AN ABSTRACT OF THE DISSERTATION OF

Maciej Maselko for the degree of Doctor of Philosophy in Molecular and Cellular Biology presented on April 27, 2011
Title: Molecular Characterization of an HIV-1 Drug Target and Antiviral Compound Development.

Abstract Approved:

Manoj K. Pastey

The Human Immunodeficiency Virus -1 (HIV-1) has been the source of substantial human misery since it was first discovered in the early 1980s. Despite remarkable progress that has been made towards understanding HIV-1, there is no cure, no vaccine and life-prolonging therapies are beyond the reach of millions in need. Our research sought to both gain insight into potential therapeutic targets as well as the preclinical testing of drug candidates.

We demonstrate that a RhoA derived peptide is an effective inhibitor of HIV-1 entry. Although this peptide inhibits HIV-1 due to its poly-anionic nature, it nevertheless demonstrates that endogenous host proteins may be repurposed for novel therapeutic uses. We also characterized the mechanism and effectiveness of Basant, a polyherbal topical microbicide candidate for the

prevention of HIV-1 transmission. Our data demonstrate that it inhibits the entry of both CCR5 and CXCR4 tropic HIV-1 at non-toxic concentrations.

Finally, data is presented on the characterization of a novel HIV-1 protein expressed from an alternative reading frame of the HIV-1 polymerase gene. We demonstrate that this protein is localized to the nucleolus and is likely expressed from a novel HIV-1 transcript. This work lays the foundation for further studies to target this protein for drug development.

Molecular Characterization of an HIV-1 Drug Target and Antiviral Compound Development.

by
Maciej Maselko

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented April 27, 2011
Commencement June 2011

Doctor of Philosophy dissertation of Maciej Maselko presented on April 27, 2011

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I understand that my dissertation will become part of the permanent collection of Oregon State University Libraries. My signature below authorizes release of my dissertation to any reader upon request.

Maciej Maselko, Author

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to my family, friends, colleagues and mentors. None of this would have been possible without their support throughout the years.

CONTRIBUTION OF AUTHORS

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Chapter 1: General Introduction

The Human Immunodeficiency Virus -1 (HIV-1) has been the source of substantial human misery since it was first discovered in the early 1980s. Despite the remarkable progress that has been made towards understanding HIV-1 biology there is no cure, no vaccine and life prolonging therapies are beyond the reach of millions in need. It is crucial that those who research HIV-1 continue to investigate the fundamental mechanisms of HIV-1 pathogenesis in search of unknown viral targets and to further our understanding of host-virus interactions. Such research must also be balanced with investment into translational research where our incomplete understanding of HIV-1 can be used with urgency to address the humanitarian crisis that has unfolded in the last thirty years.

BIOLOGY OF THE HIV-1 LIFECYCLE

HIV-1 belongs to the *Lentivirus* genus of the Retroviridae family of viruses. At the center of the spherical HIV-1 virion lay two copies of a positive sense RNA genome packaged in the nucleocapsid (NC) protein and bound to the reverse transcriptase enzyme. Moving outward, the genome and its associated proteins are encased in a conically shaped protein shell made of the capsid (CA) protein. This viral core is further surrounded by the matrix (MA) protein

which links the core to a host cell derived lipid bilayer. Trimers of the transmembrane fusion protein (gp41) pass through this bilayer and are bound to the attachment proteins (gp120) that stud the surface of the virion (Acheson, 2007). See table 1.1 for a description of HIV-1 genes and proteins.

Attachment of HIV-1 to target cells is mediated by gp120 which binds to the CD4 receptor found primarily on T-cells as well as macrophages. This interaction leads to a conformational shift in gp120 that exposes a region that can then bind to a co-receptor. Typically, this co-receptor will be CCR5, however, it may also be CXCR4. The co-receptor preference designates the virus as either R5 or X4 tropic. Individuals homozygous for a CCR5 allele that has a 32bp deletion are resistant to R5 virus (Samson, et al., 1996). Upon co-receptor binding, gp41 is inserted into the cell membrane and catalyzes viral envelope-cell membrane fusion. This allows the viral core to enter into the cell cytoplasm (Levy, 2007).

Once inside, the viral RNA sheds its capsid protein coat and the single stranded viral RNA is converted to double stranded DNA. The reverse transcriptase (RT) enzyme utilizes a lysine tRNA as a primer to initiate this

process, resulting in a DNA/RNA hybrid molecule as the initial product. The RNAse H activity of RT degrades the RNA strand and then the DNA dependent DNA polymerase activity of RT generates the complementary DNA strand (Freed, 2001). During the process of reverse transcription the Viral Infectivity Factor protein (Vif), counteracts the action of APOBEC3G, a cellular cytidine deaminase whose activity leads to hypermutation of the HIV-1 genome (Goila-Gaur & Strebel, 2008).

The dsDNA viral genome is then imported across the nuclear membrane into the nucleus as part of a preintegration complex (PIC). This ability to enter the nucleus despite the presence of an intact nuclear membrane allows HIV-1 to infect non-dividing cells. In addition to the viral genome, the PIC contains the integrase enzyme (IN), MA, RT and viral protein R (Vpr) (Sherman & Greene, 2002). IN then inserts the viral DNA into the host genome by creating a two nucleotide 5' overhang at both ends of the viral genome and catalyzing a trans-esterification in which the 3'-OH attacks the phosphodiester backbone of the host DNA (Delelis, Carayon, Saib, Deprez, & Mouscadet, 2008). Although this integration is not sequence specific, it is targeted towards transcriptionally active regions (Marshall, et al., 2007; Schroder, et al., 2002).

Depending on the activation status of the infected cell, the proviral DNA may quiescently persist in the cell and be copied whenever the cell duplicates its own DNA. If, however, the cell is stimulated appropriately it will produce virus. The 5' region of the HIV-1 genome contains numerous promoter binding sites including those for NF-κB and NFAT which are excluded from the nucleus of resting T-cells (Colin & Van Lint, 2009).

Within an activated and infected cell, transcription of viral DNA is initially non-processive and results mostly in short non-coding transcripts. This situation is remedied by the presence of the HIV-1 protein Tat which is among the first proteins made from the few full length transcripts. Tat binds to a short hairpin structure found on the 5' end of nascent transcripts. Tat also recruits additional transcription factors that phosphorylate the C-terminal domain of RNA polymerase II which dramatically improves its processivity (Jeang, Xiao, & Rich, 1999).

Regulation of protein synthesis is accomplished by alternative splicing of the full genomic length transcript, however, there is some evidence of transcripts generated from transcription of the antisense strand (Carrera, Pinilla, Perez-Alvarez, & Thomson, 2010). The initial transcripts become fully spliced and encode Tat, Rev and Nef. As the concentration of Rev increases, it binds to

the rev response element (RRE) which is a stem loop structure present in the *env* gene region. Rev then facilitates the nuclear export of unspliced (coding for Gag & Pol) and partially spliced (coding for Env, Vpr, Vpu, Vif) HIV-1 mRNA (Suhasini & Reddy, 2009).

Assembly of HIV-1 virions can take place either at the cell membrane in T-cells (Ivanchenko, et al., 2009) or at poorly defined cytoplasmic compartments in macrophages (Orenstein, Meltzer, Phipps, & Gendelman, 1988). Targeting of assembly to the plasma membrane takes place via the cellular ESCRT pathway and the association of Gag and Gag-Pol with the membrane is mediated by the myristoylation of the N-terminus of Gag (Benaroch, Billard, Gaudin, Schindler, & Jouve, 2010). Genomic viral RNA reaches the site of virion assembly via binding of Gag to a packaging signal that is only present in unspliced genomic RNA (Harrison & Lever, 1992). The envelope protein peptides gp120 and gp41 reach the membrane through the secretory pathway and are generated by cleavage of their precursor protein, gp160 (Moulard & Decroly, 2000).

Budding from the cell membrane involves both the multimerization of Gag proteins associated with the membrane as well as the cell's ESCRT proteins which are usually involved in the formation of multivesicular bodies (Ganser-

Pornillos, Yeager, & Sundquist, 2008; Morita & Sundquist, 2004). However, before the virus can leave the cell surface it must overcome the host restriction protein Tetherin, which binds virions to the membrane. The HIV-1 accessory protein Vpu is thought to counteract Tetherin by inducing its degradation (Dube, Bego, Paquay, & Cohen, 2010). The final step in the viral lifecycle is maturation. The *pol* encoded Protease protein proteolytically cleaves the Gag and Gag-Pol polyproteins during or shortly after budding to form the mature, infectious virion (Adamson, Salzwedel, & Freed, 2009).

DISEASE PROGRESSION

Disease progression follows an established pattern that can be divided into acute phase, asymptomatic phase and AIDS (Acquired Immune Deficiency Syndrome) (figure 1.1). During the acute phase the adaptive immune system is yet to mount a competent defense and viral replication is very robust (Pilcher, et al., 2007). Once HIV-1 specific antibodies and $CD8^+$ T-cells are present in sufficient quantities, they provide some control over viral replication and viral titer decreases. This marks the entry into the asymptomatic phase which may last for several years.

The asymptomatic phase is a protracted battle between the immune system and virus. HIV-1 has a remarkable capacity to generate escape mutants to avoid immune suppression. The HIV-1 polymerase has an error rate of about 0.2 mutations per genome (Rambaut, Posada, Crandall, & Holmes, 2004). However, the integrated proviral genome is also replicated by the host's RNA Polymerase II which has an error rate of about one mutation per genome (Alberts, 2008). An infected individual can produce over 10^{10} virions per day (Perelson, Neumann, Markowitz, Leonard, & Ho, 1996).

The ultimate outcome of untreated HIV-1 infection is the progressive loss of $CD4^+$ T-cells leading to a profoundly immune-compromised state referred to as AIDS. Paradoxically, infected cells are only a small portion of the total number of $CD4^+$ T-cells and the majority of $CD4^+$ T-cell depletion results from apoptosis of uninfected cells via poorly understood mechanism mediated by gp41 (Finkel, et al., 1995; Garg, Joshi, Ye, Shankar, & Manjunath, 2011). There is also recent evidence to suggest that these cells may in fact be undergoing apoptosis prior to HIV-1 genome integration (Doitsh, et al., 2010). Once the $CD4^+$ T-cell population is depleted, the infected individual can no longer mount a competent adaptive immune response; death is frequently caused by opportunistic infections (Kaplan, et al., 2009).

SEXUAL TRANSMISSION OF HIV

It is estimated that there were 2.7 million new HIV-1 infections in 2009 (UNAIDS. & World Health Organization., 2009). The majority of new cases are acquired by heterosexual intercourse and of these, women are more likely than men to get infected (UNAIDS. & World Health Organization., 2008). In order for HIV-1 to be transmitted to a woman it must overcome the obstacles presented by the vaginal mucosa. This is a not a trivial task. Semen contains an average viral load of ~11,000 viral RNA copies per/ml (Pilcher, et al., 2007) yet the transmission risk per act of sexual intercourse (male to female) is only approximately .08% (Boily, et al., 2009).

The vagina has a thick epithelial lining with few cells susceptible to HIV-1. Its low pH environment is destabilizing to the virus and the mucus secreted can trap and expel virions. Furthermore, there are antiviral proteins and the production of hydrogen peroxide by normal flora (Haase, 2005). Virions are thought to breach the vaginal barrier in several ways. The first is by circumventing the barrier altogether via abrasions that commonly occur during intercourse (Shattock & Moore, 2003). Another favorable route is via inflamed or ulcerated mucosa due to pre-existing infections that greatly increase the risk beyond that stated above (Galvin & Cohen, 2004). Since HIV-1 infects both macrophages and $CD4^+$ T-cells; the recruitment of these cells during inflammation provides easy access for HIV-1.

The intact and healthy mucosa is not impervious to infection. Intraepithelial dendritic cells can bind virions and become productively infected or can transport virus to CD4+ T-cells (Pope & Haase, 2003). Although the epithelial cells cannot become infected they have been found to bind free virus. Whether these cells can trancytose bound virus to the lamina propria, which is rich in CD4+ T-cells, is disputed (Miller & Shattock, 2003), however, there is mounting evidence that this is a genuine path of infection (Bobardt, et al., 2007; Stoddard, et al., 2009).

TOPICAL MICROBICIDES

The vast majority of people infected with HIV-1 live in the developing world and sub-Saharan Africa in particular (UNAIDS. & World Health Organization., 2009). Even when antiviral drugs are given away for free, the travel costs to receive them can be prohibitively high (S. Rosen, Ketlhapile, Sanne, & DeSilva, 2007). Therefore, implementing effective prevention measures is critical. Preventing the sexual transmission of HIV-1 is mostly limited to the "ABC" approach of abstinence, being faithful and condom use (Collins, Coates, & Curran, 2008). This approach has several drawbacks. It is impossible to know for certain whether a sexual partner is engaged in monogamous behavior and condom use within the context of a supposedly monogamous relationship is often considered a sign of distrust. Furthermore,

cultural norms regarding the benefits of mixing of fluids, the refusal of some men to use condoms even when visiting prostitutes and the inability of women to negotiate for condom use are all factors that contribute to the limited success of the ABC model (East, Jackson, O'Brien, & Peters, 2011; Shannon & Csete, 2010; Varga, 1997).

Topical microbicides are compounds which when applied to the genital mucosa prevent the transmission of HIV-1 and (ideally) other sexually transmitted infections. By enabling a woman to apply a prophylactic substance to her vaginal mucosa before or shortly after intercourse, topical microbicides give women greater control over their safer sex practices. This is underscored by the desirability of microbicides that can be used covertly (R. K. Rosen, et al., 2008). Since the application of substances for a variety of purposes to the vagina is fairly common in some cultures, topical microbicides may also be more acceptable than condoms (Gafos, et al., 2010).

