vITK or vNTC as a control. Cells were infected, washed and incubated for 48h then harvested for actin staining and flow cytometry after which remaining cells were mounted in ProLong Gold with DAPI for confocal microscopy.

Knockdown of ITK achieved with vITK infection was lethal in this cell line

Knockdown of ITK was attempted in several cell lines and with different methods including by the use of knockdown virus and electroporation. Our observation was that in each of the methods attempted the knockdown of ITK activity was eventually lethal to the cells.

Discussion

The observation that the reduction in ITK activity achieved by knocking down the gene using a lentiviral vector resulted in static cultures and eventual cell death limited our ability to conduct extensive phenotypic characterization of the cells. In future studies, if pursued, a drug or method that reduced the ITK activity by a lesser amount might allow for successful model development. ITK levels would need to be quantified beforehand so that a fold- or percent-reduction tipping point couldl be determined. It may very well be that the range of reduction would be too narrow to practically be used to study this mechanism *in vitro*.

CHAPTER THREE: BCR/ABL INHIBITION

Introduction

Bcr protein is encoded by the Breakpoint Cluster Region (BCR) an oncogene associated with development of chronic myeloid leukemia (CML) (Heistercamp, 1991). Bcr/Abl protein is the product of a translocation mutation of the Abelson (Abl) kinase domain from one chromosome and the specific breakpoint cluster region (Bcr) of another resulting in this constitutively active oncogenic tyrosine kinase. Over activity of the Bcr/Abl kinase may result in the development of Chronic Myeloid Leukemia (CML), ALL, autoimmune arthritis, and gastrointestinal stromal tumors (GIST) (Alimena, 2009). Because perturbations in the phosphorylation/de-phosphorylation pathways of the cell have been discovered in our lab to be a rich mechanism by which HIV hijacks cellular processes, existing compounds generated in cancer research laboratories for similar pathways were a natural direction. Examples from a class of small molecules that are used in the treatment of GIST were an interesting possibility. Drugs all end in the suffix –inib and were identified for their tyrosine kinase inhibition. **Imatinib** mesylate (tradename: Gleevec) is used in GIST caused by a mutation in the *Kit* oncogene which encodes a growth factor receptor with tyrosine kinase activity that ultimately results in an activation cascade increasing survival and proliferation in the cells (Sommer, 2003). **Nilotinib** (tradename: Tasigna®) is a derivative of imatinib demonstrating greater activity

against wild-type and mutant Bcr/Abl than the parent drug. **Dasatinib** (tradename: Sprycel®) is an even more potent second-generation TK inhibitor showing efficacy in the treatment of (CML), autoimmune arthritis. It inhibits both the SRC, BCR/ABL and Lyn tyrosine kinases, even in CML with mutations that confer resistance (O'Hare, 2005) (Golemovic, 2005). Samples of the above inhibitors were acquired and tested against an indicator cell line for anti-HIV activity.

Materials and Methods

Inhibitor preparation

The compounds Imatinib (LC Laboratories, Cat #I-5508, lot #BCK-104), Nilotinib (LC Laboratories, Cat #N-8207, lot #BNL-107), and Dasatinib (LC Laboratories, Cat #D-3307, lot #BDS-106) were obtained and prepared for use as described below.

Imatinib: Powdered compound was re-suspended in dimethylsulfoxide (DMSO)(Fisher Scientific, Cat #BP231-100, lot #116070) as a vehicle to ensure solubility to a 100X working stock concentration. Re-suspension concentrations were as follows: Imatinib 10mM, Nilotinib 1.25mM, and Dasatinib 750 μM. To minimize the potential cytotoxic impact of DMSO itself, all further dilutions were carried out in sterile RPMI cell culture media. The indicator cell line was used to screen the compounds for evidence of anti-HIV activity. Uninfected cells were maintained as a negative control and cells treated with only DMSO served as a vehicle control for the compounds. Unless otherwise indicated, environmentally cells are maintained at 37°C in an incubator supplemented with 5% CO_2.

Virus preparation and cell infections

Infectious HIV-1_{NL4-3} was prepared by Lipofectamine 2000 (Life Technologies, Cat #L3000008, lot #1345608) transfection of HEK293T cells with purified proviral DNA, pNL4-3 as described in (Wu Y. , 2001). The supernatant was harvested at 48 hours post-transfection, filtered using a 0.45µm syringe-driven nitrocellulose membrane and stored at -80°C until use. The concentration of p24 in the resulting supernatant was determined by ELISA performed in triplicate. Virus titers used in all experiments unless specified otherwise were $10^{3.5}$ to $10^{4.5}$ $TCID_{50}$ of HIV-1 in 1 x 10^6 cells.

Cell lines used

Two mammalian cell culture lines were utilized for this series of experiments. Unless otherwise indicated, environmentally cells are maintained at 37°C in an incubator supplemented with 5% CO_2.

HEK293T cells: Adherent cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen) added, to confluence, trypsinized and plated in sterile, round 50mm petri dishes. Lipofectamine transfection, harvesting and storage of viral particles as described was performed.

Rev-CEM-GFP Cells: CEM-SS cells (NIH, Cat #776) were stably transfected with integrating constructs containing an HIV-Rev-dependent site, the Rev response element (RRE), coupled to green fluorescent protein (GFP) gene. Cells were cultured in RPMI Medium 1640 (Gibco, Cat #11875119, lot #1315436) with 10% heat-inactivated FBS, 50 U/mL penicillin, and 50 ug/mL of streptomycin.

Results

Bcr/Abl inhibitors demonstrate varying toxicity and impacts on HIV-1 infection

To determine if the inhibition of the Abl tyrosine kinase activity is sufficient to reduce infectivity of HIV-1, Rev-dependent indicator cells were treated with imatinib mesylate, nilotinib and dasatinib. The same experimental conditions were used to treat and infect similar number of cells without adding the drugs back to the cells. The results were consistent with what we observed here, there was little to no negative effect on the

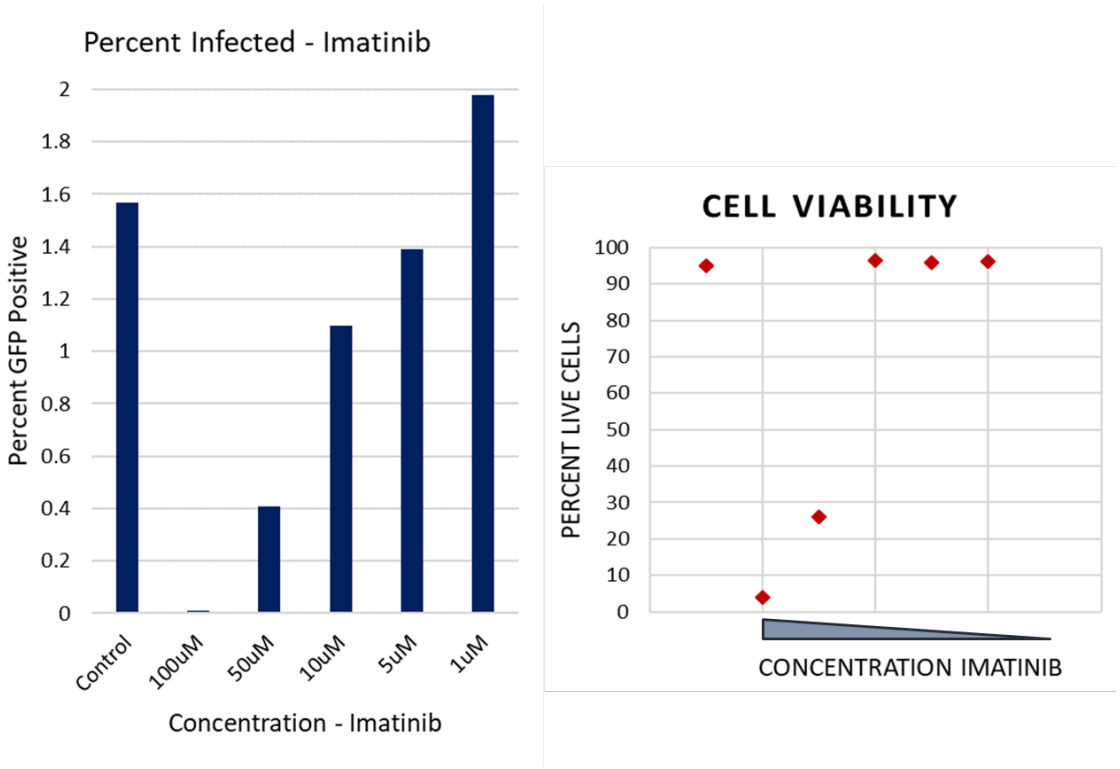


Figure 7 - Screening of imatinib with indicator cells:

High concentration of imatinib does inhibit expression of GFP but also is cytotoxic to them at the higher of the two concentrations.

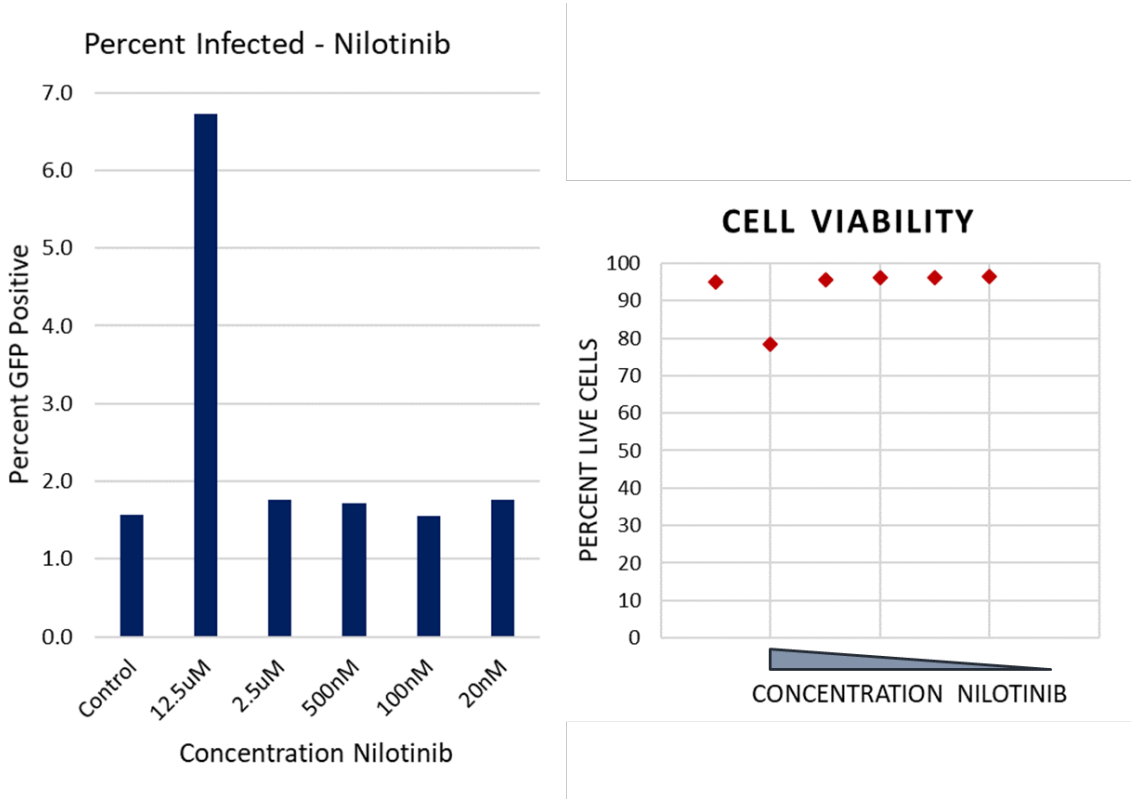


Figure 8 - Screening of nilotinib with indicator cells:

2.5 x 10^5 Rev-CEM-GFP-Luc cells were pre-treated for 1h with 7.5µM, 1.5µM, 300nM, or 60nM then infected with equal p24 HIV-1_{NL4-3} for 2h, washed and incubated for 72h. Cells were analyzed by flow cytometry for GFP production at 48 and 72h post-infection.