Despite the poor transmission efficiency and relatively delicate nature of the HIV-1 virion the development of a topical microbicide has been fraught with disappointment. One of the first topical microbicide candidates to be tested in a clinical trial was nonoxynol-9, a spermicidal detergent which was found to actually increase HIV-1 transmission because of irritation caused to the vaginal mucosa which led to the recruitment of susceptible cells (Van Damme,

et al., 2002). It is now recognized that an acceptable microbicide must have a number of properties beyond inactivation of virus including: gentle on the vaginal/rectal mucosa with repeated use, acceptable in appearance, odor and taste to the user, stable at temperatures encountered in the developing world, low cost, culturally tolerated, and compatible with latex (Balzarini & Van Damme, 2007). A recent clinical trial of a gel containing 1% of the reverse transcriptase inhibitor Tenofovir demonstrated a 54% reduction in HIV-1 seroconversion among women who used the gel for more then 80% of sexual encounters. This was the first and only successful field trial of a candidate microbicide to date (Abdool Karim, et al., 2010). This is encouraging, however, if a microbicide has a lower protective effect than condoms there is some concern that people who normally use condoms will switch to microbicides and increase their risk of transmitting or contracting HIV-1 (Foss, Vickerman, Heise, & Watts, 2003).

DISCUSSION

It has been nearly 30 years since the HIV-1 virus has been discovered. Despite a massive and pan-disciplinary research effort, there is little hope that a vaccine or cure will be developed within the next decade sparking calls for a return to basic research (Cohen, 2008; Senior, 2008). However, there have been remarkable successes in the battle against HIV-1. A person with access to antiretroviral therapy has a life expectancy approaching that of the

uninfected (Collaboration, 2008). The incidence of vertical transmission of HIV-1 from mother to child *in utero* or during breastfeeding have both been substantially mitigated (Thorne & Newell, 2003). There is also a decline in the number of new infections, with a greater than 25% drop in the incidence in many sub-Saharan nations between 2001 and 2009 (UNAIDS. & World Health Organization., 2010).

Our approach has been to investigate candidate therapeutics while simultaneously engaging in basic research to identify and characterize a novel target for the next generation of antiviral compounds. This thesis describes the products or our work.

Table 1.1. Description of HIV-1 Genes and Proteins.

Gene Name	mRNA Species	Protein Function
Group Specific Antigen (gag)	Full Length	Structural, synthesized as polyprotein. Targets viral assembly to cell membrane. Proteolytically cleaved post-budding as part of viral maturation into capsid, nucleocapsid, matrix and p6 peptides. (Benaroch, et al., 2010)
Polymerase (pol)	Full Length	Codes for viral enzymes. Expressed as a Gag-Pol fusion by ribosomal frameshift. Proteolytically cleaved post-budding into protease, reverse transcriptase and integrase.(Freed, 2001)
Envelope (env)	Partially Spliced	Transmembrane surface proteins gp120 and gp41 needed for viral attachment and entry. Synthesized as gp160 and cleaved by cellular proteases in the ER.(Moulard & Decroly, 2000)
Viral Infectivity Factor (vif)	Partially Spliced	Accessory protein. Packaged into virion. Counteracts cellular cytidine deaminase APOBEC3G. (Goila-Gaur & Strebel, 2008)
Viral Protein R (vpr)	Partially Spliced	Accessory protein. Promotes G2 arrest in infected cells. Boosts viral transcription. (Andersen, Le Rouzic, & Planelles, 2008)
Viral Protein U (vpu)	Partially Spliced	Accessory protein. Causes internalization and degradation of CD4 from cell surface. Counteracts the host protein tetherin to facilitate viral release from cell surface.(Dube, et al., 2010)
Regulator of Virion (rev)	Completely Spliced	Regulatory protein. Binds to rev response element in full length and partially spliced viral RNA to facilitate nuclear export.(Suhasini & Reddy, 2009)
Negative Effector (nef)	Completely Spliced	Accessory protein. Downregulates CD4, MHCI. Additional roles in infection poorly understood. (Foster & Garcia, 2008)
Transcriptional Activator (tat)	Completely Spliced	Regulatory protein. Binds to TAR stem loop structure on nascent viral RNA. Recruits cellular factors necessary for processive transcription. (Jeang, et al., 1999)

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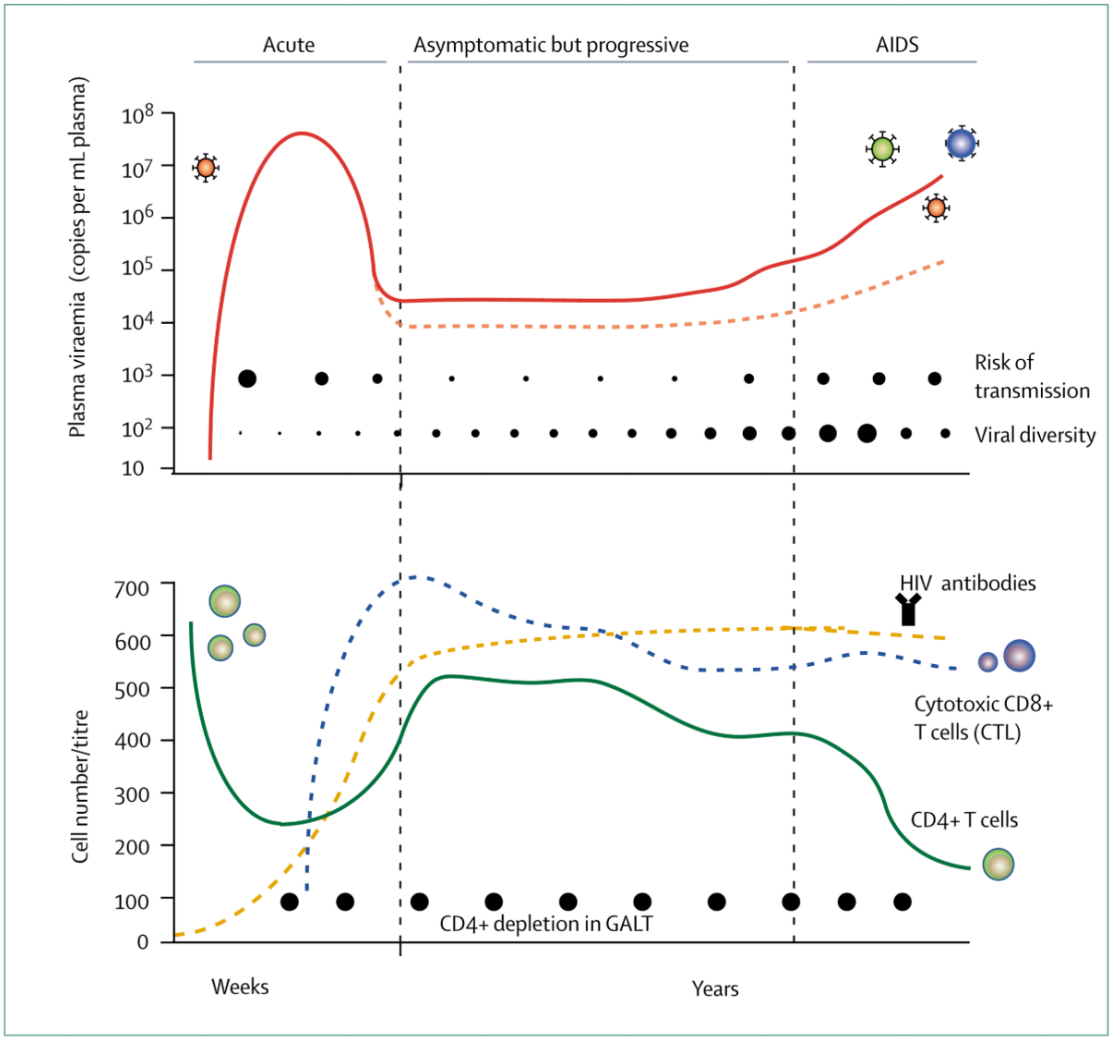

Figure 1.1 Viral and Immune Dynamics of HIV-1 Infection (Used with permission from (Simon, Ho, & Abdool Karim, 2006). HIV-1 infection can be divided into three phases: acute, asymptomatic but progressive and AIDS. During acute infection, viremia is high but is brought under control by the adaptive immune system to a "viral set point" that varies between individuals. Over time viral diversity increases as HIV-1 mutates to escape immune control. $CD4^+$ T-cell levels eventually drop to levels where there is pronounced immune-deficiency. Transmission risk is known to correlate with viral titer which is highest during acute infection and AIDS. Although there is some recovery to the circulating $CD4^+$ T-cell count after acute infection, $CD4^+$ T-cell levels in the GALT remain depressed.

Chapter 2:

A RhoA-Derived Peptide Inhibits Human Immunodeficiency Virus-1 Entry *in vitro*.

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Current HIV Research

Bentham Science Publishers Ltd.

P.O. Box 446

Oak Park, IL 60301-0446

USA

Volume 9, Number 1, January 2011

ABSTRACT

RhoA-derived peptides have been shown to have antiviral activity against both human respiratory syncytial virus and human parainfluenza virus-3. The present study investigates the toxicity, anti-HIV-1 activity and mechanism of action of a RhoA-derived peptide ($RhoA_{77-95}$) *in vitro*. The efficacy of this peptide was compared to a scrambled peptide of the same amino acid composition and Enfuvirtide, a HIV-1 entry inhibitor. Our data show that this RhoA-derived peptide is a non-toxic and effective inhibitor of a CXCR4 tropic strain of HIV-1. We also demonstrate that the mechanism of entry inhibition is likely mediated by polyanionic properties and is dependent on the dimerization of peptides.

INTRODUCTION

There have been approximately 25 drugs approved for use in the treatment of human immunodeficiency virus-1 (HIV-1) (De Clercq, 2009). Despite the presence of multiple drug combinations, the emergence of resistant viral strains remains a serious threat for which there is a constant need of additional treatment options. A small RhoA-derived peptide has been previously shown to have potent anti-viral activity against respiratory syncytial virus (RSV) and parainfluenza virus type-3 (PIV-3) (Pastey, Gower, Spearman, Crowe, & Graham, 2000). Such RhoA-derived peptides are being considered for development as antiviral therapeutics for RSV (Budge & Graham, 2004).

RhoA is a host protein that belongs to the Ras superfamily of small GTPases, and is important for the regulation of cytoskeletal dynamics (Wheeler & Ridley, 2004). An interaction between the envelope fusion (F) protein of RSV and RhoA was initially discovered by a yeast two-hybrid screen and subsequently confirmed by various means (Pastey, Crowe, & Graham, 1999). This interaction was mapped to amino acids 77-95 of RhoA. A short peptide composed of this sequence ($RhoA_{77-95}$, TDVILMCFSIDSPDSLENI) inhibited RSV and PIV-3 *in vitro.* $RhoA_{77-95}$ also exhibited strong antiviral activity in a mouse model of RSV infection (Pastey, et al., 2000). Since there is structural and functional homology between the HIV-1 fusion protein (gp41) and the RSV

F protein (Colman & Lawrence, 2003), we investigated if $RhoA_{77-95}$ also had activity against HIV-1 infection.

Initially, the antiviral activity of $RhoA_{77-95}$ was attributed to its ability to interfere with a putative transmembrane interaction between viral fusion peptides and host RhoA. However, it was found that the anti-RSV activity of $RhoA_{77-95}$ is dependent on its dimerization via disulfide bonds which can form by oxidation during freeze-thawing (Budge, Lebowitz, & Graham, 2003). We found that oxidation also dramatically improved the anti-HIV activity of $RhoA_{77-95.}$ The primary rationale for this study was to determine if $RhoA_{77-95}$ has anti-HIV-1 activity *in vitro.* Once this was established we investigated some of the properties that contribute to its activity by comparing inhibition to a scrambled sequence peptide ($RhoA_{scrmbl}$). We determined which stage of the viral lifecycle $RhoA_{77-95}$ interferes with and compared it to the FDA approved entry inhibitor, Enfuvirtide (Manfredi & Sabbatani, 2006).

MATERIALS AND METHODS

Cells. TZM-bl and Ghost (GHOST (3) R3/X4/R5) and Ghost X4(GHOST (3) X4) cells were obtained from the NIH AIDS reference and reagent program. Cells were grown in Dulbecco's Minimum Essential Media (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% Fetal bovine serum (FBS) with or

without 100U/mL penicillin G, 100µg/mL streptomycin, and 0.25µg/mL amphotericin B. Cells were grown at 37°C with 5% CO_2.

Virus. HIV_{4-3} virus stocks were generated by transfecting Ghost cells with pNL4-3 plasmid (NIH AIDS reference and reagent program) using Turbofectin 8.0 (Origene, Rockville, MD). After 5 days post transfection, the media was collected, centrifuged briefly to pellet any cell debris, aliquoted and stored at -80°C.

Peptides. Enfuvirtide (Trimeris/Roche T-20, Fusion Inhibitor, N-acetylated derivative) was obtained from NIH AIDS reference and reagent program. $RhoA_{77-95}$ (TDVILMCFSIDSPDSLENI) and a control peptide HA (PKSSWSDHEASSGV) were obtained from Biomatik (Wilminton, DE). A scrambled version of $RhoA_{77-95}$, $RhoA_{scrmbl}$, (DDMSVISELICTSPLDFIN) was obtained from Sigma (St Louis, MO). All peptides were dissolved in PBS. $RhoA_{77-95}$ and $RhoA_{scrmbl}$ were subject to five freeze-thaw rounds prior to use.

Toxicity Assay .TZM-bl cells were plated at a density of 25,000 cells per well in a 96-well plate. The following day the media was removed and each well received 3.125 to 50µM of either $RhoA_{77-95}$ or $RhoA_{scrmbl,}$ and 62.5nM to 1µM of Enfuvirtide in 10µL dissolved in Dulbecco's PBS (DPBS; Invitrogen) or DPBS control, and then 90µL of fresh media. Each condition was performed in

triplicate. After 48hrs, the cells were assayed for viability using the Cell Titer Blue assay (Promega, Madison, WI) according to manufacturer instructions.

HIV-1 cytopathicity inhibition assay. Ghost X4 cells seeded in a 12-well plate were infected with HIV_{4-3} virus and concomitantly treated with 10μM of $RhoA_{77-95}$ or $RhoA_{scrmbl}$ dissolved in DPBS, or DPBS control. The next day, the media was replaced with 1mL of DMEM supplemented with 10% FBS, 100U/mL penicillin G, 100μg/mL streptomycin, and 0.25μg/mL amphotericin B. Each condition was performed in triplicate. After five days, the cells were examined for syncytia formation.

Time Series To evaluate drug inhibition dynamics, 96-well cell culture plates were seeded with 25,000 TZM-bl cells per well in 100μL media. The next day the media was replaced with stock virus diluted in 95μL media. Immediately thereafter and at 1, 2, 4, and 6hrs post-infection 5μl of treatment compound or delivery control was added in triplicate. After 48hrs β-galactosidase activity was measured using Pierce's Mammalian β-galactosidase assay kit (Thermo Fisher Scientific, Rockford, IL) by spectrophotometer (Ultramark. Biorad. Hercules, CA) at an optical density of 405nm. The compound treated β-galactosidase activity data was analyzed by two-way ANOVA with Bonferroni post tests. The data from the media replacement experiments was analyzed by one-way ANOVA and Tukey's post-test.

Establishment and comparison of IC_{50} data. Cell culture 96-well plates were seeded with 25,000 TZM-bl cells per well in 100μL media. The next day the media was replaced with 90μL of media containing virus. The wells then received varying concentration of antiviral compound in 10μL of PBS or control. A standard curve of virus in culture media containing 10% DPBS was included to calculate inhibition based on β-galactosidase activity. Three replicates were included for each point in the standard curve. The concentration of drug necessary to reduce the viral replication by 50% was then interpolated from the standard curve.

Statistical Analysis All statistical analysis was performed using Graphpad Prism 5.03 software.

RESULTS AND DISCUSSION

$RhoA_{77-95}$ and $RhoA_{scrmbl}$ are non-toxic and inhibit HIV-1 syncytia formation

The Cell Titer Blue assay was used to investigate the toxicity of $RhoA_{77-95}$ and $RhoA_{scrmbl}$ as compared to Enfuvirtide, which is an anti-HIV-1 peptide currently in clinical use. TZM-bl cells were treated with drug concentrations ranging from 3.125 to 50μM of either $RhoA_{77-95}$ or $RhoA_{scrmbl,}$ and 62.5nM to 1μM of Enfuvirtide. None of the compounds demonstrated any cytotoxicity even at their highest concentrations (Fig 2.1).

HIV_{4-3} utilizes the CXCR4 co-receptor for infection and is known to be more cytopathic *in vitro* than CCR5 co-receptor HIV-1 virus (Valentin, Trivedi, Lu, Kostrikis, & Pavlakis, 2000). Cytopathicity manifests as multinuclear syncytia formed by cellular fusion. We infected CXCR4 expressing Ghost cells and concomitantly treated them with 10μM of $RhoA_{77-95}$, $RhoA_{scrmbl}$, or PBS delivery control. After five days the cells were examined for virus-induced syncytia (Fig. 2.2). There was no evidence of syncytia in the $RhoA_{77-95}$ treated-infected wells. Although, the $RhoA_{scrmbl}$ treatment did not appear as effective as $RhoA_{77-95}$, there was very little syncytia formation. This demonstrated that both peptides effectively inhibit HIV-1 and supports prior work that indicated antiviral activity is due to the polyanionic nature of the peptide rather than any competitive interference of a fusion protein and cellular RhoA (Budge, Li, Beeler, & Graham, 2004).

<u>$RhoA_{77-95}$ inhibition of HIV-1 is improved by freeze-thawing but is not entirely dependent on charge.</u>

Budge *et al.*(Budge, et al., 2003) demonstrated that the inhibition of RSV by $RhoA_{77-95}$ was greatly improved by peptide dimerization which takes place via disulfide bonds formed by oxidation during freeze-thaw cycles. We subjected $RhoA_{77-95}$ and $RhoA_{scrmbl}$ to five freeze-thaw cycles before infecting TZM-bl

cells, which express β-galactosidase in the presence of the HIV-1 Tat protein. We found that this freeze-thaw enhancement of antiviral activity also holds for HIV-1 (Fig. 2.3A).

Since both $RhoA_{77-95}$ and $RhoA_{scrmbl}$ were able to inhibit syncytia formation we obtained their IC_{50} values to determine if the charge of the peptide is the only determinate of inhibition. We also compared the IC_{50} values to Enfuvirtide. Cells were infected and immediately treated with the same concentrations as used in the toxicity study above. After 48hrs we assayed for β-galactosidase activity (Fig 2.3B). The calculated IC_{50} values and 95% confidence intervals from this experiment are 7.8 (6.1-11.0) μM for $RhoA_{77-95,}$ 27.0 (24.7-30.4) μM for $RhoA_{scrmbl}$ and 550 (528-572) nM for Enfuvirtide. Although Enfuvirtide is clearly the most potent inhibitor, $RhoA_{77-95}$ is more effective inhibitor of HIV compared to $RhoA_{scrmbl}$. The observed difference between $RhoA_{77-95}$ and $RhoA_{scrmbl}$ may be due to differences in their ability to form dimers as suggested by Budge *et al.* (Budge, et al., 2003) or the presence of a motif in $RhoA_{77-95}$ that allows for better interaction with the virus. In any case, this data suggests that the charge of the peptide alone cannot entirely explain the inhibitory effect of $RhoA_{77-95.}$ A separate experiment was conducted with an unrelated control peptide HA (PKSSWSDHEASSGV) which had no effect on HIV-1 infection (Fig 2.3C).

RhoA$_{77-95}$ inhibits HIV-1 entry

Since polyanionic compounds act as entry inhibitors (Moulard, et al., 2000) we hypothesized that this may be RhoA$_{77-95}$'s mechanism of action as well. A time series experiment was performed in which cells were treated with RhoA$_{77-95}$, Enfuvirtide or PBS at multiple time points post infection. By comparing the inhibition dynamics of RhoA$_{77-95}$ to Enfuvirtide, a known fusion inhibitor (Manfredi & Sabbatani, 2006), we can determine whether RhoA$_{77-95}$ inhibits HIV-1 before or after it enters the cell. The results show that RhoA$_{77-95}$ had an inhibitory effect when used to treat cells up to 2hrs post infection and Enfuvirtide was effective up to 4hrs post infection (Fig. 2.4A). This indicates that RhoA$_{77-95}$ acts at a pre-entry stage. In order to further characterize the inhibition mechanism we also pretreated virus prior to infection and were able to demonstrate that RhoA$_{77-95}$ efficiently neutralizes cell free virus (Fig 2.4B).

In this study we have demonstrated that RhoA$_{77-95}$ is a non-toxic and effective inhibitor of CXCR4 tropic HIV-1 in a cell culture model. RhoA$_{77-95}$ appears to inhibit HIV-1 infection prior to entry. These findings broaden the antiviral scope of RhoA$_{77-95}$. The inability of RhoA$_{scrmbl}$ to inhibit HIV-1 with the same efficacy as RhoA$_{77-95}$ argues that the observed effect is dependent on more than just the charge of the peptide; however, it is also clear that the dimerization that occurs during freeze-thaw cycles is necessary for efficient inhibition. This further supports the idea that the anti-viral activity is not based on the

interference of a necessary interaction between HIV and cellular RhoA as demonstrated in prior work (Budge, et al., 2003; Budge, et al., 2004). Nevertheless, investigating host derived compounds that have the necessary chemical characteristics to be of therapeutic value, even if not biologically predicted to have such activity may be fruitful. As a host derived peptide, $RhoA_{77-95}$ poses little risk of an adverse immune response. Our future work will focus testing if $RhoA_{77-95}$ can inhibit HIV-1 in a mouse model of infection as a topical microbicide and if we can engineer endogenous vaginal flora to secrete this peptide *in situ.*

ACKNOWLEDGEMENTS

We would like to thank Brittany Sterling for technical assistance and Kelsi Sandoz for critical reading of the manuscript. We would also thank NIH AIDS reference and reagent program for providing TZM-bl, Ghost (GHOST (3) R3/X4/R5) and Ghost X4(GHOST (3) X4) cells, pNL4-3 HIV-1 plasmid and Enfuvirtide drug.

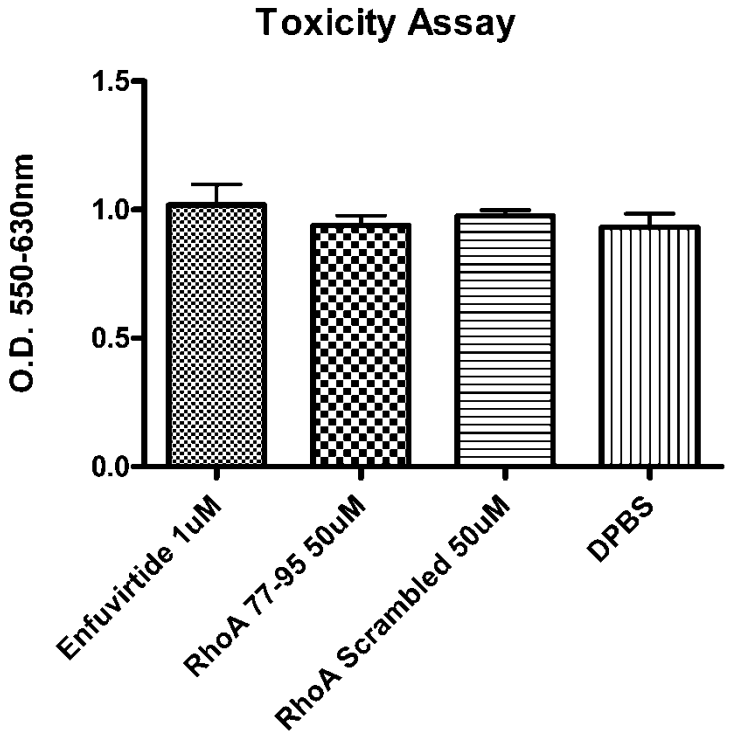


Figure 2.1. Toxicity Assay: Antiviral peptides are non-toxic *in vitro* at the highest levels tested. Cells were treated with peptides or DPBS delivery control and after 48hrs the cells were assayed for viability according to manufacturer instructions using the Cell Titer Blue assay. A one-way ANOVA revealed no significant differences ($p>.05$) Error bars represent S.E.M. n=3

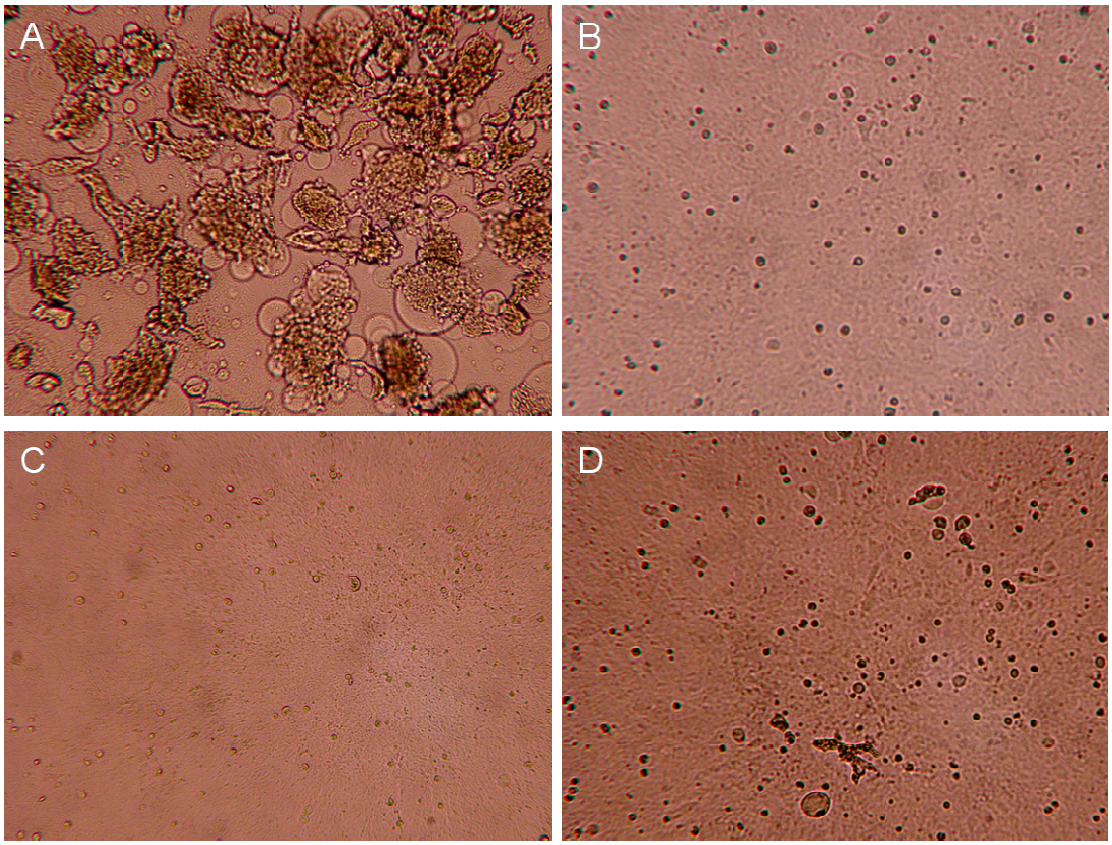


Figure 2.2. $RhoA_{77-95}$. and $RhoA_{scrmbl}$ inhibit HIV-1 in Ghost cells. Cells were concomitantly given virus and 10μM peptide drug. Photographs were taken 5 days post infection with HIV-1 at 200X magnification. **A.** HIV-infected cells treated only with DPBS delivery control exhibiting characteristic syncytia. **B.** Uninfected cells. **C.** HIV-infected, $RhoA_{77-95}$ treated cells showing no syncytia. **D.** HIV-infected, $RhoA_{scrmbl}$ treated cells showed little syncytia.