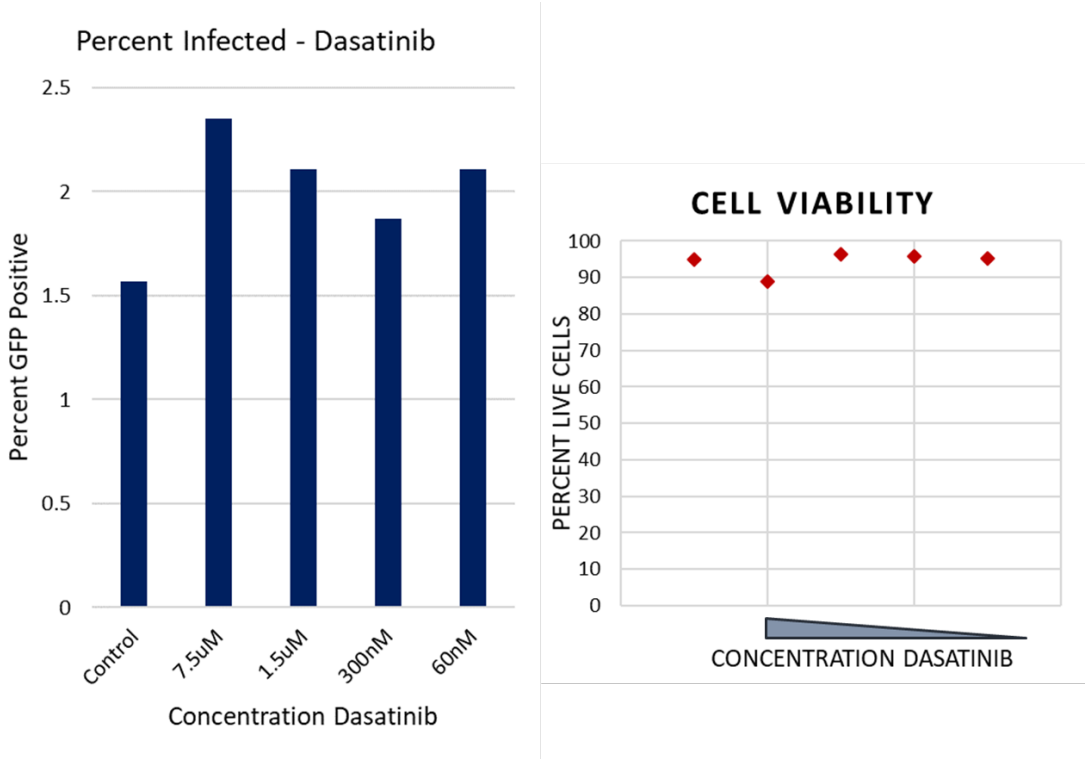


Figure 9 – Dasatinib increases GFP expression in indicator cells:

2.5 x 10^5 Rev-CEM-GFP-Luc cells were pre-treated for 1h with 7.5µM, 1.5µM, 300nM, or 60nM dasatinib then infected with equal p24 HIV-1_{NL4-3} for 2h, washed and incubated for 72h. Cells were analyzed by flow cytometry for GFP production at 48 and 72h post-infection.

Discussion

Imatinib treatment of cells resulted in a dose dependent response, and also indicated a sharp cytotoxicity concentration cut off. As seen in **FIG 7**, 100µM and 50 µM treatment concentrations were highly toxic to the Rev-CEM-GFP-Luc cells, so the GFP inhibition is very likely an artifact. At lower concentrations (below 10 µM) there is a

stark rebound in the viability of the cells back to equal to untreated, uninfected cells. However, there is also a narrow concentration range in which imatinib appears to reduce HIV-1 replication. Once that concentration is down to 1 μM, the trend appears to begin to increase replication.

Nilotinib did not inhibit HIV-1 replication in this testing system. At the highest concentration tested (12.5 μM), nilotinib treatment resulted in some cytotoxicity but, more surprisingly, it multiplied GFP expression by nearly 4-fold.

Dasatinib did not demonstrate significant cytotoxicity in Rev-CEM-GFP-Luc cells during these experiments. However, at all concentrations, dasatinib also appeared to enhance HIV-1 replication in this model to at least some degree.

CHAPTER FOUR: HEAT SHOCK PROTEINS

Introduction

Heat Shock Proteins (HSPs) exist in both prokaryotic and eukaryotic organisms and since their identification have been shown to be highly conserved across phylogenetic studies in structure, antigenicity and function. Members of the heat shock family of proteins play important roles in both normal cellular processes as molecular chaperones as well as during the cellular response to stressors such as temperature alterations from which they gain their name (Lindquist, 1988). The chaperone and molecular stabilizing ability allows them to prevent catastrophic changes in conformation in their nascent chaperoned charges. HSPs are generally differentiated by their molecular weight in kilodaltons (kD). HSP70 refers to the heat shock protein of 70,000 Daltons when resolved by polyacrylamide gel electrophoresis. In human cells the family consists of multiple species indicated by their size: HSP110, HSP100, HSP60, HSP40, HSP70, HSP90, and HSP20, otherwise known as Ubiquitin. HSPs can also exist in multiple isoforms.

Heat Shock Proteins have been shown to be involved in multiple steps in the replication cycles of many viruses, from involvement in complexes that function as viral receptors (Valle, 2005) (Arias, 2002) the mediation of entry and replication (Tsou, 2013) McClellan, 2007) to the induction of the innate immune responses in the host (Kim,

2012). Another set of studies revealed that HSP90 forms part of cluster of cell homeostasis-related factors that are upregulated during HIV infection, suggesting its manipulation post-infection (DeBoer, 2015).

HSP70 can be found as a membrane-bound or a cytosolic molecule and is a molecular chaperone which is crucial for cellular protein homeostasis. It has two distinct and highly conserved domains, the 40 kDa N-terminal nucleotide binding domain (NBD), and the 25 kDa C-terminal substrate-binding domain (SBD) (Lackie, 2017). Homeostasis is controlled by ATP, which binds to the NBD. As the ATP binds, the SBD changes and is then able to interact with hydrophobic regions of the target substrate. Changes in the substrate then trigger ATPase, which decreases the amount of ATP bound to the NBD, which inhibits further substrate binding (Zhuraleva, 2012). HSP90 is mostly found in the cytoplasm and was initially discovered as a steroid hormone receptor binding protein by Catelli et al in 1985 (Catelli MG, 1985).

HSP90 is also a highly conserved homodimeric unit whose function is essential for proper protein processing (Lackie, 2017). HSP90 has an N terminal domain that binds nucleotides. The C terminal domain is responsible for dimerization and has a conserved sequence which interacts with the middle domain which is important in ATP hydrolysis. Although there is a constitutive level of HSP90 expression in the cell, as its name indicates, increased temperature upregulates the activity of the protein. In this response, HSP90 activates a group of transcription factors in the cytoplasm, among these are NFκ-B, NFAT and STAT.

We screened several HSP inhibitors for HIV suppressive activity using the Rev-dependent CEM-SS based indicator cell line and identified a derivative of the antibiotic geldanamycin with a demonstrable antiretroviral effect in vitro. Geldanamycin is naturally produced by the bacterium *Streptomyces hygroscopicus* (Calcul, 2012). Its chemical activity binds the ATP-binding site of HSP90, preventing its activation. In clinical trials the drug proved potent, but highly hepatotoxic, and so was modified by displacing the methoxy group with an amine, resulting in the derivative tanespimycin or 17-alkyl-allylo-geldanamycin (17-AAG) (Pasqua, Wilding, Cheeseman, & Jones, 2017).

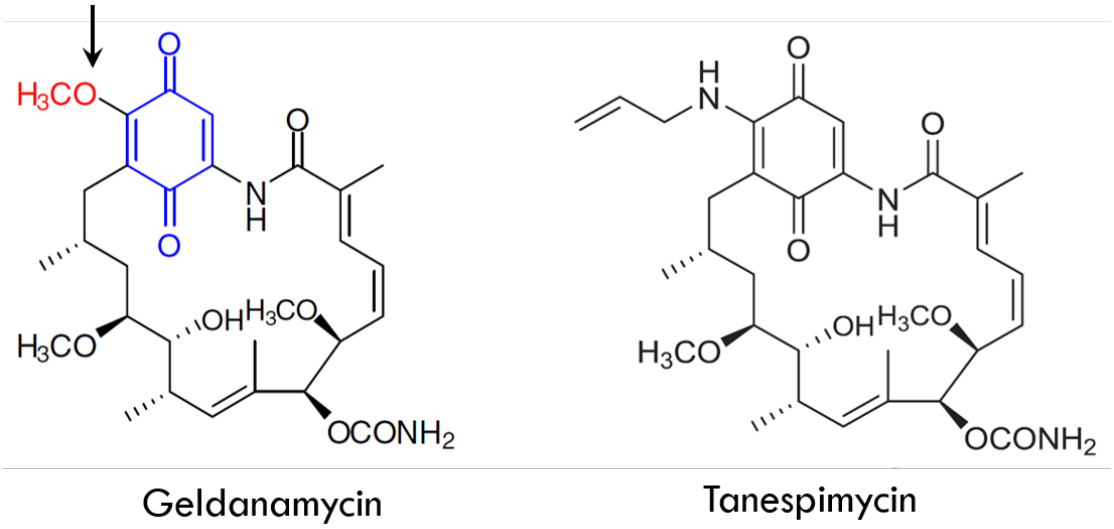


Figure 10 – *Chemical structure models*: the parent, geldanamycin, and derived inhibitor tanespimycin (17-AAG). Arrow indicates the location of the substituted methoxy group that is replaced by an amine to gain solubility and lower toxicity.

Section 2: Potential HIV-1 Inhibitors

Initial screening of HSP inhibitors began with three candidate compounds: The known heat shock protein inhibitor KNK-437 (3-[1,3-Benzodioxol-5-ylmethylene]-2-oxo-1-pyrrolidinecarboxaldehyde), the calcium-disrupting chelator BAPTA-AM (1,2-bis-[2-aminophenoxy] ethane-N,N,N',N'-tetraacetic acid tetra[acetoxymethyl]), and 17-AAG (17-[allylamino]-17-demethoxygeldanamycin), also known as tanespimycin. The Rev-dependent indicator cell line Rev-CEM was used to screen a limited panel of concentrations to assess if any will be appropriate for further study of anti-HIV activity.

Materials and methods

Inhibitor preparation: Samples of the compounds 17-AAG, KNK-437 and BAPTA-AM were graciously provided by Dr. Ramin Hakami (George Mason University) suspended in dimethylsulfoxide (DMSO, Fisher) as a vehicle. To minimize cytotoxic impact of DMSO itself, all further dilutions were carried out in RPMI cell culture media. Stock concentrations were diluted to final treatment concentrations of (10μM, 2μM, 400nM, 80nM, and 16nM). The HIV infection indicator cell line, Rev-CEM, was used for screening the compounds for anti-HIV activity. Uninfected cells were maintained as a negative control and cells treated with only DMSO will serve as a vehicle control for the inhibitors.

Virus preparation and cell infections: Infectious HIV-1_{NL4-3} was prepared by Lipofectamine 2000 (Life Technologies) transfection of HEK293T cells with purified proviral DNA, pNL4-3 as described (Wu Y. , 2001). The supernatant was harvested at 48h post-transfection, cleared of gross debris by centrifugation then filtered through a

Whatman 0.45μm syringe-driven nitrocellulose membrane, aliquoted and stored at -80°C until use. The concentration of p24 in the resulting supernatant was determined by ELISA performed in triplicate and $TCID_{50}$ was performed using. Virus titers used in all experiments unless specified otherwise were $10^{3.5}$ to $10^{4.5}$ $TCID_{50}$ of HIV-1 in 1 x 10^6 cells. Infections were performed with or without spinoculation enhancement as described (Guo, 2011).

Cell Lines Used: Two cell culture lines were utilized for this series of experiments, a transfection cell line to produce the desired form of HIV and an indicator cell line with the potential to be used for high-throughput screening. Unless otherwise indicated, environmentally cells are maintained at 37°C in an incubator supplemented with 5% CO_2.

HEK293T Cells. Adherent cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) with 10% heat-inactivated fetal bovine serum (FBS) added, to confluence, trypsinized and plated in sterile, round 50mm petri dishes. Lipofectamine transfection as described by Wu and Marsh was performed (Wu Y. , 2001).

Rev-CEM-GFP Cells. CEM-SS cells (NIH, #776) were stably transfected with integrating constructs containing an HIV-Rev-dependent site, the Rev response element (RRE), and the green fluorescent protein (GFP) gene (Wu, Beddall, & Marsh, Rev-Dependent Indicator T Cell Line, 2007) Cells were cultured in RPMI 1640 medium with 10% heat-inactivated FBS, 50 U/mL penicillin, and 50 ug/mL of streptomycin added.