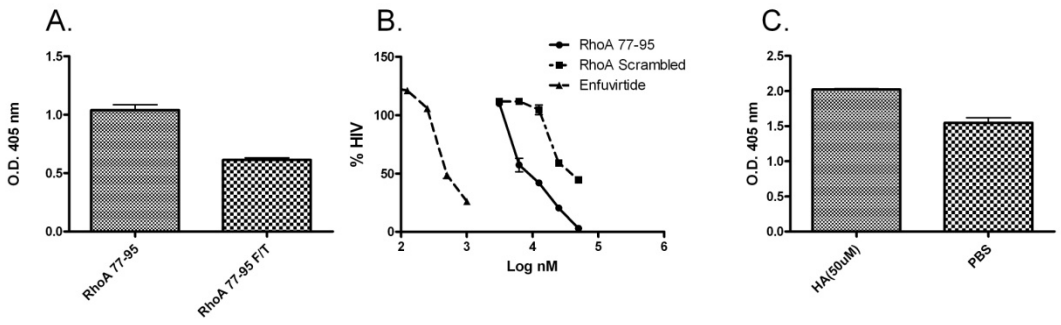


Figure 2.3. TZM-bl cell β-galactosidase assay of HIV-1 inhibition by antiviral peptides. A. HIV-1 infected cells were treated with 3.5μM of $RhoA_{77-95}$ that was prepared and frozen once or freeze-thawed five times (F/T) to cause the formation of peptide dimers. The freeze-thawed peptide performed significantly better ($p<0.001$) as determined by unpaired, two-tailed t-test. **B.** TZM-bl cells were infected with HIV-1 and treated with a range of concentrations of Enfuvirtide, $RhoA_{77-95}$, or $RhoA_{scrmbl}$. β-galactosidase activity was assayed 48hrs later and HIV-1 inhibition was determined from a standard curve. The IC_{50} value for each peptide was calculated (see text).**C.** HA peptide control does not inhibit HIV-1. All experiments performed with three replicates.

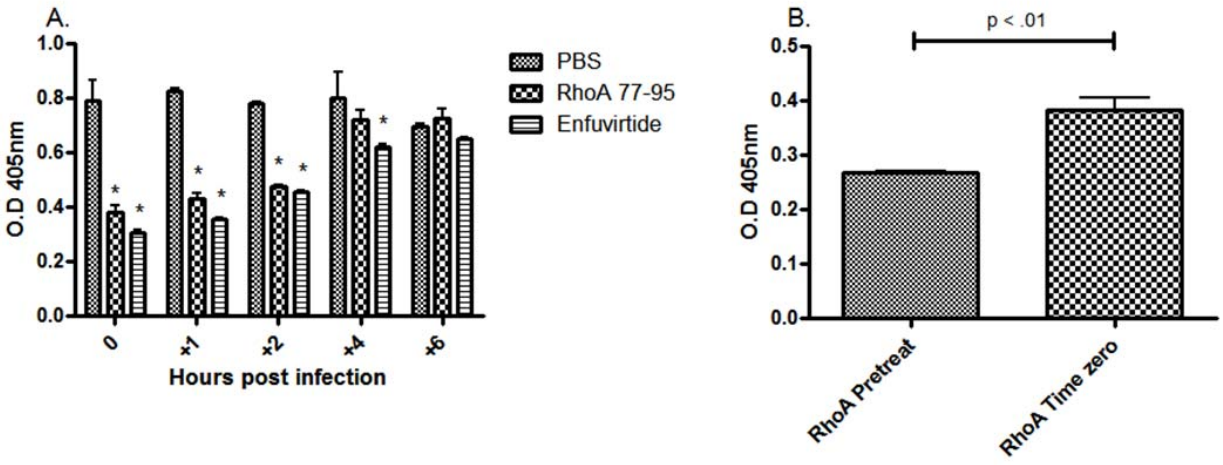


Figure 2. 4. Inhibition of virus before and after exposure to cells. A. TZM-bl cells were treated with 10μM $RhoA_{77-95}$, or $RhoA_{scrmbl}$ or .5μM Enfuvirtide or PBS at multiple time points post infection to investigate which stage of infection is inhibited. After 48hr cells were assayed for β-galactosidase expression. Data was analyzed by two-way ANOVA and Bonferroni posttest. Asterisks denote significant difference ($p < .05$) compared to PBS treatment. $RhoA_{77-95}$ inhibits virus when added up to two hours post infection. Enfuvirtide is effective up to four hours post infection. **B.** Cell free virus in culture media was pretreated with $RhoA_{77-95}$ for 1.5hr at 37°C before infecting cells or concomitantly treated upon infection at time zero. Pretreating HIV-1 with $RhoA_{77-95}$ increases viral inhibition. Data was analyzed by two-tailed t-test.PBS treatments showed no difference between time points. Both experiments were performed with three replicates.

Chapter 3:

Basant, a polyherbal topical microbicide candidate inhibits both CCR5 and CXCR4 tropic Human Immunodeficiency Virus-1 infection *in vitro* by interfering with viral entry.

Maciej B. Maselko, Rupali S. Joshi, Smita Kulkarni, G.P. Talwar and Manoj K. Pastey

ABSTRACT

Basant is a polyherbal topical microbicide candidate with activity against CXCR4 tropic HIV-1 as well as fungal and bacterial sexually transmitted infections. The current study confirms that Basant is effective against HIV-1_{NL4-3} and extends its activity to the CXCR4 tropic HIV-1_{UG070} and CCR5 tropic HIV-$1_{Ba-L,}$ HIV-1_{VB59} and HIV-$1_{96USNG58}$ strains demonstrating that it is effective against both lab strains and primary isolates regardless of co-receptor use. We also investigated the kinetics of Basant inhibition as compared to the fusion inhibitor Enfuvirtide and report that Basant's primary mechanism of HIV-1 inhibition is interfering with the viral lifecycle at a pre-entry stage.

INTRODUCTION

In the absence of an effective vaccine or prophylactic measure that can be used at the discretion of women, there has been a significant effort towards the development of a topical microbicide that can prevent Human Immunodeficiency Virus-1 (HIV-1) transmission. Despite multiple disappointments, a recent clinical trial of a gel containing 1% Tenofovir (Karim, et al.) has yielded favorable results and demonstrated that this goal of an effective microbicide is attainable.

Basant is a promising poly-herbal topical microbicide candidate. Basant is formulated with diferuloylmethane (curcumin),purified extracts of *Emblica officinalis* (Amla), saponins from *Sapindus mukorossi, Aloe vera* and rose water. The excipient is composed of glycerol, PEG-400, alginic acid, xanthan gum, lactic acid, citric acid, potassium-sodium tartrate and benzoic acid buffered at a pH of 3.5. Basant has potent activity against, gonorrhea, Candida *spp.*, HPV16 and CXCR4 tropic HIV-1 (Talwar, et al., 2008). It inhibits both preinfection as well as post infection *Chlamydia trachomatis* (cell free bacteria as well as in infected cells) (Bhengraj, Dar, Talwar, & Mittal, 2008). Basant also has spermicidal activity (G.P. Talwar, unpublished data). After a preclinical toxicology study in rabbit vagina, as per USFDA recommended protocol, Basant has successfully undergone a Phase I clinical safety trial in 30 women of reproductive age with due permission of The Drugs Controller

general of India and Institutional Ethics Committee. It is currently in Phase II b therapeutic trials in women at early stage of cervical dysplasia, molecularly positive for HPV 16/18.

A previous report demonstrated that Basant is an effective inhibitor of HIV-1_{NL4-3} and HIV-1_{IIIB} virus (Talwar, et al., 2008). Both of these strains predominately utilize the CXCR4 co-receptor. The vast majority of sexually transmitted cases of HIV-1, however, are the result of the transmission of HIV-1 virions that preferentially utilize the CCR5 co-receptor (Haase, 2005). Since Basant is being developed as a topical microbicide, this study demonstrated that it is also effective against CCR5 tropic HIV-1. The current study also demonstrates the effectiveness of Basant against primary isolates of HIV-1. Finally, we investigate the kinetics of HIV-1 inhibition by Basant in comparison to Enfuvirtide, to establish that Basant's anti-HIV-1 effect can be primarily attributed to its interference with a pre-entry step in the viral lifecycle.

MATERIALS AND METHODS

Viral stocks and cell lines

HIV-1_{Ba-L}, HIV-$1_{96USNG58}$, HIV-1_{UG070}, HIV-1_{VB59}, pNL4-3 plasmid , TZM-bl cells which express β-galactosidase activity in an HIV-1 Tat dependent manner and PM-1 cells were all obtained from the NIH AIDS Research & Reference reagent program (Germantown, MD).

Treatment Compounds

Basant cream was formulated as described in (Talwar, et al., 2008). Enfuvirtide was obtained from the NIH AIDS Research & Reference Program.

Toxicity assay

A 96-well plate was seeded with 2.5×10^4 TZM-bl cells per well. The next day the media was replaced with 5µL of a 50:50 dilution series of KY jelly or Basant in DPBS and 95µL of fresh culture media. After two days, toxicity was assayed using Promega's Cell Titer Blue assay (Promega, Madison, WI) and an Ultramark spectrophotometer (Biorad, Hercules, CA). For PM-1 cells, CC_{50} was determined using the trypan blue cytotoxicity assay after a five day incubation with Basant or KY jelly. The duration of the toxicity assays were identical to the HIV-1 inhibition assays.

TZM-bl assay for HIV-1 inhibition

A 96-well plate was seeded with 2.5×10^4 TZM-bl cells per well. The next day the medium was replaced with 95µLof HIV-1 stock in culture media. Each well was immediately treated with 5µLof Basant or KY jelly diluted in DPBS (Invitrogen, Carlsbad, CA). An HIV-1 standard curve with each viral strain was made by performing a 2:1 dilution series of virus in cell growth media with 5% DPBS. All conditions were performed in triplicate. After two days, cells were

assayed for β-galactosidase activity using Pierce's Mammalian β-gal assay kit (Rockford, IL) by measuring absorbance at 405 nm. IC_{50} was determined by curve fitting the inhibition data to the standard curve followed by interpolation.

PM-1 assay for inhibition

For cell free virus inhibition, sub toxic concentrations of Basant or KY jelly were incubated with primary isolates of CXCR4 tropic HIV-1_{UG070} and CCR5 tropic HIV-1_{VB59}. The virus-drug mixture was then added to PM-1 cells. After five days, the inhibition of virus growth was monitored by p24 antigen and compared with the virus growth in the absence of drug. For cell to cell virus inhibition, cells were first infected overnight with virus, and then washed three times with PBS prior to treatment.

Time Series Experiments

TZM-bl cells were infected as above with HIV-1_{NL4-3} and then treated at multiple time points post-infection with final concentrations of 0.1% for Basant and KY jelly and 2.5μg/mL for Enfuvirtide (obtained from the NIH AIDS Research & Reference Program) delivered in 5μL. Cell pretreatment experiments were performed with 5μL of drug in 95μLgrowth medium for 2hr or 4hr and then rinsing the cells three times with DPBS prior to infection. Virus pretreatments were performed by incubating viral stock solution in growth

media for 2hr or 4hr with drug at 37°C prior to addition to cells. A two-way ANOVA followed by Bonferroni post-tests was used to identify differences between treatments at each time point.

RESULTS

Basant inhibits both lab adapted and primary isolates of CXCR4 and CCR5 tropic HIV-1 at non-toxic levels

Basant was non-toxic to TZM-bl cells at concentrations of 0.3% or below as compared to KY jelly (Fig. 3.1A) or PBS delivery control (not shown). This was used as the starting concentration for determining IC_{50} (half-maximal inhibitory concentration) values for inhibition of CCR5 tropic HIV-$1_{B\text{-}al}$, HIV-$1_{96USNG58}$, and CXCR4 tropic HIV-$1_{NL4\text{-}3.}$ Basant effectively inhibited all three viral strains, although the effectiveness was variable (Fig 3.1B-D and Table 3.1). IC_{50} values of Basant for CCR5 tropic HIV-$1_{Ba\text{-}L}$, HIV-$1_{96USNG58}$, and CXCR4 tropic HIV-$1_{NL4\text{-}3}$ were 0.162%, 0.089%, and 0.017%, respectively.

We additionally tested Basant using the human T-cell derived PM-1 cells and primary isolates of CCR5 (HIV-1_{VB59}) and CXCR4 (HIV-1_{UG070}) tropic virus. In PM-1 cells the CC_{50} value for BASANT was found to be .032% and .11% for KY jelly. Basant inhibited both HIV-1_{UG070} and HIV-1_{VB59} after one hour incubation with virus, with IC_{50} values of .0045% and .0035%, respectively.

Basant also inhibited cell to cell transmission in cells treated after an overnight incubation with virus with IC_{50} values of .0044% for HIV-1_{UG070} and .011% for HIV-1_{VB59}. (Table 3.2)

Basant inhibits HIV-1 prior to viral entry.

We next sought to determine which stage of the HIV-1 lifecycle Basant interferes with. This was done by performing several time series experiment in which the kinetics of Basant inhibition was compared to that of Enfuvirtide, an established HIV-1 fusion inhibitor (Manfredi & Sabbatani, 2006). First, we tested how long after virus was added to cells could Basant and Enfuvirtide exhibit an antiviral effect. We found that Basant was able to inhibit infection if added up to 3hr post infection (Fig 2a). In contrast, Enfuvirtide continued to be effective 4hr post incection. Next, we determined if the observed antiviral effect was as result of direct action on virions or if Basant induces an antiviral state in the cells. There was no observed viral inhibition when cells were pretreated with Basant or Enfuvirtide, however, treatment of virus prior to infecting cells demonstrated inhibition by both drugs.