Fluorescence Activated Cell Sorting (FACS): CD4 T cell derived Rev-CEM-GFP-Luc (Yi & al, 2017) cells were infected as described (Wu Y. , 2001) with HIV and infection was measured via GFP production by flow cytometry detection using the FACSCalibur instrument (BD Biosciences). To measure cytotoxicity concurrently in the cells, 1µl of 2 µg/ml propidium iodide (Fluka) was added to the cells prior to flow cytometry. By excluding from the analysis by gating, cells that took up the PI, only viable cells in the sample were measured for GFP expression.

(KNK-437)

KNK-437 a synthetic benzylidene lactam compound that effectively inhibits the activation of heat shock protein in human colon carcinoma cells (Yokota, 2000). KNK-437 has also been shown to induce apoptosis and inhibit accumulation of heat-induced HSPs (Sharma C, 2018)

(BAPTA-AM)

BAPTA-AM is an intracellular calcium ion chelator. It has been shown to prevent apoptosis in cancer cells by suppression of the Ca^{2+} buildup that leads to initiation of the apoptotic pathway. HSP70 downregulation results in increased intracellular Ca^{2+}. (Dudeja, 2009)

(17-AAG)

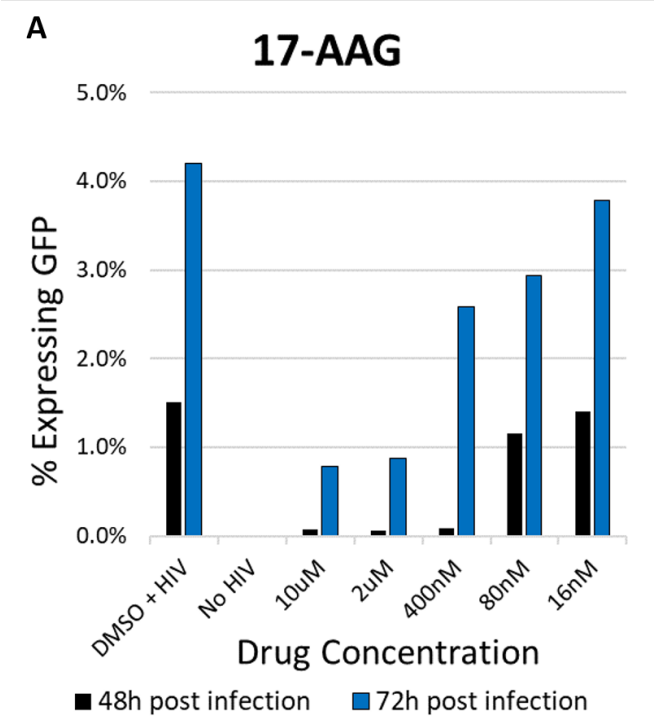
17-N-Allylamino-17-demethoxygeldanamycin (17-AAG), also called tanespimycin, is a small HSP90 inhibitor that shows promise inhibiting the growth of cancerous tumors (Ho SW, 2013). 17-AAG works by preventing HSP90 from forming a stable complex, but instead to a form that is easily targeted by proteasomes (Bharadwaj S,

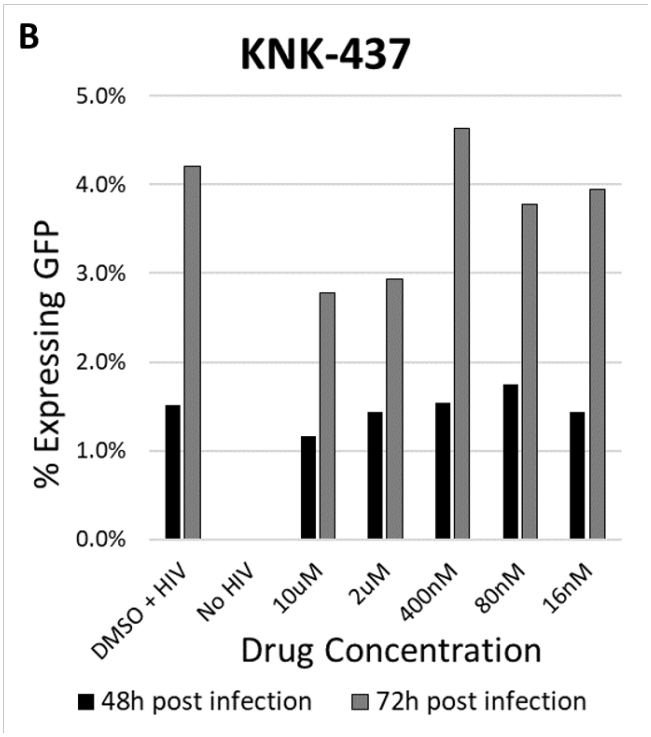
1999). 17-AGG also blocks the association between HSP90 with HSF-1, which causes transcription of other HSPs (Waza M, 2006).

Results

17-AAG, but not KNK-437 or BAPTA-AM, reduce expression in Rev-CEM-GFP

Initial drug preparation and screening study of these HSP inhibitors was undertaken in the Wu laboratory, data is shown (Fig-11). Rev-CEM cells were treated with serial 5-fold dilutions 10 µM, 2 µM, 400 nM, 80 nM, or 16 nM of the inhibitors 17-AAG, KNK-437, or BAPTA-AM to determine whether these compounds reduce the HIV Rev-driven expression of GFP.




B
KNK-437
5.0%
4.0%
3.0%
2.0%
1.0%
0.0%
% Expressing GFP
DMSO + HIV
No HIV
10uM
2uM
400nM
80nM
16nM
Drug Concentration
48h post infection
72h post infection

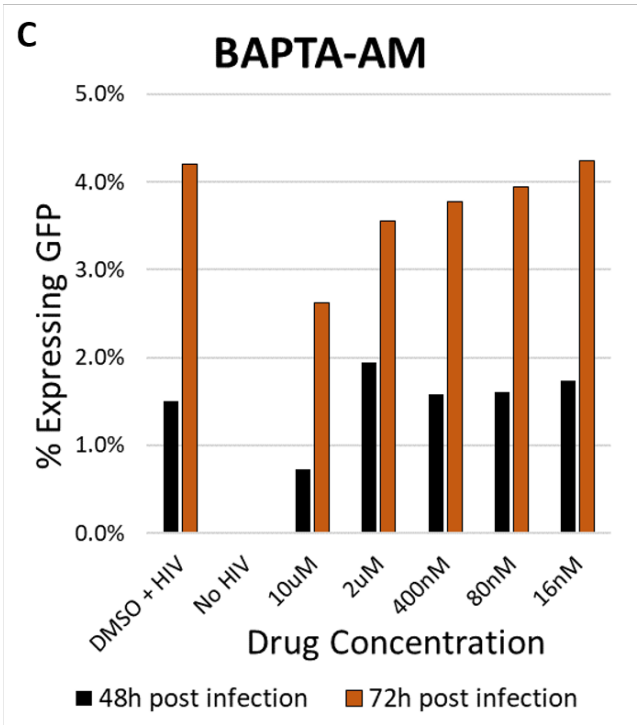


Figure 11 – 17-AAG (tanespimycin), but not KNK-437 or BAPTA-AM, reduces the HIV Rev-driven expression of GFP.

2.5 x 10^5 Rev-CEM-GFP-Luc cells were aliquoted to individual sterile culture tubes (Falcon). Cells were pre-treated for 1h with 5-fold serial dilutions of each inhibitor, suspended in DMSO as a vehicle. DMSO-only treated cells were a control. After infection with replication-competent wild-type HIV-1_{NL4-3} for 2h, cells were washed, and incubated in RPMI 1640 with inhibitors at the indicated concentrations. *Panel A* – the specific HSP90 inhibitor 17-AAG (tanespimycin) results are shown. *Panel B* – inhibitor of activation of heat shock proteins KNK-437 results are shown. *Panel C* – the calcium ion chelator BAPTA-AM results are shown. Data is seen as graphical results at 48h and 72h post-infection from a FACSCalibur flow cytometer. Data courtesy of Joy Deng.

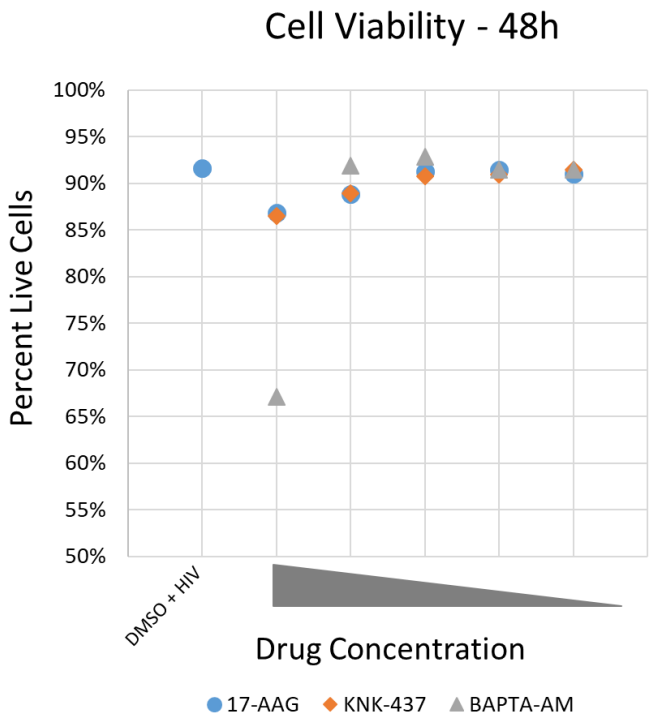


Figure 12 – BAPTA-AM treatment results in greater cytotoxicity than KNK-437 or 17-AAG at highest concentration.

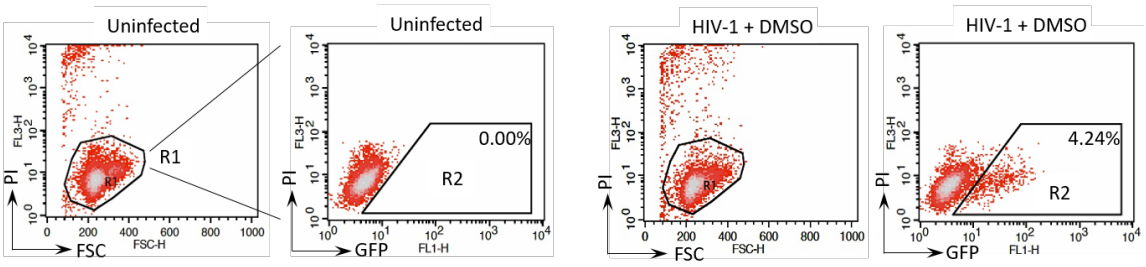
From the same experimental set of cells, 1µl of propidium iodide is added prior to flow cytometry to stain cell debris, dead, and dying cells for gating from our data. Debris is excluded and viability of the cell population is ascertained at 48h post-infection for the three HSP inhibitors vs. control cells. The same data was collected at 72h post-infection and was consistent. Data courtesy of Joy Deng.

The results shown in Fig 11 suggest that the panel of heat shock protein inhibitors seem to demonstrate a dose-dependent impact on the number of GFP expressing cells in this system. By exposing the cells to a range of concentrations of the inhibitors, infecting

them with WT HIV-1_{NL4-3}, then analyzing the cells by flow cytometry at 48 and 72 hours post infection, we show a reduction in the measurable number of GFP-expressing cells. In particular, tanespimycin treatment results in a definite reduction of HIV infected cells. In the tanespimycin cohort, we also saw the lowest percentage of cell death by propidium iodide staining (Fig 12), suggesting that this range of concentrations of tanespimycin was reasonably well-tolerated by the cells.

Tanespimycin (17-AAG) reduces HIV-induced GFP expression in indicator cells

Due to the cytotoxicity of BAPTA-AM at effective concentrations, it was excluded from further study in this project. Similarly, KNK-437 was excluded due to its apparent ability to actually cause an increase in GFP expression at one concentration, and therefore HIV replication, in this indicator cell line. 17-AAG, however, appeared to be able to reduce GFP expression and caused little in the way of cytotoxic effects, except at its highest concentration. Therefore, this inhibitor was selected for further characterization in our laboratory.



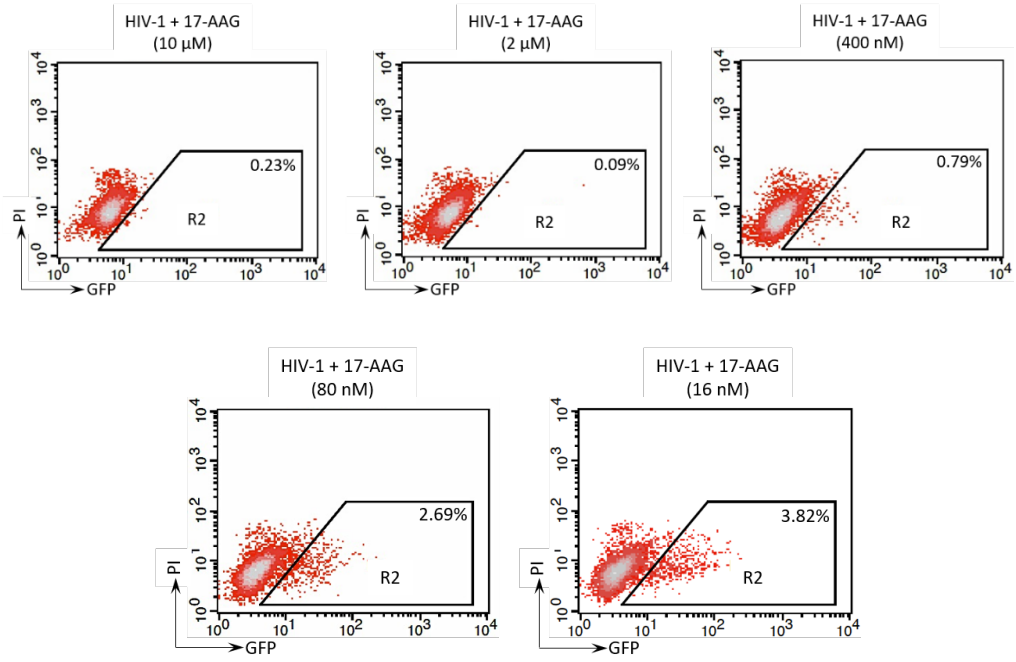


Figure 13 – Tanespimycin reduces HIV-1-driven GFP expression in indicator cells.

Dot plots showing the gating strategy for flow cytometry of tanespimycin-treated, HIV-infected Rev-CEM-GFP-Luc indicator cells at 48h post-infection. A repeat screen was consistent with this initial observation (data not shown).

It was verified that the HIV inhibition seen in these indicator cells was not due to the drug affecting the GFP itself in the system. Jurkat cells were transfected with p-Egfp-Cl plasmid and the treated with tanespimycin. Cells were harvested for analysis by flow cytometry at 24, 48, and 72h post-transfection (Fig 14). It appears in these cells that treatment with tanespimycin is actually capable of enhancing the transfection efficiency of the reagent at the 2µM concentration. However, the presence of tanespimycin does not reduce the expression of GFP in this model.

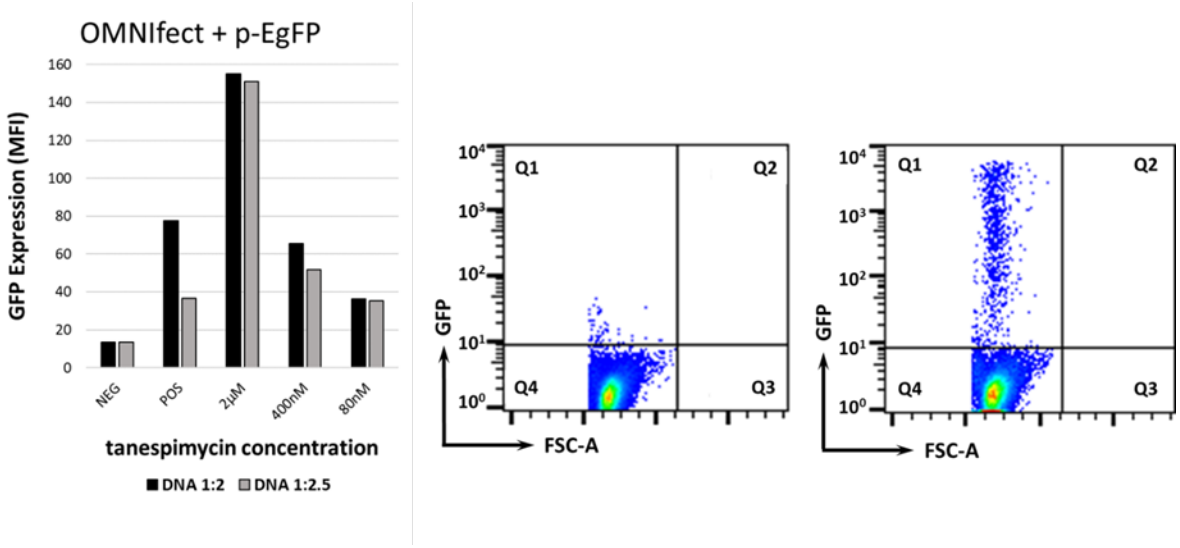


Figure 14 – tanespimycin treatment does not interfere with GFP expression in transfected Jurkat cells

Jurkat cells were maintained at a density of 5 x 10^5 c/ml or less in RPMI 1640 and 3.5 x 10^5 cells were pre-treated for 1h with 2µM, 400nM, or 80nM tanespimycin, transfected using OMNIfect (TransOMIC Technologies) transfection reagent and Cl-pEgfp plasmid (courtesy Dr. Elke Bergmann-Lightner). Transfected cells were analyzed on the MACSQuant 16 flow cytometer (Miltenyi Biotec) at 24, 48, and 72 hours post-transfection.

Discussion

The conclusion of the initial screening work was that pre-treatment of the aforementioned indicator cell line with tanespimycin appeared to reduce the number of cells generating the GFP indicator in response to HIV infection. As a result, a project was proposed to characterize this inhibition more thoroughly. The entry and infection cycle of HIV will be investigated using various techniques in concert with inhibitor treatment to determine if possible which portions of the lifecycle are most impacted by tanespimycin

treatment and therefore are most likely dependent on HSP90 activity. Production of nascent viral proteins will be examined to determine if there is a specific protein that is most affected by disruption of HSP90 activity.

CHAPTER FIVE: STUDIES OF HSP 90 INHIBITOR

Introduction

After screening several potential inhibitors for HIV-1 suppressive activity using a Rev-dependent indicator cell line, we identified tanespimycin as the most promising of the candidates with a demonstrable antiretroviral effect in vitro. Tanespimycin, as a specific inhibitor of the activity of HSP90, was selected for further study in an effort to characterize what portions of the viral lifecycle the presence and function of this protein may be critical.

Tanespimycin is has been demonstrated as well-tolerated in a clinical monotherapy dose-escalation study in multiple myeloma patients (Richardson P. e., 2010). Patients were cohorted to receive doses of tanespimycin via IV infusion over the course of 11 days and at concentrations ranging from 5 mg/kg up to 17.5 mg/kg. In the pharmacokinetic analysis they found that the half-life of the drug varied from 2.2-4.4 hours with T_{MAX} (time to maximum plasma concentration) occurring between 1 and 2 hours post-infusion and at a concentration approaching 1×10^4 ng/ml (Richardson P. e., 2010).

Materials and Methods

Inhibitor preparation

Tanespimycin (17-(allylamino)-17-demethoxygeldanamycin, Sigma-Aldrich, cat #A8476) suspended in dimethylsulfoxide (DMSO, Fisher) to a working concentration of 3mM as a vehicle. To minimize cytotoxic impact of DMSO itself, all further dilutions for experimental purposes were carried out in RPMI cell culture media. Stock concentrations were diluted to final treatment concentrations of 10μM, 2μM, 400nM, 80nM, and 16nM in suspended cells. Uninfected cells were maintained as a negative control and cells treated with only DMSO will serve as a vehicle control for the inhibitors.

Virus preparation and cell infections

Wild-type HIV: Infectious HIV-1_{NL4-3} was prepared by Lipofectamine (Life Technologies) transfection of HEK293T cells with purified proviral DNA, pNL4-3 as described (Wu Y. , 2001). The supernatant was harvested at 48 hours post-transfection, filtered using a 0.45μm syringe-driven nitrocellulose membrane and stored at -80°C until use. The concentration of p24 in the resulting supernatant was determined by ELISA performed in triplicate. Virus titers used in all experiments unless otherwise indicated were $10^{3.5}$ to $10^{4.5}$ $TCID_{50}$ of HIV-1 in 1 x 10^6 cells. Infections were performed with or without spinoculation enhancement as described (Guo, 2011).

Single-cycle HIV: Single-cycle virus was prepared by Lipofectamine (Life Technologies) transfection of HEK293T cells with 1:1 ratio of 12μg purified plasmid pNL4-3(KFS), co-transfected with 12μg pNL4-3ΔΨgp160 as described by Yu et al, (Yu, 2009). The supernatant was harvested at 48 hours post-transfection, filtered using a

0.45µm syringe-driven nitrocellulose membrane and stored at -80°C until use. The concentration of p24 in the resulting was determined by ELISA performed in triplicate.

VSV-G-pseudotyped HIV: NL4-3 virus which enters the cell via endocytosis was prepared by Lipofectamine (Life Technologies) transfection of HEK293T cells with a 1:1 ratio of the purified plasmids pNL4-3(KFS), lacking a functional *env* gene, and co-transfected with pHCMV-G expressing the envelope glycoprotein of the vesicular stomatitis virus, VSV-G (Yee, 1994). The concentration of p24 in the resulting supernatant was determined by ELISA performed in triplicate.

Non-integrating HIV: virus capable of infecting the cell, but unable to integrate into the host genome was prepared by Lipofectamine 2000 (Life Technologies) transfection of HEK293T cells with the purified plasmid pNL4-3D116N lacking a functional integrase, and co-transfected with pHCMV-G envelope. The concentration of p24 in the resulting supernatant was determined by HIV p24 ELISA performed in triplicate.

Cell Lines Used: Two cell culture lines were utilized for this series of experiments, a transfection cell line to produce the desired form of HIV and an indicator cell line with the potential to be used for high-throughput screening. Unless otherwise indicated, environmentally cells are maintained at 37°C in an incubator supplemented with 5% CO_2.

HEK293T Cells. Adherent cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) with 10% heat-inactivated fetal bovine serum (FBS) added, to confluence, trypsinized and plated in sterile, round 50mm petri dishes.

Lipofectamine transfection as described (Wu Y. , 2001) with purified plasmid DNA, detailed in virus descriptions above, was performed to generate all of the viruses used in this work.

*Rev-CEM-GFP-Luc Cells***.** CEM-SS cells were infected with integrating lentiviruses assembled from constructs containing an HIV-Rev-dependent site, the Rev response element (RRE) and the gene encoding green fluorescent protein (GFP) **(Wu Y. , 2001)** along with an additional indicator gene, luciferase (Luc) **(Yi & al, 2017)**. In the presence of HIV Rev, transcripts containing the RRE are not multiple spliced and degraded, so translation occurs and both GFP and Luciferase are produced in the cell. Cells were cultured in RPMI 1640 medium with 10% heat-inactivated FBS, 50 U/mL penicillin, with or without 50 ug/mL of streptomycin added.

Protein detection and quantification

Western Blot: To determine the relative levels of HIV-1 proteins generated in CEM-SS and in PBMCs whole cell lysates were generated from one million pelleted cells re-suspended in 1X LDS sample lysis buffer. Samples were then run on SDS-PAGE gel (NOVEX Nu-PAGE, 4-12%) and electrophoretically transferred to 0.45 μm pore nitrocellulose membrane. Membrane was probed for HIV proteins using HIV immunoglobulin-human (NIH cat #3947, lot #110180) with DyLight 800 goat anti-human (KPL, cat #072-07-10-06, lot #081289) secondary antibody. Membranes were stripped with Stripping Buffer (Thermo) and re-probed for levels of GAPDH with polyclonal goat anti-GAPDH (AbCam, cat #ab9483, lot #GR38228-11) and DyLight IR 800 rabbit-anti-goat IgG (KPL cat #072-06-13-06, lot #111117) as a loading control to

normalize for uneven loading. Protein levels were calculated and plotted for expression in untreated vs. inhibitor-treated cells.