DISCUSSION

The present study has demonstrated that Basant inhibits HIV-1 at a pre-entry stage and that it is effective against both lab adapted and primary isolates of CXCR4 and CCR5 tropic HIV-1. The latter being a critical attribute for a topical

microbicide. A study by Talwar et al. (Talwar, et al., 2008) found that the primary ingredient with anti-HIV-1 activity is *Aloe vera*. Our literature review did not find any other studies that demonstrated that *Aloe vera* has anti-HIV-1 effects. It is plausible that inhibition is mediated by anthraquinones within *Aloe*, which have previously been found to inhibit multiple other enveloped viruses, presumably by disrupting their lipid envelopes (Alves, Perez-Fons, Estepa, & Micol, 2004) and it will be interesting to determine if this is indeed the case for HIV-1. Circumin, which is present in Basant, can inhibit HIV-1 by inhibiting transcription of the viral genome by acting as an inhibitor of p300/CREB binding protein (CBP) (Balasubramanyam, et al., 2004). Our experiments did not indicate that Basant acts at a post-entry step, however, due to the toxicity of Basant above 0.3% to TZM-bl cells the final concentration of curcumin was low. The CBP study also used DMSO as a solvent for curcumin. Since DMSO is known to permeabalize cells, less curcumin may have entered the cells in our experiments. On the other hand, a phase I clinical trial and a toxicity assay utilizing rabbits found that full strength Basant is well tolerated *in vivo.* Therefore, we cannot rule out the possibility that curcumin may play an important role in a clinical setting. It will be interesting to determine if the topical application of Basant is able to inhibit CBP activity in human vaginal mucosa. Such pre and post entry inhibition would certainly be desirable for a topical microbicide and may extend its usefulness to post-exposure prophylaxis.

Basant is a microbicide candidate with activity against a broad range of sexually transmitted infections (STIs) (Talwar, et al., 2008). Many of these STIs are known to increase the probability of transmission of HIV-1 due to inflammation of the genital mucosa (Galvin & Cohen, 2004). By inhibiting these STIs, Basant may exert an additional indirect impact on HIV-1 transmission which is not present in many other microbicides currently in development. Basant is compatible with Probiotic strains of *Lactobacilli*, normally resident in the vagina. It does not exercise any inhibitory effect on their replication, nor interferes with their survival (GP Talwar, unpublished data) and is a polyherbal formulation composed of plant extracts which have been a part of the Asian pharmacopeia for ages and are accepted by the population and are considered safe for human use. We will next test Basant's efficacy as an effective vaginal microbicide in preventing HIV transmission in a humanized mouse model prior to clinical efficacy trials.

ACKNOWLEDGEMENTS

The authors would like to thank Meagan Prescott, Jonathan Ward, Jonathan Gallion and Casey Ward for technical assistance.

This research was funded by NIH Grant 1R21AI079564.

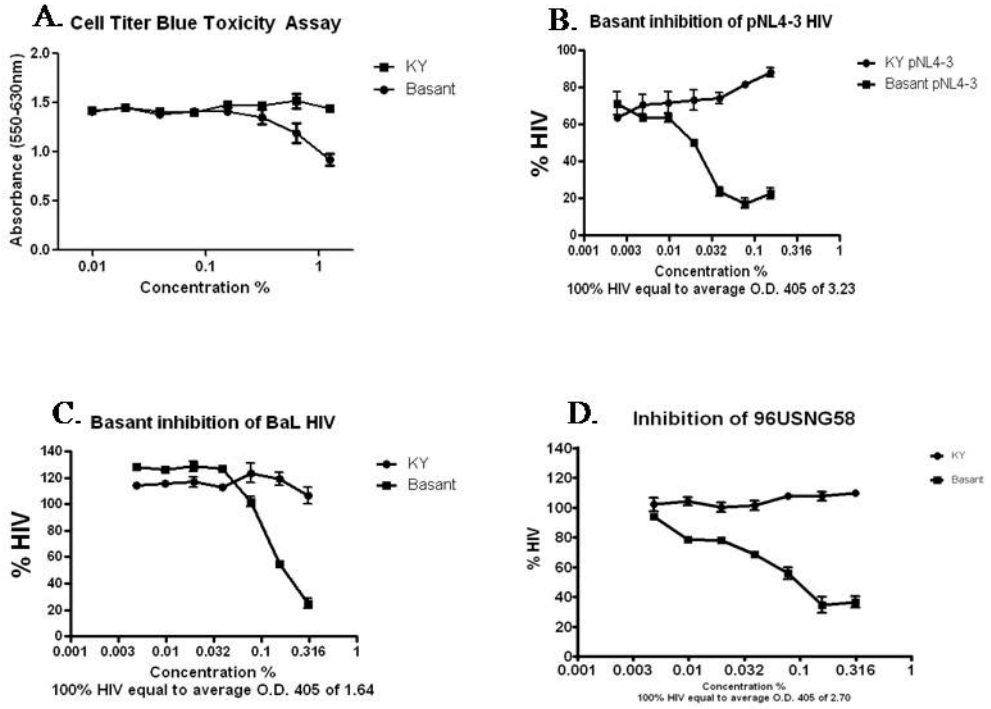


Figure 3.1.Basant toxicity and inhibition. A. Toxicity assay was conducted by treating TZM-bl cells with varying concentrations of Basant or KY jelly. A Cell Titer Blue Assay was performed after 2 days. Basant concentrations below 0.3% are non-toxic. **B-D**. Inhibition assays of CXCR4 (B) and CCR5(C & D) tropic HIV-1. TZM-bl cells were infected and concomitantly treated with a dilution series of Basant or KY jelly. After two days, Tat dependent β-galactosidase activity was assayed. A standard curve was used to determine the inhibition as a function of treatment concentration. Basant inhibits all strains, albeit with varying efficiencies. Graphs display the average of three replicates. Error bars indicate S.E.M.

Table 3.1. Basant IC_{50} values**.** Values presented for each HIV-1 strain tested on TZM-bl cells treated at time of infection.

HIV-1 Strain	Basant IC_{50}
HIV-1_{NL4-3}	.017%
HIV-1_{BaL}	.162%
HIV-1_{96USNG}	.089%

Table 3.2. Basant inhibitory concentration values for inhibition of cell to cell spread of HIV-1 and pretreated HIV-1 in PM-1 cells.

	Cell to Cell Assay		One Hour Pretreatment Assay	
HIV-1	UG070	VB59	UG070	VB59
CC50	.032%	.032%	.032%	.032%
IC50	.0045%	.0035%	.0044%	.011%
IC80	.012%	.016%	< .0078%	.030%
TI	7.1	9.1	7.3	2.9

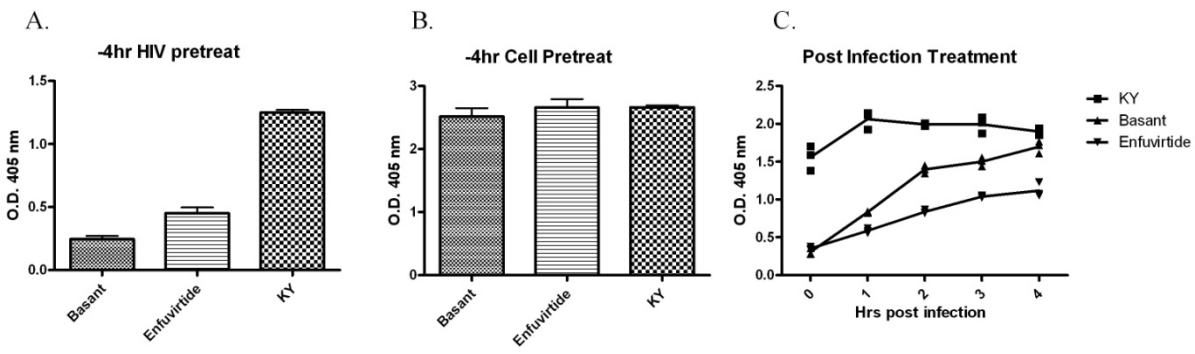


Figure 3.2. Kinetics of HIV-1 Inhibition A. HIV-1_{NL4-3} was pretreated for four hours at 37°C with Basant, Enfuvirtide or KY jelly and then added to TZM-bl cells. HIV-1 Tat dependent β-galactosidase activity was assayed after two days. Both Basant and Enfuvirtide inhibited HIV-1 replication. **B**. TZM-bl cells were treated for four hours and then rinsed with PBS prior to infection. None of the compounds induced an antiviral state in the cells. **C**. Cells were infected with HIV-1_{NL4-3} and then treated at multiple time points. Treatment of cells with Basant is effective for up to three hour post-infection. Enfuvirtide continues to be effective at four hours post infection.

Chapter 4:

The characterization of a novel HIV-1 open reading frame within the polymerase gene.

Maciej Maselko, Casey Ward, Meagan Prescott, Timothy Putman and Manoj Pastey

ABSTRACT

HIV-1 employs a multitude of non-canonical approaches to effectively express fifteen proteins from nine genes. This includes the use of alternative splicing, ribosomal frame-shifting and post-translational cleavage. We describe a well conserved and previously unstudied alternative open reading frame in the polymerase gene that is required for HIV-1 replication in multiple cell lines. The small and highly basic protein expressed from this reading is synthesized in a Rev independent manner and is localized in the nucleolus.

INTRODUCTION

The expression of the proteins from the Human Immunodeficiency Virus -1 (HIV-1) proviral genome utilizes a diverse set of molecular approaches. Transcription is regulated by both the activation state of the infected cell as well as the concentrations of the HIV-1 regulatory proteins Tat and Rev. Expression of viral mRNAs proceed from multiple spliced forms followed by single and finally unspliced mRNA species (Kingsman & Kingsman, 1996). In addition to cap dependent translation, HIV-1 utilizes non-canonical translational strategies such as an internal ribosomal entry site (IRES) and ribosomal frame shifting (Brasey, et al., 2003; Mazauric, Seol, Yoshizawa, Visscher, & Fourmy, 2009).

In addition to the fifteen proteins that are part of the established repertoire of HIV-1, CD8+ T-cells have been found circulating in infected individuals that recognize epitopes from alternative reading frames (ARF)s whose products have not been characterized (Cardinaud, et al., 2004). RNA from infected patients has also revealed numerous unusual HIV-1 RNAs, including transcripts that show evidence of trans-splicing and transcription from the anti-sense strand (Carrera, et al., 2010).

The initial discovery of a novel ORF was made by Manoj Pastey (unpublished data) using Vector NTI software (Invitrogen, Carlsbad, CA) to annotated the pNL4-3 infectious HIV-1 plasmid. Two novel candidate reading frames of interest were discovered and named non-structural-1 (NS1) and non-structural-2 (NS2). Mutagenesis of NS1 did not produce a replication defect and was not further investigated. The NS2 reading frame is found in the +3 position within the integrase region of the polymerase gene (Fig 4.1). The translated reading frame encodes a 66 amino acid protein (Fig 4.2) with a molecular weight of 7961.02 daltons and is very basic (p.I.=11.26).

Since the first nucleotide of the NS2 reading frame is in the third position of the *pol* gene reading frame, many mutations in this position will not change the amino acid sequence of *pol* and may be considered neutral. Furthermore, the context of the NS2 start codon with an adenine in the -3 position (relative to AUG_{NS2}) and a guanine in the +3 position corresponds to a strong Kozak sequence for efficient translational initiation (Kozak, 1987).

Site directed mutagenesis of the NS2 start codon while preserving the overlapping *pol* codon resulted in a highly replication defective phenotype. An in frame GFP fusion to the end of the NS2 reading frame of pNL4-3 resulted in fluorescence that was also dependent on the NS2 start codon. These prior

experiments established the NS2 reading frame as expressed and required for robust replication of HIV-1 to high titers in a variety of cell lines.

The results presented in this present study document further work to characterize NS2. Utilizing site directed mutagenesis, recombinant protein and HIV-1 replication experiments we find that this reading frame likely expresses a nucleolar protein that is expressed in a Rev-independent manner from a novel species of mRNA. We also present data from our attempts to purify the protein from which we conclude that the protein is highly unstable.

RESULTS

Computational Analysis of NS2

NS2 is located in the +3 position of the *pol* reading frame. Mutations in this position are least likely to alter the amino acid translation of *pol*. Analysis of 8,680 HIV-1 sequences from the Los Alamos Sequence Database (Leitner T, 2005) revealed that the NS2 open reading frame is conserved in 96% of the isolates. A comparison of amino acid identity between HIV-1 clades demonstrates that NS2 is highly conserved at almost every position (Table 4.1). Overall, there is 81.7% identity to the NS2 consensus sequence averaged across all isolates. The overlapping *pol* reading frame only shows 59.9% identity across isolates and perhaps most strikingly, regions of *pol* that show substantial divergence still retain NS2 sequence identity. For

comparison, the HIV-1 accessory protein vpr shares 90.7% identity with all isolates. We also performed an analysis of the frequencies of each amino acid at each position once again demonstrating the high level of conservation (Table 4.2).

Querying multiple databases with the NS2 sequence indicates that it is not homologous with any known proteins at the sequence or predicted structural level. Structural predictions using Psipred indicated that NS2 may contain two helix domains with moderate confidence (Fig 4.3).

NS2 exhibits strong nucleolar localization

In order to better characterize the function of NS2 we sought to gain an understanding of the subcellular localization of the protein. HeLa cells expressing the necessary receptors for HIV-1 infection (TZM-bl cells) were transfected with a pNL4-3 construct which expressed GFP fused to the C-terminus of NS2. Examination of these cells by fluorescence microscopy indicated that NS2-GFP exhibits nucleolar localization (Fig 4.4A).

NS2 is generated from a novel HIV RNA

The NS2 ORF is in an ARF of the *pol* gene and previous characterization of the mRNA species generated during infection did not find an mRNA that corresponded to an independent NS2 transcript (Carrera, et al., 2010). We

hypothesized that NS2 may be generated from the *pol* transcript. This might be possible with a ribosomal frameshifting mechanism or an internal ribosomal entry site. We tested this hypothesis by knocking out the Rev protein in our pNL4-3 NS2-GFP construct. Since Rev is required for the nuclear export of the unspliced RNA that contains the *pol* transcript, knocking out Rev should also eliminate any NS2-GFP that is made from the same transcript. To our surprise, the Rev mutant also produced NS2-GFP (Figure 4.4B) which indicates that NS2 is translated from a previously undescribed and fully spliced mRNA.

All of the protein coding subgenomic length HIV-1 RNA species are produced by splicing of the full genomic RNA. And all of the RNA species share a common 5' region (Purcell & Martin, 1993). We sought to identify the NS2 RNA and 5' splice junction by RT-PCR using a forward primer that bound to this common 5' region and a reverse primer that bound to an internal portion of the NS2 coding region. We were expecting to find a genomic length RNA which codes for the HIV-1 polymerase and a shorter NS2 RNA, but we were only able to detect the genomic length RNA species. These results imply that NS2 may be translated from a novel transcript species that does not include the 5' region common to other transcripts.

NS2 is Recalcitrant to Purification Attempts

We attempted to confirm the presence of NS2 in HIV-1 infected cells using previously generated rabbit antiserum against NS2 overlapping peptides. We found that NS2 cannot be readily isolated using typical protein extraction methods (Figure 4.6A). The isolelectric point of NS2 is predicted to be 11.26 and the charge at pH 7 was calculated to be +13.1. Since it is localized to the nucleolus we reasoned that it likely has a strong association with the negatively charged genome. We therefore developed a nuclear protein extraction protocol very similar to that used for chromatin protein isolation followed by a Na-deoxycholate/acetone precipitation step (see materials and methods). We used cells transfected with NS2 tagged with a V5 peptide in order to optimize the protocol.

Rabbit antiserum lacks sufficient specificity to discriminate presence of NS2

Once an effective extraction protocol was developed and we identified which antiserum reacted with tagged NS2 we investigated if WT NS2 could be detected from HIV-1 infected cells. We infected MT-2 cells, a human T-cell line, with HIV-1. Four days after infection, HIV-1 induced cytopathicity was present and protein was isolated according to our extraction protocol. We included uninfected MT-2 cells as well as protein from non-transfected 293T cells and those transfected with tagged NS2 as controls. The rabbit antiserum was able to detect the tagged NS2 and a corresponding band was present in

the infected MT-2 cells (Figure 4.6B). Unfortunately, numerous subsequent western blots revealed that we could not rule out that the band was not caused by background since it would also be present in some of our negative controls. We also tried to use the antiserum for immunoprecipitation experiments, but were unable to pull down NS2 from transfected cells.

NS2 was not be identified by mass-spectroscopy.

Since our rabbit antiserum is not sufficiently specific, we decided to try to identify NS2 by mass-spectroscopy. Lysate from NS2-V5 transfected cells was subject to SDS-PAGE and coomassie stained or transferred to PVDF membrane and probed with anti-V5 antibodies. A band in the coomassie gel that corresponded to the location of the band in the antibody probed blot was excised and analyzed by mass-spectroscopy. The result did not identify NS2, but rather histone H4. Since there was no unique band in the coomassie stained lysate from NS2-V5 transfected cells when compared to non-transfected cells we tried to improve the separation of NS2 from other proteins. 2D gels are not appropriate since the isoelectric focusing buffers available are not effective at the high isoelectric point of NS2. We instead tried alternative running buffers, native gels, and positively charged acid-urea gels. In all cases, we could not recognize a unique band. We concluded that NS2 is either covalently linked to a histone protein, which is plausible because of its

nuclear localization or it is too similar to other proteins to be effectively separated by gel electrophoresis.

Attempted Purification of Recombinant NS2 from Bacterial Cells

Purified NS2 protein can be used to generate high quality antibodies. A pure protein can also be used to investigate T-cell responses from HIV-1 infected patients. Since we could not isolate NS2 from mammalian cells prior attempts to purify his-tagged NS2 failed (Manoj Pastey, personal communication) we decided to utilize an bacterial expression system. We selected the IMPACT system (NEB, Ipswich, MA) where *E.coli* would be IPTG induced to express NS2 fused to an intein which is connected to a chitin binding protein(CBP). Bacterial lysate is then loaded onto a chitin bead column and proteolytic intein cleavage is induced with a reducing agent, allowing for the elution of a NS2 without any tags.

We first cloned NS2 into the pTYB11 plasmid to generate an N-terminal fusion. IPTG induction followed by coomassie staining of SDS-PAGE separated lysate confirmed that the cells expressed the desired fusion protein (Figure 4.7A). The NS2-intein-CPB readily bound to chitin beads, however, we could not find conditions that would allow for efficient cleavage of NS2 from the column (Figure 4.7B). Western blot analysis using rabbit NS2 antiserum confirmed that the induced *E. coli* was expressing NS2, which bound to the

chitin beads. Even with the improved sensitivity of western blot over coomassie stain, the cleaved NS2 was difficult to detect (Figure 4.7C). We also cloned NS2 into pTXB1 which formed a C-terminal fusion construct, however the resulting protein did not bind to the chitin beads.

MATERIALS AND METHODS

NS2 Protein Isolation and Blotting Protocols

Two protocols were developed to isolate NS2. With the exception of the blot in figure 4.6A, all experiments utilized the first method. HIV-1 infected or NS2 transfected cells were collected by centrifugation, pelleted and lysed using BugBuster lysis reagent with protease inhibitors (Novagen) according to instructions. The lysate was then freeze-thawed three times in an ethanol dry ice bath and then sonicated followed by a 15min centrifugation step at 16,100 RCF at 4C. The supernatant was discarded and the pellet was solubilized in 1ml of 1M NaCl solution with protease inhibitors and sonicated. This was centrifuged at 5,000 RCF for 5min and the supernatant was transferred to a fresh tube. Sodium deoxycholate was added to a concentration of .02% and incubated at 4ºC for 30min. Afterwards, 100µL of Trichloroacetic acid was added, vortexed and incubated for 15min at 4ºC. This solution was centrifuged for 15min at 16,100 RCF, 4ºC. The supernatant was replaced with 1ml of -20ºC acetone, vortexed, pelleted. This acetone wash was repeated

once. Samples were then placed on a 70ºC heat block for 15min to dry. The pellet would be dissolved in SDS sample buffer prior to SDS-PAGE.

A far simpler method was later developed using a solution of 3% SDS, 150mM NaCl, 25mM Tris HCl pH 8.0, .1% Beta-mercaptoethanol, and protease inhibitors. This solution was added to pelleted cells, incubated at room temperature for 5min, sonicated until no longer viscous, heated for 5min at 90C and then microcentrifuged at 15,000rpm for 5min. The supernatant was transferred to a fresh tube, however, this step is usually not necessary since there is no visible insoluble fraction.

Antibodies used in this study: polyclonal rabbit anti-NS2 were generated from overlapping 20 amino acid peptides derived from NS2. Serum samples that detected NS2-V5 were pooled. Rabbit anti-V5 antibodies (#600-401-378, Rockland, Gilbertsville, PA), mouse anti-p24 antibodies (#6521, NIH AIDS Research & Reference Reagent Program, Germantown, MD). HRP-conjugated mouse anti-Rabbit (#A 2074, Sigma, St. Louis, MO). Fluorescent donkey anti-mouse (#610-732-124, Rockland, Gilbertsville, PA).

Mass Spectroscopy

Protein isolated from 293T cells transfected with CMV/R CD5-NS2-V5-HIS plasmid was loaded onto a 10% Bis-Tris gel and electrophoresed. The gel was

then stained with coomasie blue solution and a band corresponding to the expected location of NS2 was excised. The gel slice was washed with 50μL of deionized water and then destained four times with 100μL of a 50:50 solution of acetonitrile: 50mM ammonium bicarbonate at 37ºC for 30min with rocking. This was followed by 15min incubation with 100μL of acetonitrile. The gel slice was dried by vacuum. Trypsin digestion was performed by adding 20μL of proteomics grade trypsin (Sigma) solution and incubating overnight at 37ºC. The sample was then sent to the Oregon State University Environmental Health Sciences Center Mass-Spec facility for analysis.

Fluorescent Microscopy

Cells grown on glass coverslips were rinsed with cold PBS and fixed for 10min with 4% paraformaldehyde. Coverslips were then rinsed three times with PBS and mounted onto glass slides using vectasheild mounting media with DAPI nuclear stain (Vector Labs) prior to imaging.

Construction of IMPACT Plasmid and Protein Extraction

Cloning of NS2 into the pTYB11 intein vector was performed using the following primers: Forward primer with SapI restriction site, 5’-GGTGGTTGCTCTTCCAACATGGCAGCTAGATTGTAC-3’ and reverse primer with EcoRI restriction site, 5’-GGTGGTGAATTCTTAACTGTAGTACTGG-3’. We used a codon optimized

version of NS2 as a template. The completed plasmid was sequenced to ensure that the NS2 insert did not contain any mutations and was in the correct reading frame. Intein expression and isolation protocols were performed according to manufacturer instructions (NEB, Ipswich, MA).

Construction of pNL4-3 NS2-GFP ΔRev Plasmid

An ATG→ CTG mutation was introduced into the start codon of Rev in the pNL4-3 NS2-GFP by site directed mutagenesis followed by PCR ligation. Three PCR reactions were performed. Reaction one forward primer: 5'-ATC TCC TAC GGC AGG AAG AA-3'. Reaction one reverse primer: 5'-AAT TTG CTA GCT ATC TGT TTT AAA GT-3'. Reaction two forward primer: 5'-TAA TAA GAA TTC TGC AAC AAC TGC-3'. Reaction two reverse primer: 5'-TCC TGC CGT AGG AGA TGC CTA C-3'. Reactions one and two used pNL4-3 NS2-GFP plamsid as template and high fidelity pfx polymerase (Invitrogen, Carlsbad, CA). The products of the first two reactions were gel purified and PCR ligated using the reaction one forward primer and reaction two reverse primer which contain NheI and EcoRI restriction sites, respectively. The PCR ligated product was then cloned into pNL4-3 using standard proceadures.

RT-PCR of HIV-1 RNA

Total RNA from pNL4-3 transfected TZM-bl cells was isolated using Prepease RNA isolation kit (Affymetrix, Santa Clara, CA). cDNA synthesis was

performed using Invitrogen's Superscript III First Strand synthesis kit (Calsbad, CA) and random hexamer primers. PCR amplification used Phusion Flash high fidelity PCR master mix (Finnzymes, Espoo, Finland) with the following cycling parameters: 98ºC for 10s then 30 cycles of 98ºC 1s, 56ºC 5s, 72ºC 3min followed by a final 72ºC extension step for 3min. Forward primer: 5'-GCTCAAAGTAGTGTGTGCCCGTCTG-3' Reverse primer: 5'-GTATGCTGTTTCTTGCCCTGTCTCTGC-3'.

DISCUSSION

We present here evidence for the existence of a novel HIV-1 protein expressed from a unique RNA species from an alternate reading frame of the *pol* gene. The NS2 reading frame was found to be highly conserved across all clades of HIV-1. Remarkably, this reading frame had a higher degree of conservation than the overlapping polymerase reading frame despite the ability of the NS2 reading frame to mutate substantially without altering the amino acid sequence of polymerase.

We hypothesize that NS2 is expressed early during HIV-1 infection because it is made in the absence of Rev. We also found evidence that NS2 may not contain the 5' splice site that is common to all other HIV-1 RNA which is formed by splicing of the full length transcript (Kingsman & Kingsman, 1996). If the NS2 transcript does not originate from the full length transcript, then one

interesting possibility raised by this finding is that NS2 may be transcribed independently of Tat which could be significant for latency.

The nucleolar localization of NS2 is interesting since the nucleolus has functions that play important roles during viral infections such as regulation of the cell cycle, apoptosis, mRNA trafficking and innate immunity (Hiscox, Whitehouse, & Matthews, 2010). Nucleolar trafficking of HIV-1 mRNA and Tat is critical for HIV-1 infection (Michienzi, Li, Zaia, & Rossi, 2002). The isoelectric point of NS2 is comparable to that of histone proteins and its strong positive charge under physiological conditions suggests that NS2 interacts with DNA and/or RNA.

At the moment, there are many more questions about NS2 than answers. It is hard to imagine how NS2 could not be a genuine protein with the multiple lines of evidence that we have found. Nevertheless, short of isolating NS2 from a natural infection, the data remains equivocal. Our experiments were frequently stymied by the apparent degradation of NS2 from our protein samples despite taking numerous precautions. We would work with GFP tagged constructs whose expression could be verified by fluorescent microscopy prior to protein isolation and then not be able to detect it in lysates. Numerous attempts at purification of tagged protein have failed. Developing

high quality antibodies and a reliable method to purify NS2 therefore remains a priority of our research.

It may seem incredible that there is an undescribed protein made by HIV-1 in the face of so much intense investigation. However, as recently as 2001 a novel Influenza A protein (PB1-F2) was discovered in an overlapping reading frame of PB1 (Chen, et al., 2001). Evidence of trans-splicing and novel immunogenic peptides from HIV-1 (Cardinaud, et al., 2004) underscore the idea that there are likely to be many more surprises. Furthermore, if NS2 is as unusual as our data suggests, then how it has remained hidden from science for so long is perhaps less mysterious.