HIV-1 p24 ELISA: To determine the concentration of released viral p24 antigen from infected cells 150µL of re-suspended cell culture was removed to a microcentrifuge tube, centrifuged at 13,000 RPM for 30 seconds, then 135 µL of supernatant was removed to a new microcentrifuge tube. Supernatants were lysed using 15 µL p24 lysis buffer (5% Triton-X in phosphate buffered saline). Released p24 was then assessed by modified sandwich ELISA protocol. Briefly, a polyclonal p24 antibody (NIH AIDS Reagent Program, Cat #3537) was used to coat the wells of a 96-well ELISA plate overnight. It was then washed and blocking was accomplished using PBS with 2.5% FBS. The above-mentioned p24 lysate samples were then added to the appropriate wells and bound with a biotinylated, polyclonal anti-HIV antibody. Horseradish peroxidase-conjugated streptavidin was then used with the chromogenic substrate 3,3',5,5'-tetramethylbenzidine (KPL, Rockville MD) and the chromogenic reaction was read at A_{630} on a plate-capable spectrophotometer.

Quantitative real-time Polymerase Chain Reaction (PCR)

Samples for real-time PCR analysis were obtained from 1 mL re-suspended culture at the following time points: 2h, 4h, 12h, 24h, and 48h post-infection.

Nucleic Acid Extraction: Cells were pelleted, lysed and the total viral DNA and RNA extracted using the SV Wizard RNA Purification Kit (Promega, cat #Z3100)) as described (Yoder, 2008). Briefly, DNA was captured in a spin column while RNA is eluted after cell lysis; DNA-capture column was then processed per product insert to

clean and elute the DNA. Eluted RNA-containing solution is applied to a second RNA-capture column and DNA was digested. RNA was then washed and eluted with molecular biology grade water (Sigma, MO). The nucleic acid content of each eluate was then measured on a Nano-drop instrument (ThermoFisher, MA)

Quantification of total viral DNA: DNA was extracted from experimentally treated cells and PCR performed with primers and probe (IDT) were acquired with sequences as follows: forward primer 5'LTR-U5 (sequence: 5' – AGATCCCTCAGACCCTTTTAGTCA – 3'), reverse primer 3'GAG (sequence: 5' - TTCGCTTTCAAGTCCCTGTTC – 3') and probe FAM-U5/gag (sequence: 5' – (FAM) – TGTGGAAAATCTCTAGCAGTGGCGCC – (BHQ) – 3')

Quantification of nef transcripts: Total RNA was extracted from treated and infected cells and reverse transcribed to cDNA via the addition of SuperTaq Plus buffer (cat #8707G), random decamers (cat #5722G), dNTPs (cat #8228G5), RNAse inhibitor (cat #2682) and using M-MLV reverse transcriptase (cat #AM2044) all from Ambion, as described (Wu Y. , Selective transcription and modulation of resting T cell activity by preintegrated HIV DNA, 2001). Real-time PCR was performed on cDNA using the following primers and probe all obtained from IDT: forward primer 5'Nef (sequence: 5' – GGCGGCGACTGGAAGAA – 3'), 3'Rev (sequence: 5' – AGGTGGGTTGCTTTGATAGAGAAG – 3'), and probe Nef/Rev (sequence: 5' – FAM – CGGAGACAGCGACGAAGAGCTCATC-TAMRA – 3')

Fluorescence Activated Cell Sorting (FACS)

CD4 T cell derived Rev-CEM-GFP-Luc cells were infected as described above with HIV and infection was measured via GFP production by flow cytometry detection using the FACSCalibur instrument (BD Biosciences, NJ). To measure cytotoxicity concurrently in the cells, 1µl of 2 µg/ml propidium iodide (Fluka, GER) was added to the cells prior to flow cytometry. By excluding from the analysis by gating, cells that took up the PI, only viable cells in the sample were measured for GFP expression. To ensure tanespimycin was not preventing folding or another aspect of GFP function, CEM-SS cells were transfected with p-EgFP using OMNIfect (TransOMIC Technologies, AL) and read on a MACSQuant (Miltenyi Biotech, GER) flow cytometer with 1µl of 2 µg/ml acridine orange/propidium iodide (Vitascientific, MD) for viability.

Results

HIV-1 inhibitory concentration of tanespimycin is well-tolerated

To determine whether there is a breakpoint concentration of tanespimycin where cytotoxicity reaches an unacceptable level when considered against HIV inhibitory effect, Rev-CEM T cells were used. In this instance, cells were pre-treated for 1 hour before infection with 10 µM, 2 µM, 400 nm, 80 nM, 16 nM, or DMSO alone as a positive control. Cells were harvested at 48h post-infection and analyzed by flow cytometry. Inhibition of HIV replication occurred in a dose-dependent manner between 10 µM, 2 µM, and 400 nM with little impact on replication observed at the lowest dilutions. Some cytotoxicity was observed at the 10 µM concentration, but even at that concentration, the viability of the cells were 93% of that of control uninfected cells. From this data the IC50

of tanespimycin against HIV-1 infection in Rev-CEM-GFP-Luc cells was calculated to be 402 nM (Spear & al., 2014).

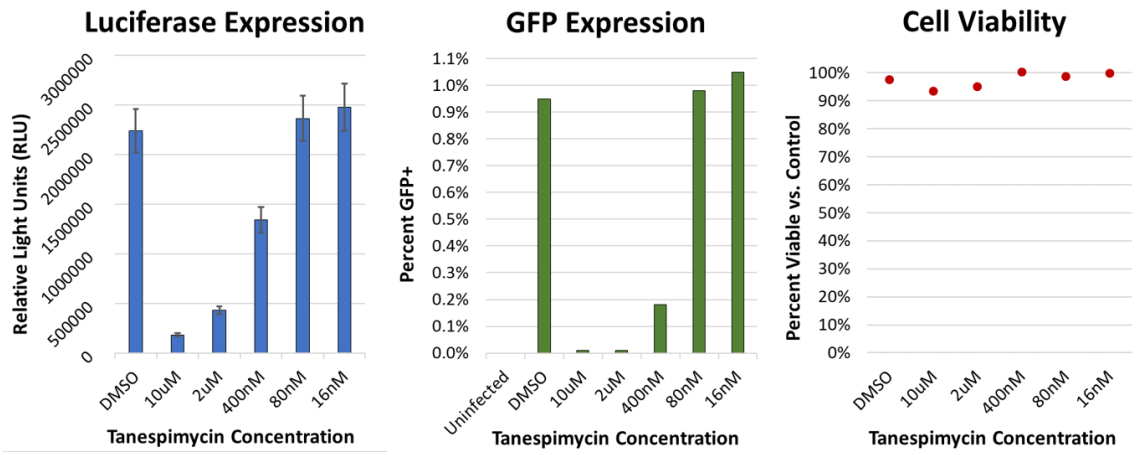


Figure 15 - HIV inhibitory concentrations of tanespimycin are well-tolerated.

Left panel, HIV-Rev driven luciferase expression in Rev-CEM-GFP-Luc cells with DMSO treated cells as a positive control. 2.5 x 10^5 cells/ml are pre-treated with 5-fold dilutions of tanespimycin in RPMI media, infected with equal p24 HIV-1_{NL4-3}. Cells were harvested and Glo buffer (Promega, WI) added, luciferase activity is measured on the GloMax Multi detection System (Promega). *Middle panel,* HIV-Rev driven GFP expression in the same cells, same experiment shown in triplicate. *Right panel*, tanespimycin pre-treatment results in little cytotoxicity in the final concentration range 10µM-16nM when compared to uninfected control cells.

Treatment time does not substantially reduce inhibition of HIV-1 replication

Tanespimycin was diluted to the concentrations 10µM, 2 µM, 400 nM, 80 nM, and 16nM and a time-of-addition (ToA) experiment was performed to determine if there

was a distinct time during the infection process that was particularly and preferentially negatively impacted by the drug treatment.

Mode of viral entry does not reduce inhibition

In order to determine if altering the mode of viral entry to the cell would allow HIV to overcome the inhibition of HSP90 activity, a VSV-G enveloped HIV was used to infect Rev-CEM cells treated over a short time course. Tanespimycin at a final concentration of 2 μM was used to either pre-or post-infection treat 2.5 x 10^5 Rev-CEM indicator cells. Cells were then infected with the viruses, washed and incubated for 48 and 72h. Aliquots of each were analyzed by flow cytometry and the data is shown below in Fig 15.

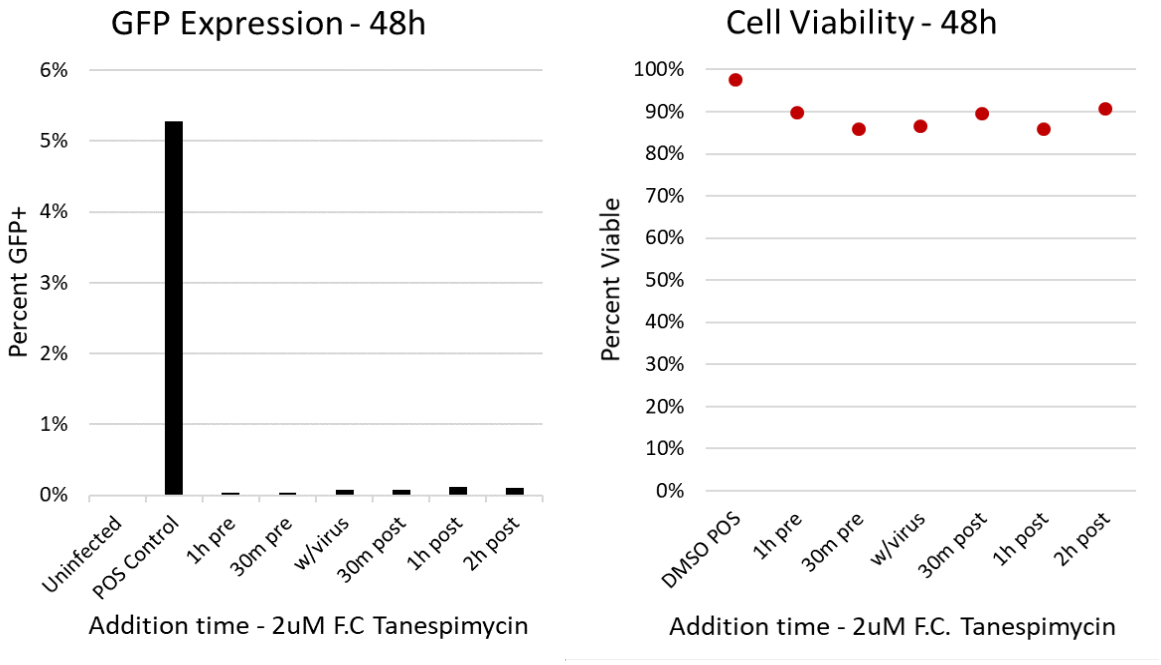


Figure 16 – Mode of viral entry does not reduce inhibition.

Left panel, HIV-Rev driven GFP expression in Rev-CEM-GFP-Luc cells with DMSO treated cells as a positive control. Cells are pre-treated or post-treated with 2 µM of tanespimycin and infected for 2h with HIV_{VSV-G}. *Right panel,* cell viability as measured against control, uninfected cells in the same experiment.

Treatment time pre- and post-infection does not alter inhibition of HIV-1

In cells treated 1h before infection, during infection and post-infection, inhibition of infection is consistent with previous results. In other words, when tanespimycin concentration is kept constant during the time surrounding infection, we still see

inhibition of GFP production in Rev-CEM-GFP-Luc cells. It was also observed that, from 4h before to 12h after infection, when cells are treated with tanespimycin, infection is almost completely prevented by the presence of the drug (0.56% → 0.01%) at 48h. Furthermore, tanespimycin remains suppressive of HIV-1 virus activity at 72h (2.24% → 0.26%). These results are representative of several experiments and are shown in Figure 16.

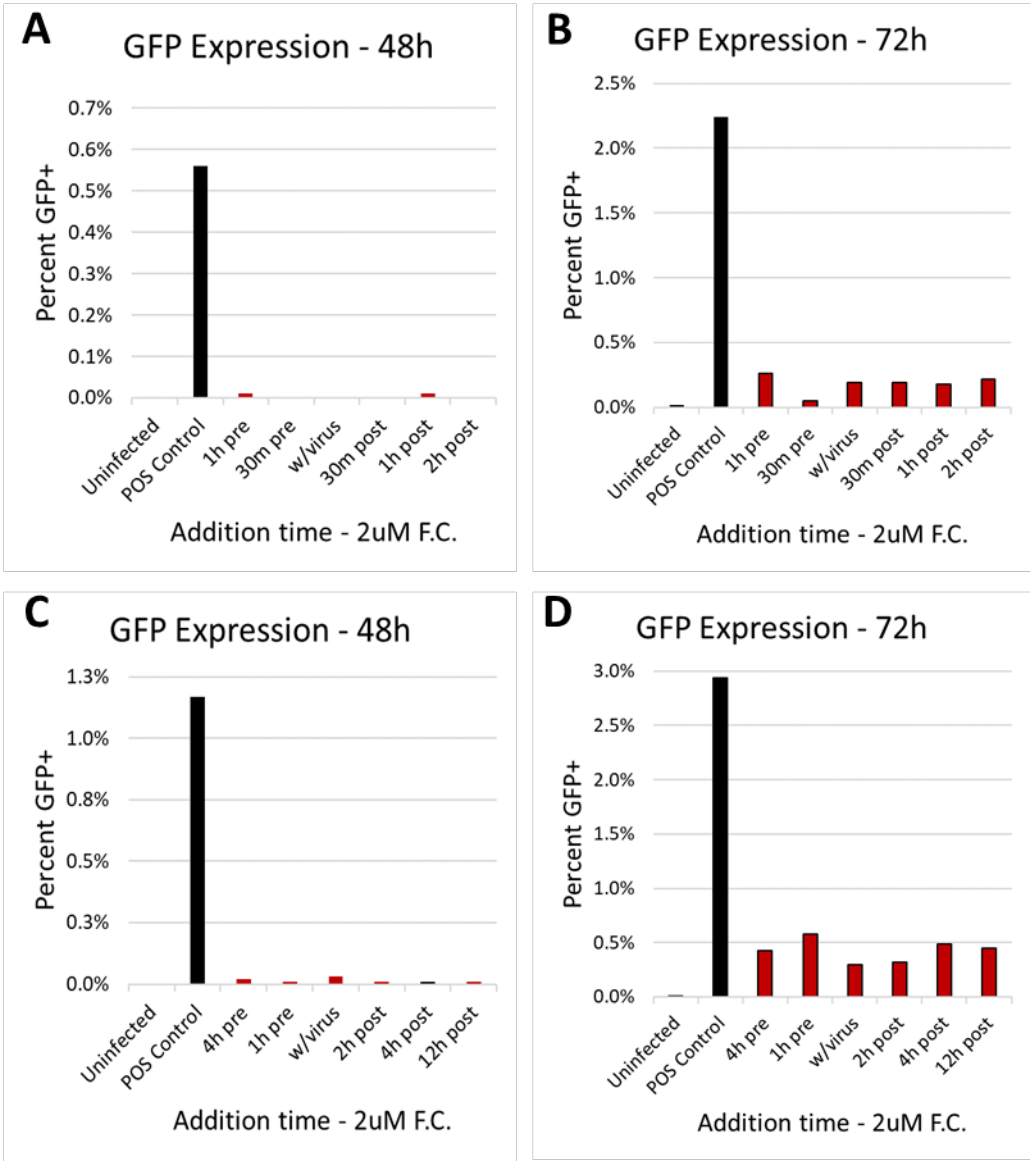

A
GFP Expression - 48h
Percent GFP+
0.7%
0.6%
0.5%
0.4%
0.3%
0.2%
0.1%
0.0%
Uninfected
POS Control
1h pre
30m pre
w/virus
30m post
1h post
2h post
Addition time - 2uM F.C.
B
GFP Expression - 72h
Percent GFP+
2.5%
2.0%
1.5%
1.0%
0.5%
0.0%
Uninfected
POS Control
1h pre
30m pre
w/virus
30m post
1h post
2h post
Addition time - 2uM F.C.
C
GFP Expression - 48h
Percent GFP+
1.3%
1.0%
0.8%
0.5%
0.3%
0.0%
Uninfected
POS Control
4h pre
1h pre
w/virus
2h post
4h post
12h post
Addition time - 2uM F.C.
D
GFP Expression - 72h
Percent GFP+
3.0%
2.5%
2.0%
1.5%
1.0%
0.5%
0.0%
Uninfected
POS Control
4h pre
1h pre
w/virus
2h post
4h post
12h post
Addition time - 2uM F.C.

Figure 17 - Treatment time pre- and post-infection does not alter inhibition of HIV-1. Healthy, growing, Rev-CEM-GFP-Luc cells were treated with tanespimycin at a final concentration of 2μM at the indicated time points, and incubated. Samples were taken for flow cytometry at 48h and 72h post-infection. *Panels A & B* show results from 1h before to 2h after infection at 48 and 72h. *Panels C & D* extended the pre-infection treatment to 4h and the post-infection treatment to 12h, results were obtained by flow cytometry at 48 and 72h.

Additional dosing does not increase inhibition of HIV-1

The mean half-life of tanespimycin in humans is 2.3 hours and it is processed to its metabolites through the liver (Gerson, Caimi, William, & Creger, 2018). As seen in Figure 14, applying only a single dose before infection is effective at suppressing HIV-1 driven GFP expression in this system. Figure 17 shows that a consistent concentration of tanespimycin, beginning at different time points surrounding infection, also is still effective at suppressing viral replication. Given this information, we wanted to determine if multiple doses of tanespimycin would result in enhanced inhibition. Based on the data presented in Figure 18, multiple dosing of cells does not appear to result in greater inhibition of HIV-driven GFP expression.

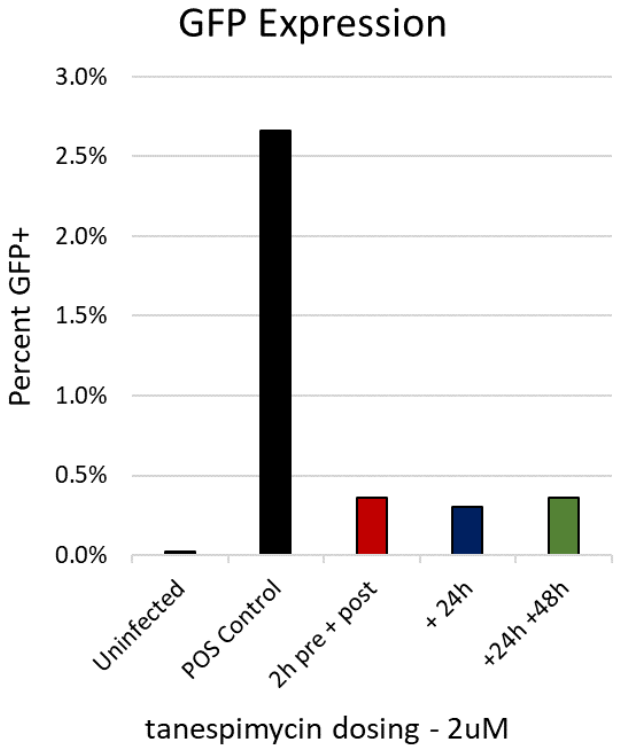


Figure 18 – A single dose of tanespimycin is sufficient to suppress replication.

Rev-CEM-GFP-Luc cells are pre-treated at a 2μM final concentration of tanespimycin for 2h pre-infection. Cells were infected with equal amounts HIV-1_{NL4-3} for two hours, washed and re-suspended in fresh media containing 2μM tanespimycin. Control cells were uninfected or treated with equal volume of DMSO alone.

Tanespimycin does not inhibit proviral DNA synthesis

Subsequent to the screening work to identify the potential of this compound, the mechanism of tanespimycin inhibition of HIV-1 was investigated by following the life cycle of the virus. The viral DNA was extracted from treated cells and qPCR was

performed to determine how tanespimycin treatment impacted its synthesis. Briefly, 10^6 CEM-SS cells were treated with 2 uM tanespimycin for 1 hour, infected with equal p24 of single-cycle HIV for 2h. Samples were harvested at the indicated time points, lysed and processed for both DNA and RNA extraction. qRT-PCR was performed on calculated equal ng input DNA and displayed as copy number per input ng DNA. The results of which are exhibited graphically in Figure 19. From these results we infer that tanespimycin does not inhibit the expression of pro-viral DNA produced as infection takes hold. In fact, tanespimycin treatment appears to enhance somewhat the total viral DNA synthesis in treated cells, and the levels consistently peak at 12h post infection as in the control.

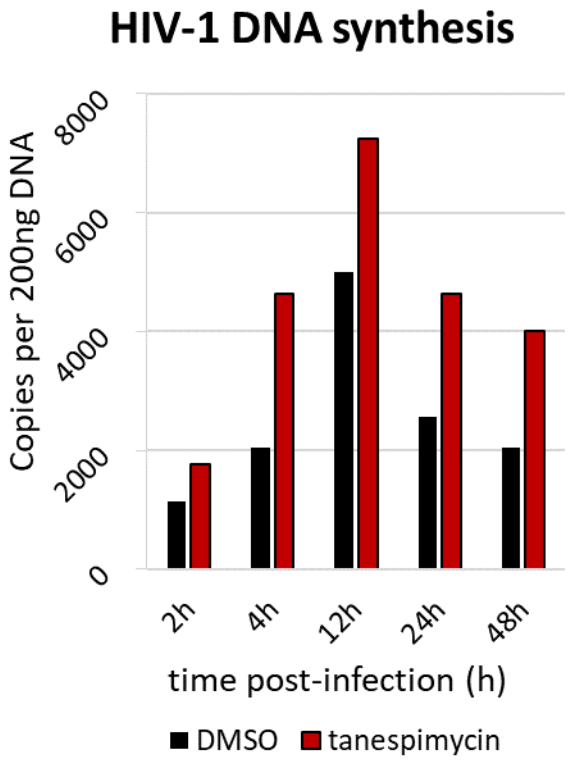


Figure 19 – Tanespimycin does not inhibit proviral DNA synthesis.

1 x 10^6 CEM-SS cells were treated with 2 uM tanespimycin for 1 hour, infected with equal p24 of single-cycle HIV for 2h. Samples were taken at the indicated time points, lysed and processed for both DNA and RNA extraction. qRT-PCR was performed on equal ng input DNA and displayed as copy number per 200 ng DNA.

Tanespimycin reduces 2-LTR circle DNA in the nucleus

The accumulation of 2-LTR circle DNA in the nucleus of the cell as a step in the lifecycle of HIV is an indicator of successful levels of nuclear import of the cDNA transcripts from the cytoplasm. The same nucleic acid isolates used to ascertain the total HIV-1 DNA copy number previously were then probed via qPCR for the levels of 2-LTR

circle accumulation at the corresponding time points. In this experiment (Figure 20) at first glance it appears that at 12h there is enhancement, 24h inhibition, and enhancement again at 48h. However when the levels of 2-LTR circles are then normalized against the copy number of total HIV-1 DNA, a pattern emerges. The ratio of 2-LTR circle to total DNA indicates that tanespimycin inhibits formation of that particular species across all time points.

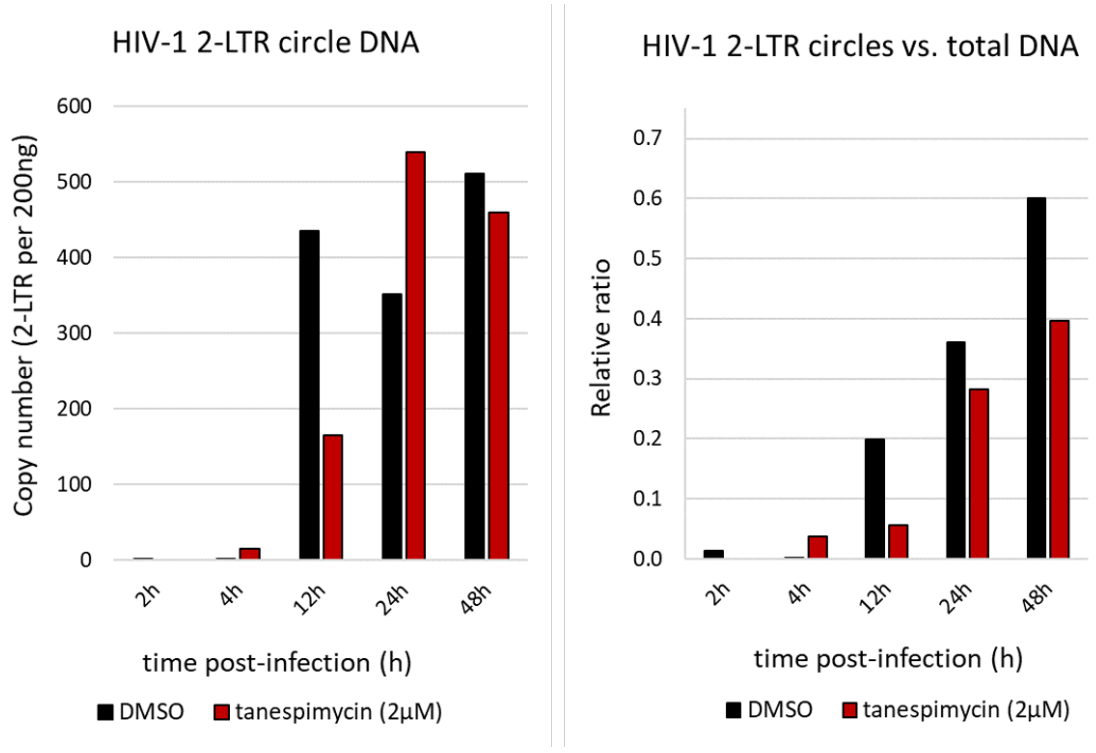


Figure 20 – Tanespimycin reduces 2 LTR circle DNA in the nucleus.