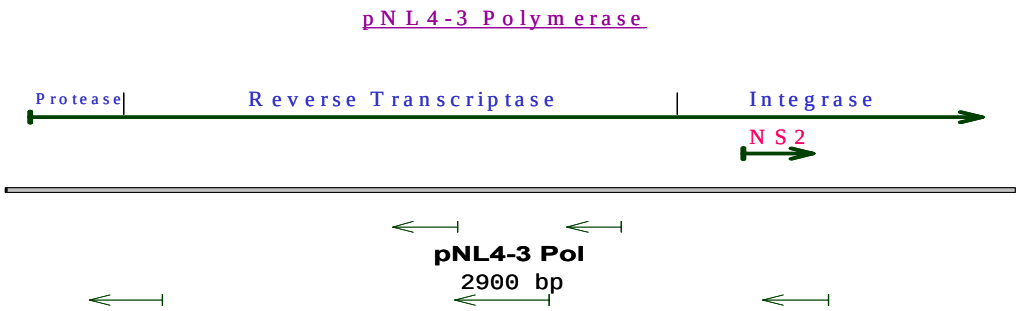


Figure 4.1. Schematic representation of pNL4-3 pol gene and encoded proteins. The *pol* gene encodes the precursor for the protease, reverse transcriptase and integrase enzymes. The NS2 open reading frame is within the integrase region.

```
Pol     · D  C  S   P  G  I  W   Q  L  D   C  T  H   L  E  G  K ·
NS2                    M   A  A  R   L  Y  T  F   R  R  K ·
2101    AGACTGTAGC CCAGGAATAT GGCAGCTAGA TTGTACACAT TTAGAAGGAA

Pol     ·  V  I  L   V  A  V   H  V  A  S   G  Y  I   E  A  E
NS2     · S  Y  L   G  S  S  S   C  S  Q   W  I  Y   R  S  R  S ·
2151    AAGTTATCTT GGTAGCAGTT CATGTAGCCA GTGGATATAT AGAAGCAGAA

Pol      V  I  P  A   E  T  G   Q  E  T   A  Y  F  L   L  K  L ·
NS2     ·  N  S  S   R  D  R   A  R  N  S   I  L  P   L  K  I
2201    GTAATTCCAG CAGAGACAGG GCAAGAAACA GCATACTTCC TCTTAAAATT

Pol     · A  G  R   W  P  V  K   T  V  H   T  D  N   G  S  N  F ·
NS2      S  R  K  M   A  S  K   N  S  T   Y  R  Q  W   Q  Q  F ·
2251    AGCAGGAAGA TGGCCAGTAA AAACAGTACA TACAGACAAT GGCAGCAATT

Pol     ·  T  S  T   T  V  K   A  A  C
NS2     · H  Q  Y   Y  S
2301    TCACCAGTAC TACAGTTAAG GCCGCCTGT
```

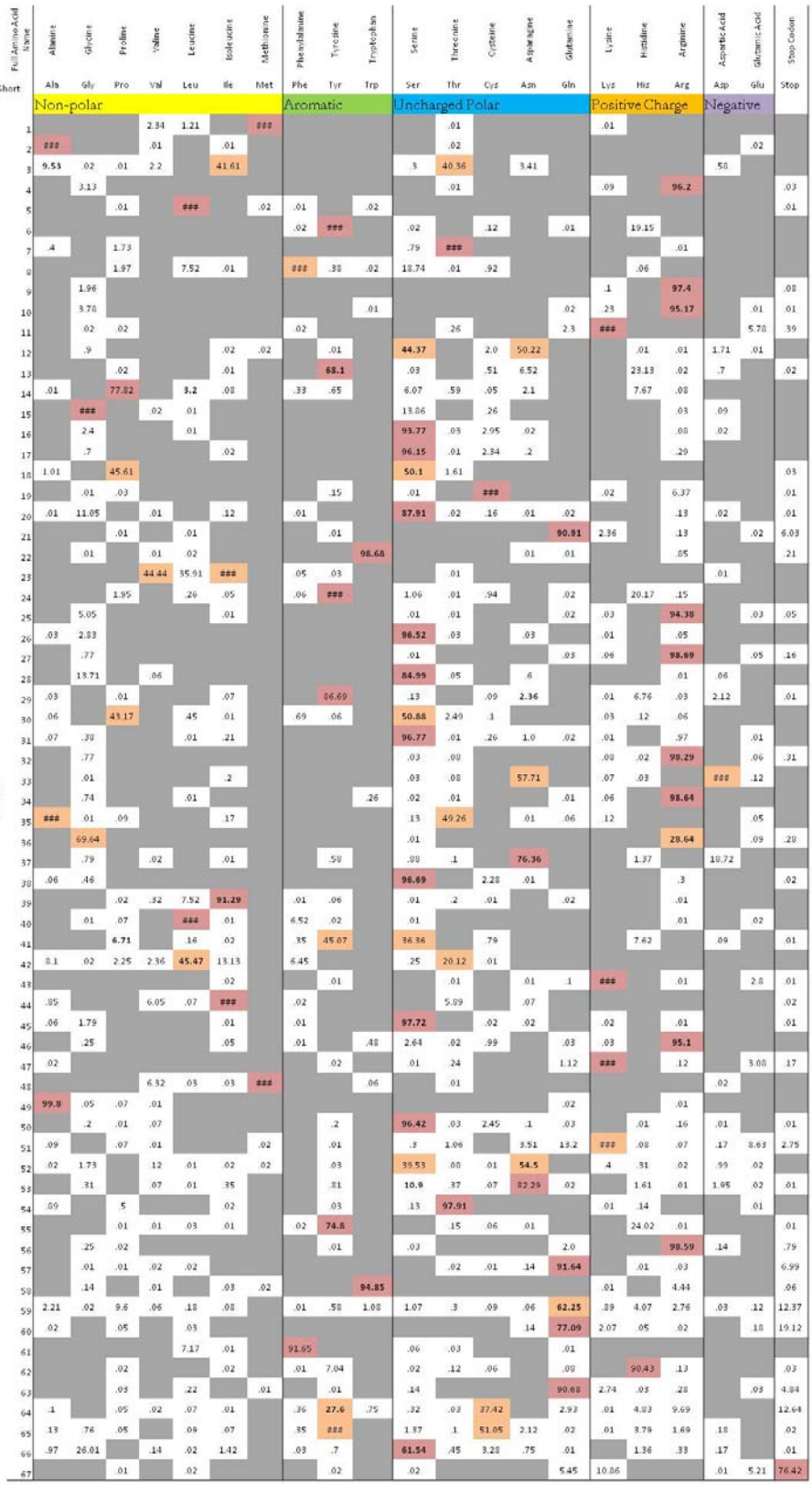
Figure 4.2. Nucleotide sequence and translation of pol and NS2 reading frames. Nucleotide numbering is according to pNL4-3 sequence. The NS2 start codon is in pink.

Table 4.1. The NS2 ORF is conserved across HIV-1 clades. Sequence data for all HIV-1 clades from the Los Alamos Sequence Database was analyzed for percent conservation of amino acid identity at each position of the NS2 reading frame compared to the consensus sequence. Columns A-P indicate the clade. The NS2 column contains the consensus sequence and the average percent identity across clades for a given amino acid position. The bottom row contains the overall conservation for the clade. NS2 is highly conserved with 81.7% cross clade identity shared with the consensus sequence. The last two columns contain the overlapping reading frame of the polymerase gene and the first 67 amino acids of Vpr. NS2 conservation exceeds that of polymerase and is comparable to Vpr.

HIV-1 Clade

Position	PNL4-3	ns2		A		B		C		D		G		N		O		P		polCDS		vprCDS	
1	M	M	96.1	M	79.5	M	97.7	M	95.2	M	99.2	M	97	V	75	M	92.5	L	100	F	99.9	M	99.5
2	A	A	99.9	A	100	A	99.8	A	99.8	A	100	A	100	A	100	A	100	A	100	F	99.8	E	99.7
3	A	I	41.6	I	85.9	T	69.9	I	91	I	92.9	I	88	V	58.3	N	73.3	T	66.7	R	99.3	Q	92.4
4	R	R	96.2	R	94.5	R	97	R	98.4	R	95.2	R	95.2	R	91.7	R	50.9	G	100	E	96.7	A	85.7
5	L	L	98.7	L	98.8	L	98	L	99.6	L	97.6	L	97.6	L	100	L	98.8	L	100	N	71.7	P	99.1
6	Y	Y	79.4	H	88.1	Y	90.8	Y	93.7	Y	95.2	Y	95.8	Y	100	H	89.4	Y	100	L	98	E	93.1
7	T	T	96.5	T	97.9	T	95.8	T	97.1	T	96.8	T	96.4	T	100	T	100	P	100	A	97.7	D	94.1
8	F	F	68.2	S	92	F	77.1	F	83.3	F	59.5	F	91.6	L	83.3	F	52.2	H	100	F	95.1	Q	97.7
9	R	R	97.4	R	97.2	R	97.3	R	97.9	R	96	R	97	R	100	R	96.9	R	66.7	P	61	G	98.5
10	R	R	95.2	R	95.7	R	96.6	R	95.8	R	96.8	R	98.2	R	83.3	R	98.1	R	66.7	Q	94.3	P	97.5
11	K	K	89.6	K	96.3	K	92.4	K	89.5	K	91.3	K	90.4	K	83.3	Q	85.7	K	100	G	88.7	Q	95.2
12	S	N	50.2	S	81.3	N	54.3	N	55.4	S	79.4	N	83.2	N	100	S	46.6	N	100	E	53.4	R	98.7
13	Y	Y	68.1	N	79.8	Y	92.7	H	91.5	Y	96.8	Y	96.4	H	100	H	85.1	Y	100	A	96.5	E	96.9
14	L	P	77.8	S	36.1	P	86.9	P	83.4	P	78.6	P	80.2	S	66.7	N	94.4	P	100	R	84.2	P	98.8
15	G	G	84.8	G	69.4	G	91.5	G	83.4	G	86.5	G	88.6	C	100	S	100	C	66.7	E	72.9	Y	80.3
16	S	S	93.8	S	96	S	93.6	S	98.4	S	96.8	S	97	S	83.3	C	93.8	S	66.7	F	81	N	92.1
17	S	S	96.2	S	99.1	S	98.2	S	98.1	S	96.8	S	98.8	G	66.7	C	99.4	S	100	S	58.1	E	92.3
18	S	S	50.1	P	83.5	S	86.1	P	92.5	S	48.4	P	90.4	P	100	P	88.8	P	100	S	82.2	W	98.5
19	C	C	93	C	99.1	C	98.3	C	96.5	C	99.2	C	99.4	C	100	C	99.4	C	100	E	92.9	T	76.1
20	S	S	87.9	S	94.8	S	95.6	S	96.3	S	93.7	S	80.2	G	100	G	80.7	S	66.7	Q	93.9	L	98.1
21	Q	Q	90.9	Q	96	Q	96.2	Q	95.7	Q	94.4	Q	96.4	Q	100	K	93.8	Q	100	T	72.1	E	96.3
22	W	W	98.7	W	100	W	99	W	97.6	W	98.4	W	99.4	W	100	W	98.8	W	100	R	88.6	L	91.2
23	I	I	58.3	L	94.8	I	89	L	89.4	L	93.7	L	94	L	100	I	80.1	L	66.7	A	84.3	L	97.6
24	Y	Y	73.9	Y	93.6	Y	86.2	H	90.4	Y	92.1	Y	95.8	L	100	H	84.5	P	66.7	N	78	E	97.7
25	R	R	94.4	R	96.9	R	96.9	R	80.1	R	96	R	97.6	R	100	R	100	G	100	S	92.8	E	92.8
26	S	S	96.5	S	96.9	S	97.2	S	91.7	S	97.6	S	98.8	S	100	S	93.8	S	100	P	91	L	97
27	R	R	98.7	R	98.8	R	98.6	R	99.3	R	96.8	R	100	R	100	R	99.4	R	100	T	83	K	98.4
28	S	S	85	S	96.6	S	96.3	G	83.3	S	96.8	S	89.8	S	100	S	90.1	S	100	S	53.2	N	41.7
29	N	Y	86.7	Y	93	Y	86.6	Y	91.3	Y	88.1	Y	89.2	Y	91.7	N	52.2	N	66.7	R	69.9	E	97.4
30	S	S	50.9	P	94.5	S	83	P	84.7	S	87.3	P	88.6	S	91.7	T	95	S	100	E	64.7	A	98.2
31	S	S	96.8	S	98.8	S	97.2	S	94.3	S	94.4	S	95.8	C	100	S	95	S	100	L	73.3	V	97.1
32	R	R	98.3	R	95.1	R	98.4	R	98.5	R	99.2	R	99.4	R	100	R	97.5	R	100	Q	45.9	R	94.3
33	D	N	57.7	N	96.3	D	69.3	N	96.5	N	96.8	N	98.2	N	83.3	N	90.1	N	100	V	54.3	H	97.4
34	R	R	98.6	R	99.7	R	98.8	R	97.4	R	98.4	R	98.8	R	83.3	R	99.4	R	100	W	37	F	98.7
35	A	T	49.3	T	94.8	A	83.2	T	95.4	A	81.7	T	92.2	T	100	T	92.5	T	100	G	68.8	P	98.1
36	R	G	69.6	G	73.1	G	80.9	R	89.6	G	92.9	G	88	G	83.3	R	60.2	R	66.7	R	42.4	R	94
37	N	N	76.4	D	74.6	N	94.8	N	91.8	N	91.3	N	82.6	N	91.7	N	85.1	N	66.7	D	57.6	P	51.6
38	S	S	96.7	S	99.7	S	99.2	S	98.6	S	97.6	S	98.8	S	100	C	96.3	C	100	N	45	W	98
39	I	I	91.3	I	97.9	I	98.1	I	96.9	I	51.6	I	94	I	100	L	94.4	I	100	N	26.9	L	97.9
40	L	L	92.1	L	93.9	L	92	L	93.7	L	88.9	L	96.4	F	100	L	64.6	F	33.3	S	41.7	H	90.4
41	P	Y	45.1	S	66.1	S	45.1	Y	79.4	S	74.6	Y	77.8	Y	100	P	95.7	C	100	S	20.2	G	60.6
42	L	L	45.5	A	48.3	L	80.1	T	84.5	L	81	I	74.3	F	100	V	93.2	A	100	S	33.2	L	96.8
43	K	K	96.2	K	97.2	K	96.7	K	95.6	K	67.5	K	97	K	100	K	100	Q	100	E	33.2	G	97.4
44	I	I	85.7	I	88.4	I	87.1	I	89.8	I	81.7	I	92.2	V	66.7	I	79.5	I	100	A	36.3	Q	96.6
45	S	S	97.7	S	98.2	S	98.5	S	98.9	S	94.4	S	86.2	S	100	S	97.5	G	100	G	35.4	H	55.2
46	R	R	95.1	R	94.5	R	97.8	R	98	R	96.8	R	97	W	91.7	C	46.6	S	66.7	E	31.5	I	95.3
47	K	K	94.3	K	94.8	K	94	K	94.3	K	96.8	K	94.6	K	100	K	98.1	K	100	D	23.7	Y	97.8
48	M	M	92.6	M	95.4	M	92.7	M	95	M	95.2	M	52.1	M	100	M	91.3	M	100	R	32.2	E	66.8
49	A	A	99.8	A	100	A	99.8	A	99.7	A	100	A	100	A	100	A	100	A	100	G	31	T	94.5
50	S	S	96.4	S	98.8	S	98.7	S	96.9	S	98.4	S	99.4	S	100	C	89.4	C	100	G	33.8	Y	97.7
51	K	K	68.1	K	96	K	84	Q	89.6	K	94.4	K	64.7	K	100	$	80.7	K	100	T	20.6	G	97.6
52	N	N	54.5	S	85	N	86.8	S	86.1	S	81	S	68.9	S	100	S	36	S	100	V	25.1	D	96.9
53	S	N	82.3	S	63	N	84.1	N	91.6	S	80.2	N	88	Y	100	N	81.4	N	66.7	S	46	T	97.4
54	T	T	97.9	T	93.3	T	98.4	T	98.4	T	73.8	T	98.8	T	100	T	99.4	T	100	F	33.8	W	96.3
55	Y	Y	74.8	H	86.5	Y	94.9	Y	85.6	Y	96	Y	71.9	H	100	Y	96.9	H	100	S	33.6	A	34.2
56	R	R	98.6	R	98.2	R	98.7	R	98.9	R	99.2	R	99.4	$	100	R	100	R	100	F	40.5	G	97.5
57	Q	Q	91.6	Q	74.6	Q	93.1	Q	93.4	Q	95.2	Q	90.4	$	100	Q	90.1	Q	100	P	46.6	V	96.9
58	W	W	94.9	W	98.8	W	99.1	W	98.9	W	100	W	98.8	W	100	W	63.4	W	100	Q	46.8	E	87.8
59	Q	Q	62.2	Q	56	Q	70.5	Q	70.2	Q	82.5	Q	82.6	T	50	A	90.1	A	100	I	45.5	A	90.5
60	Q	Q	77.1	Q	95.4	Q	89.6	$	88.2	Q	84.9	Q	92.2	Q	100	$	98.1	$	100	T	46.6	I	77
61	F	F	91.6	F	85.6	F	91.8	F	93.3	F	94.4	F	98.8	F	100	F	77.6	L	100	L	47.4	I	86.9
62	H	H	90.4	H	95.7	H	89.5	H	94.2	H	73	H	94	H	91.7	Y	91.9	Y	66.7	W	45.9	R	94.9
63	Q	Q	90.7	Q	96	Q	92.1	Q	90.3	Q	92.9	Q	92.8	$	100	K	96.3	$	100	Q	47.3	I	72.2
64	Y	C	37.4	R	48	Y	43.4	C	66.7	C	44.4	C	56.9	C	91.7	C	93.2	C	33.3	R	46	L	97.3
65	Y	C	51	C	91.7	Y	59.5	C	80.9	C	62.7	C	80.2	H	100	N	68.9	C	100	P	47.2	Q	97.1
66	S	S	61.5	S	70.9	S	46.7	S	97	S	67.5	S	97.6	C	100	H	64	S	100	L	40.1	Q	94.1
67	$	$	76.4	$	85.6	$	85.4	$	87.3	$	92.1	K	94	K	100	E	100	K	100	V	57.9	L	94.5
Overall	100.0	81.7		88.8		88.8		91.6		88.5		91.0		94.2		86.7		91.1		59.9		90.7	