1 x 10^6 CEM-SS cells were treated with 2 uM tanespimycin for 1 hour, infected with equal p24 of single-cycle HIV for 2h. Samples were taken at the indicated time points, lysed and processed for both DNA and RNA extraction. qRT-PCR was performed on equal ng input DNA and displayed

as copy number per 200 ng DNA (left panel). The right panel is expressed as the ratio of 2-LTR to total HIV-1 DNA and reveals the reduction in formation of 2-LTR circles when normalized to total DNA. Representative results shown.

Tanespimycin inhibits generation of HIV-1 *nef* mRNA transcripts

Quantification of *nef* transcript numbers, in relation to normalized amounts of the total RNA complement of the cell, indicate that mRNA transcription is enhanced, inhibited or unaffected. In our experience, the levels of Nef transcription from 100 ng of cDNA was virtually undetectable, as expected, up until the 24h p.i. time point. At both 24 and 48 h p.i. tanespimycin exposure inhibited the levels of *nef* subsequently isolated from infected cells. Nef transcripts are decreased by roughly 60% in the tanespimycin treated cells vs. the DMSO control. This result was consistent in multiple experiments, but not a critical finding as it did not directly correlate to the level of impact the treatment had on HIV replication when measured by either p24 release or indicator production.

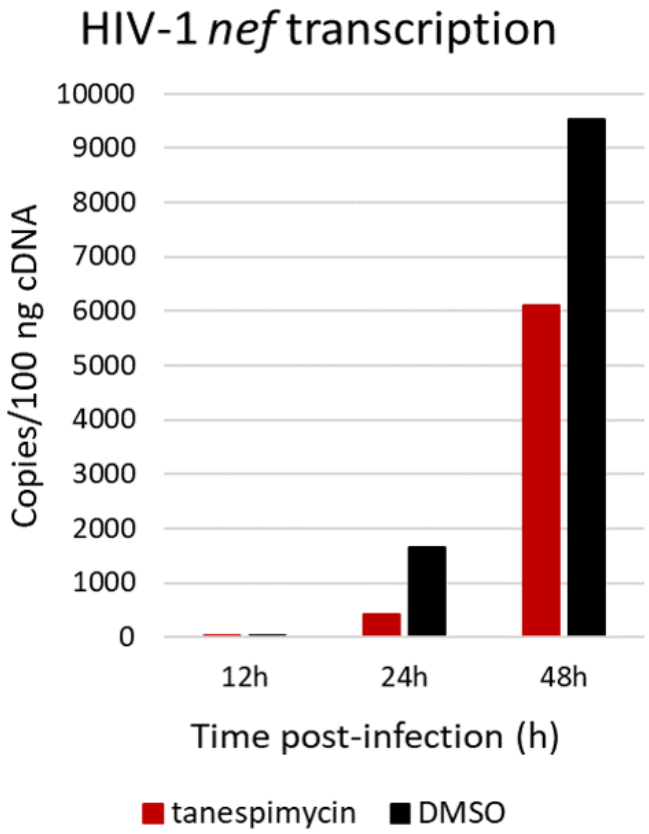


Figure 21 – Tanespimycin inhibits the production of HIV-1 nef transcripts.

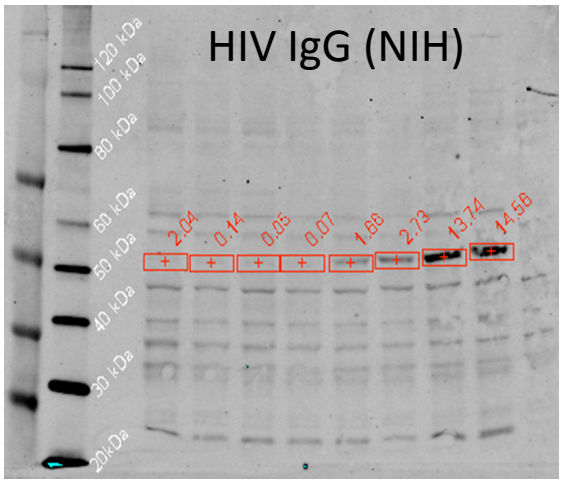
Isolated cDNA was generated by reverse transcription of RNA isolated from the same experimental set as the previously described data. 300 ng of mRNA were subject to reverse transcription with M-MLV RT (Ambion, TX) and then 100 ng cDNA was amplified for the presence of nef-specific transcripts by qRT-PCR.

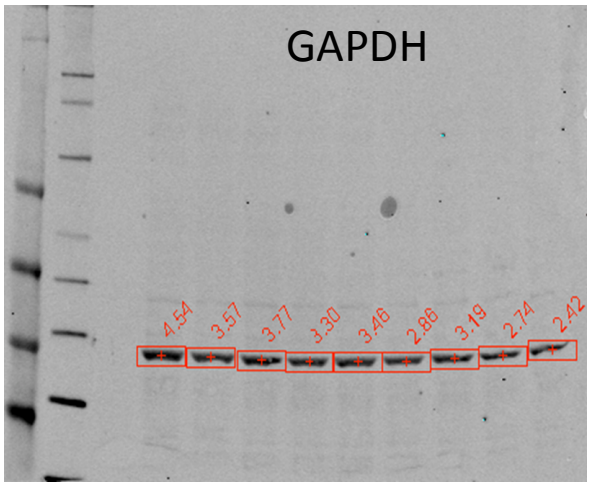
Section 6 : protein production levels

Subsequent to the nucleic acid and flow cytometry work, p24 ELISA and protein detection by Western blot of harvested, lysed cells indicated that treatment with tanespimycin resulted in a reduction of virus-specific proteins present.

Treatment of cells with tanespimycin lowers concentration of viral proteins

Relative levels of HIV-1 proteins generated in CEM-SS and in PBMCs whole cell lysates were determined by both Western blot and p24 release assays as described.





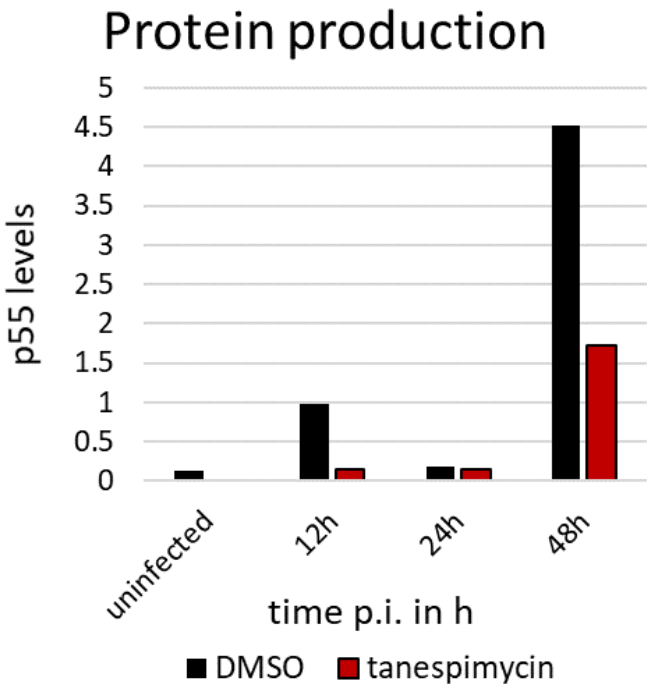


Figure 22 – Tanespimycin lowers viral protein levels in lysed cells.

Lysates generated from one million pelleted cells re-suspended in 1X LDS sample lysis buffer, run on SDS-PAGE gel and transferred to 0.45 µm pore nitrocellulose membrane. The membrane was probed using human HIV immunoglobulin and labeled goat anti-human antibodies then stripped and re-probed for GAPDH protein to use as a loading control. Normalized protein levels are indicated in the last figure.

There is a reduction in cell-bound pr55 of about 20% in the drug-treated cells (Figure 22, last panel) and, though not shown here, we probed the cell supernatant after concentration by centrifugation and it shows that there is about a 50% reduction of viral proteins on that blot. Taken together this information suggests that there should be lowered production of viral proteins within the cells and reduced release of nascent viral

particles from the infected cells when treated with tanespimycin. A further experiment, in which the supernatants from treated cells infected with a single cycle virus was also conducted to determine if our approach would yield usable information.

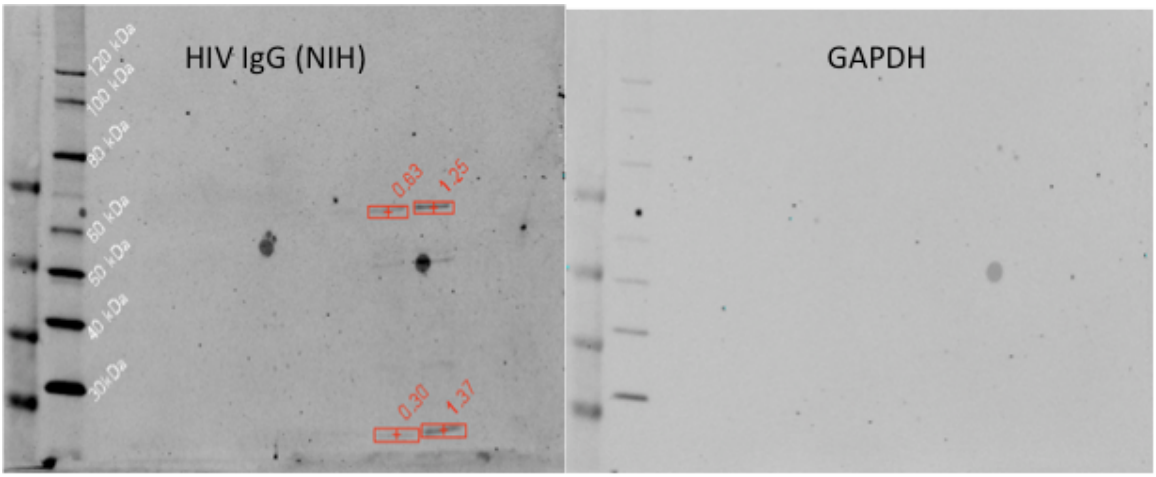


Figure 23 – Tanespimycin may reduce viral protein levels in supernatant.

Supernatant media generated from one million pelleted cells was re-suspended in 1X LDS sample lysis buffer, run on SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was probed using human HIV immunoglobulin and labeled goat anti-human antibodies then stripped and re-probed for GAPDH protein to use as a loading control. Normalized protein levels could not be calculated in this experiment due to the undetectable levels of GAPDH in the supernatant.

There was also an effort to determine the levels of viral p24 that were released during a longer-term infection process. Based on these results we deemed it likely that we would see the same type of reduction due to treatment of the cells with tanespimycin.

characterize the effect of treating virus-producing cells before transduction would provide further support for this observation.

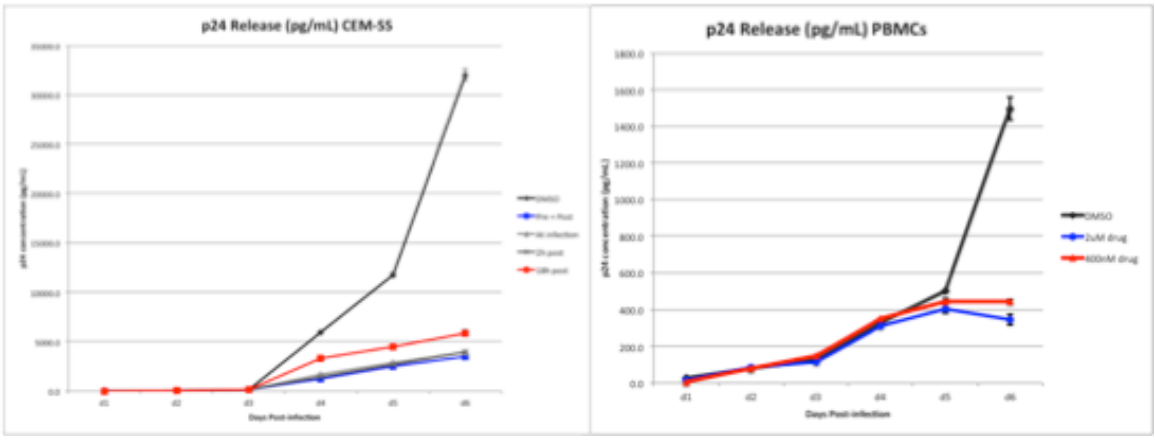


Figure 24 – Tanespimycin inhibits viral replication protein levels in vitro.

150µL of re-suspended cell culture was removed from infected cells, centrifuged and 135 µL of supernatant was removed and lysed with p24 lysis buffer. Released p24 was then assessed by modified sandwich ELISA protocol and the chromogenic reaction was read at A_{630} on a 96-well plate-capable spectrophotometer.

As shown in Figure 24 there is an impact on the replication of the virus when cells are treated with tanespimycin. Interestingly, even when the addition of the tanespimycin does not occur until 18 hours after exposure to virus there is still a significant reduction in the amount of nascent p24 released over the course of infection. Also there is little discrimination between the addition times clustered around the time of infection which is consistent with the experiments we did with the rev-dependent indicator cell line.

When the same experiment was repeated using donor-derived primary cells we see that the replication and release of viral p24 is also reduced in tanespimycin-treated cells. However, the pattern of reduction is somewhat different than in CEM-SS, the p24

levels are similar up until day 5 when viral replication increases in the control cell, but we see that in the tanespimycin-treated cells the virus is unable to reach such levels of production. Some of this difference may be due to the fact that primary cells will generally have lower levels of HSP90 and this could explain their enhanced sensitivity to its inhibition by drug treatment.

Section 8: Discussion

These data are evidence that there is a dose-dependent reduction of HIV-1 infection, both qualitatively and quantitatively, in cells when treated with tanespimycin. The indicator cells, when they are treated with the HSP90-specific inhibitor tanespimycin, produce a consistent pattern of reduced presence of the indicator gene products. HSP90 is a ubiquitous intracellular chaperone, often stabilizing nascent proteins and guiding their folding into final form. We verified that the lack of GFP expression in the treated cells was due to inhibition of HIV infection, and not simply because tanespimycin altered the function of the GFP product of the indicator construct.

Additionally, we have shown a decreased ability of HIV-1 permissive cells to generate a productive infection when treated with tanespimycin. At the point of viral entry, our data indicates that even if the viral particle does not initiate the signaling cascade and actin dynamics brought about by binding to the surface receptor CD4 and co-receptor CXCR4, tanespimycin still suppresses indicator production. This finding is further borne out by data using the mechanical technique spinoculation (Forestell, 1996) to stimulate cells treated with tanespimycin before and after infection. Guo et al showed that spinoculation of cells concurrent with HIV-1 infection consistently increased the

permissiveness of Rev-CEM indicator cells via dynamic actin rearrangement (Guo, 2011). Despite the power of this enhancement, tanespimycin treatment of spinoculated cells still resulted in a reduction of the percentage of cells successfully infected by greater than 90-fold at 48h (data not shown). This suggests the impact of tanespimycin/HSP90 inhibition to be largely independent of the enhancing activity generated during actin-dynamics.

In CEM-SS and primary cells, levels of released HIV-1 p24 antigen, was shown to be reduced with tanespimycin pre-treatment. It would stand to reason then that tanespimycin's apparent ability to inhibit the HIV-1 infection process indicates that HSP90 plays a role in that process.

CHAPTER SIX: FUTURE DIRECTIONS

Due to the hepatotoxicity of the parent drug, and the fact that this derivative is metabolized through that organ, it might be a more appropriate inhibitor for viruses that target liver cells if there is a similar viral assembly process occurring. Hepatitis viruses (B and C) cause a range of disease symptoms worldwide and are the most common risk factor for liver cancer as well as cirrhosis every year (WHO, 2013). A feature of hepatitis B that keeps it circulating is that infection can appear to resolve but, in a segment of patients, can result in chronic viral shedding. If the virus could be eliminated from this latent carrier status patient population, it could have a significant public health benefit. Hepatitis B-related complications contribute to the need for almost 7,000 liver transplants performed in the United States last year (Kim, 2015).

Evaluation of protein expression levels of HSP90 and HSP70 in cell line and any future animal models would be additive information. Assay to examine the extent to which activity of HSP90 is reduced. A feature of HSP90 inhibition that has been observed in multiple clinical trials of its inhibitors has been the concurrent increase in the expression and activity of HSP70 (Banerji, 2005) (Grem, 2005). Determination if this same pattern holds true in HIV-1 infection models would align this research with the existing body of pharmacological data for the compound.

This work looked at p24 levels present in both infected cells and in cell free form, suggesting that HSP90 activity influences the later stages of the HIV replication cycle to a much greater degree than the early steps of infection. Heat Shock Protein 70 has been found co-localized to the Gag/Pol polyprotein in HIV infected cells (Milev, 2012) and both HSP70 and HSP90 are upregulated in HIV-infected T cells (Rasheed, 2008). It would therefore not be surprising to discover that HSP90 is part of this complex. Further study to quantify the levels of individual HIV proteins at different stages and in what part of the cell there is a deviation from control HIV infections would be appropriate. There is also evidence that HSP70 associates with Gag and is incorporated into the membranes of the primate lentiviruses HIV-1, HIV-2, SIV(MAC), and SIV(AGM) (Gurer, 2002). Based on this, inhibition of HSP70 in viral culture of HIV-1 infected cells and then examination of the protein associations with Gag to determine if HSP90 fills that role during infection. This could be accomplished by use of other small-molecule inhibitors (PES-Cl) or by attempting knockout or knockdown of HSP70 in one of the models used in these studies.

Because there are multiple accessory proteins that assist with HSP90 interactions in the cytoplasm, there may also be specific accessory proteins that associate critically with the HIV infection cycle. The accessory proteins HSP40 and Tom70 could be interesting targets as they help stabilize the delivery of client proteins to complex with HSP90 in the case of the former, and in the case of the latter, assist in translocation of pre-proteins to the mitochondrial matrix (Hoter & al, 2018).

Investigating HSP90 inhibition by another method compared to tanespimycin could also indicate whether the inhibition of HIV documented here is reduced, possibly

by the counterbalance of HSP70 upregulation. The specific HSP90-inhibiting plant metabolite gedunin does not initiate a corresponding rise in HSP70 (Piaz, 2015). HIV-permissive cells treated with this small molecule should show even less replication than those treated with tanespimycin. Dual treatment and proteomic examination could indicate what pathways are vital in the absence of both of these HSPs. All of this information when taken in concert could inform the potential usefulness of tanespimycin as an adjunct treatment to current HAART therapies.

Because time of addition p24 shows that tanespimycin still has an intermediate suppressive effect in cells treated at 18h p.i., this indicates that HSP90 is critical to later stages of the HIV-1 replication cycle, most likely to the successful export of proteins (Gag-Pol) and possibly in the assembly of protein complexes and release of new viral particles from the cell. In order to demonstrate this conclusively, an experiment must be performed to show that, in 293T cells treated with tanespimycin and transfected with the pNL4-3 plasmid, generation of infectious HIV-1 is reduced. This proposed specific procedure is detailed below:

Transfection viral release assay:

Materials:

- 10 cm sterile culture dishes
- HEK 293T cells – seeded into 10 cm culture dish 24h before transfection. Seed 3.5 x 10^6 to each dish so that cells equal 90-95% confluence at the time of transfection.

- Purified plasmid DNA (need 24 μg per dish) – pNL4-3 (2 dishes, one will be treated, positive control will not) and pScrambled control plasmid.
- Transfection reagent, Lipofectamine 2000 (Invitrogen, cat #11668-027),
- DMEM cell culture medium with and without 10% Fetal Bovine Serum
- 100X penicillin/streptomycin

Procedure:

1. 24h before transfection, count cells and plate 3.5 x 10^6 per plate in 10mL antibiotic-free DMEM.
2. Next day: pre-warm 10 mls per plate of SF DMEM in a sterile, conical tube.
3. Dilute each DNA to be used in 1.5 ml of SF DMEM, mix gently.
4. Mix Lipofectamine 2000 gently before use, then dilute 180 μl in 4.5 mls SF DMEM. Incubate at room temperature for 5 minutes. Proceed to the next step within 25 minutes.
5. After 5 minutes incubation, combine the 1.5 ml diluted DNA with 1.5 ml diluted Lipofectamine. Mix gently and incubate for 20 minutes at room temperature. The resulting complexes are stable for up to 6h at room temperature.
6. Remove serum-containing media from cells and rinse very gently with ~4 mls SF DMEM, remove and discard. Add 5 mls fresh SF DMEM to each dish of cells.
7. Add the 3 mls of complexes to each dish drop-wise, very gently. Mix gently by rocking the plate back and forth several times.

8. Incubate at 37° C for 5h, then change the media to DMEM + 10% FBS & 1X pen/strep, 10 mls per plate.
9. Add tanespimycin to a final concentration of 400 nM to one of the HIV_{NL4-3}-containing plates and equal volume DMSO to control plate.
10. Incubate plates at 37° C for 48h
11. Harvest supernatant from the cells to a labeled 15 ml conical tube.
12. Centrifuge at 1,200 RPM for 5 minutes to pellet out cell debris. Transfer supernatant to a new sterile tube by filtering through a 0.45µm filter.
13. Aliquot harvested supernatant to sterile screw cap freezer tubes and store at -80°C.
14. Quantify the concentration of virus-associated p24 in harvested supernatants to use in characterization experiments.
15. Assess the infectivity of harvested viruses by infection of Rev-CEM-GFP-Luc cells with the resulting virus and quantification of GFP expression in the cells by flow cytometry.
16. Additionally, conduct a p24 ELISA on supernatants harvested from CEM-SS cells infected as described previously with virus from treated vs. untreated producer cells.

Expected results of the above experiments are that tanespimycin treatment of virus-producing cells will inhibit the assembly and release of new virus particles (as measured by p24 ELISA) so we should see reduced p24 concentration in treated cohorts. If there is

not a statistically significant reduction of p24 concentration, it will be necessary to test the viability of the released viruses. This should be accomplished both by use of the Rev-CEM-GFP-Luc cell line followed by flow cytometry. Additionally, replication competence should be assessed by infecting CEM-SS cells with treated cell-generated virus and determining, by another p24 ELISA, if produced virus is infectious and if it is capable of producing another generation of released viral particles.

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BIOGRAPHY

Sarah L. Learmonth Pierson graduated from Stephen F. Austin High School, Austin, Texas, in 1990. She received her Bachelor of Science in Microbiology from Texas Tech University, Lubbock, Texas in 1999. She was employed as a molecular laboratory technician at the United States Department of Agriculture, Agricultural Research Service's Cropping Systems Research Laboratory in Lubbock, Texas for two years and received her Master of Science in Biotechnology from Texas Tech University in 2003. After completion of her internship in the research and development laboratories of Avid Bioservices in Tustin, California she was commissioned as an officer in the United States Army. She began her Army career as the Chief of Virology and Immunology at Brooke Army Medical Center in Fort Sam Houston, Texas. She was then assigned overseas where she oversaw operations for the Microbiology and BSL-3 laboratories at the 121st Combat Support Hospital in Yongsan, South Korea. She returned to the U.S. as the Officer in Charge of the Endemic Disease and Biowarfare Threat Assessment section of the 1st Area Medical Laboratory, the Army's only forward deployable threat assessment laboratory. After her assignment to the 1st Area Medical Laboratory was completed, she was selected by the Army to attend the Long Term Health Education and Training program. Sarah entered the Biosciences PhD program at George Mason University with a concentration in Microbiology and Infectious Diseases investigating small molecule inhibitors of Human Immunodeficiency Virus (HIV) under Dr. Yuntao Wu, graduating in May, 2020. Her military awards include the Meritorious Service Medal, 2 Army Commendation Medals, The National Defense Service Medal, The Global War on Terrorism Service Medal, and Korean Defense Service Medal. Sarah currently holds the rank of Major in the Medical Service Corps. She is married to Lieutenant Colonel Tony Pierson, of Los Angeles, California, mother to their daughter, Amanda, of Bethesda, Maryland, and step-mother to Melissa, Emily, and Tyler Pierson, of San Antonio, Texas.