Table 4.2 Analysis of NS2 amino acid conservation. All 8,600 HIV-1 sequences available from the Los Alamos Sequence Database were analyzed for amino acid conservation. The rows are labeled with the amino acid position number of NS2 and each column is labeled with an amino acid grouped by chemical property. When a single amino acid was conserved in greater than 70% of the sequences the cell was highlighted in red. Otherwise, the predominate amino acids are seen in orange.


Full Amino Acid Name
Short
Position
Alanine
Glycine
Proline
Valine
Leucine
Isoleucine
Methionine
Phenylalanine
Tyrosine
Tryptophan
Serine
Threonine
Cysteine
Asparagine
Glutamine
Lysine
Histidine
Arginine
Aspartic Acid
Glutamic Acid
Stop Codon
Ala
Gly
Pro
Val
Leu
Ile
Met
Phe
Tyr
Trp
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Asp
Glu
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Negative

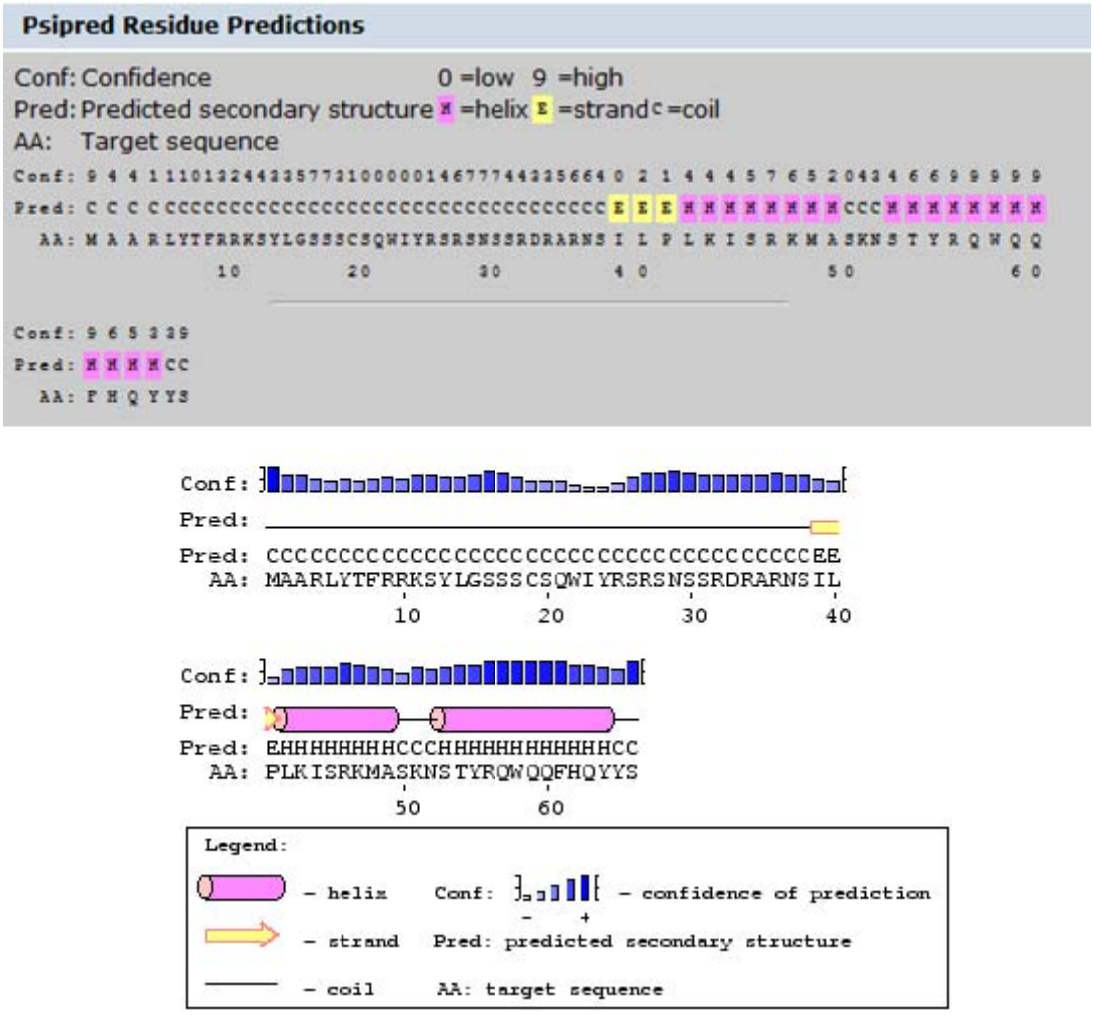


Figure 4.4 Structural Prediction of NS2. PSI-PRED v3 (McGuffin, Bryson, & Jones, 2000) was used to generate a model of NS2 based on position specific scoring from PSI-BLAST. The model predicts two C-terminal helix domains with substantial confidence, but the remainder of NS2 appears disordered.

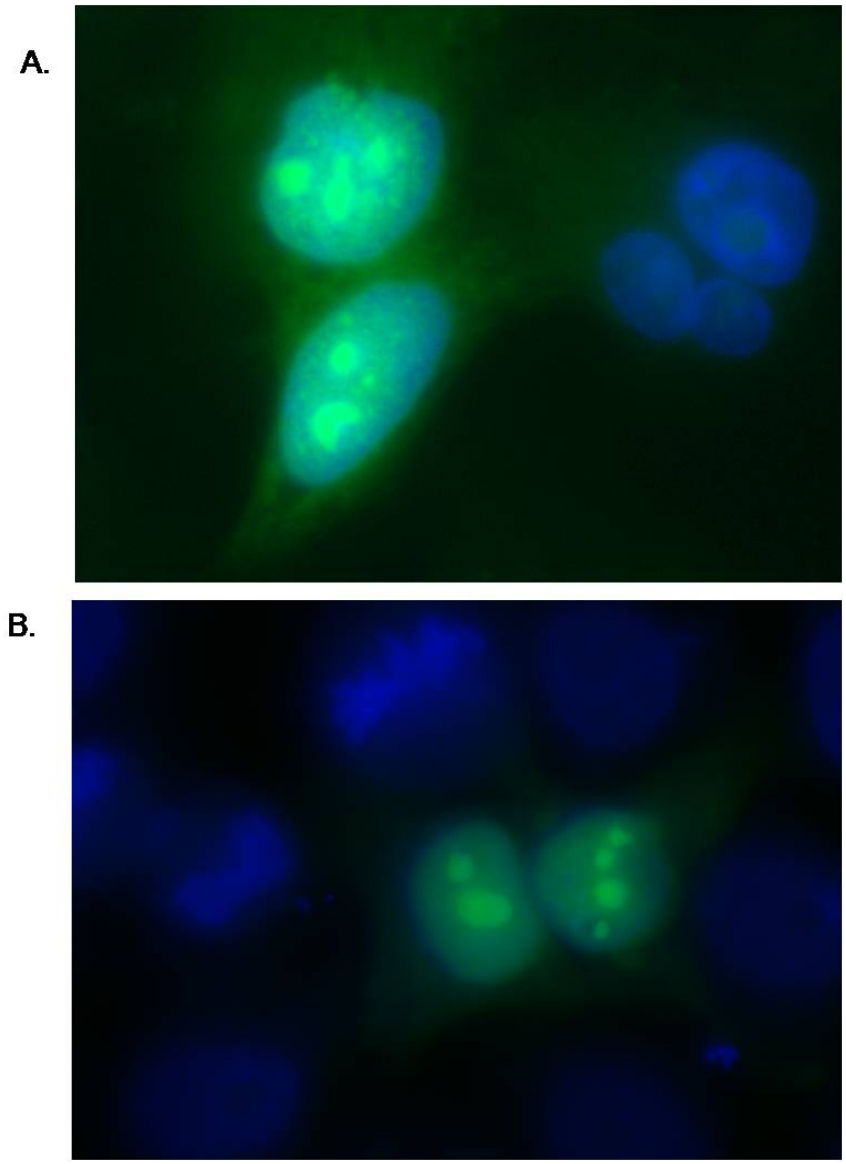


Figure 4.5. NS2 Localizes to the Nucleoli Independent of Rev. A. TZM-bl cells were transfected with pNL4-3 NS2-GFP. After 24hr, cells were stained with DAPI (shown in blue) and observed by fluorescent microscopy. The green foci are demonstrate the nucleolar localization of NS2-GFP. **B**. TZM-bl cells transfected with pNL4-3 NS2-GFP ΔRev.These cells also express NS2-GFP indicating that NS2 translation is Rev independent.

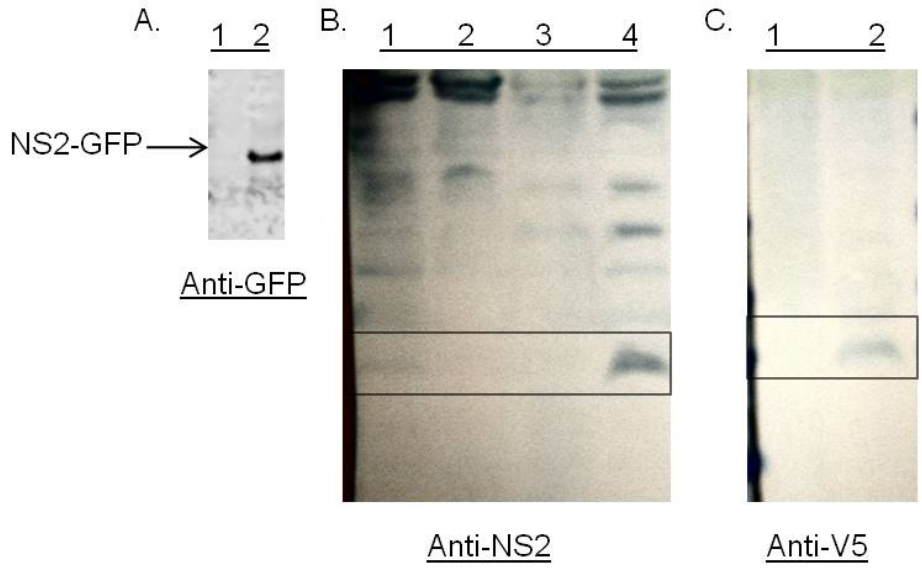


Figure 4.6. Western blot of HIV-1 infected and NS2 transfected cells. **A**. Typical extraction protocols are not sufficient for NS2. TZM-bl cells were transfected with a NS2-GFP plasmid. Lane 1 contains the soluble lysate obtained by treatment with RIPA buffer followed by sonication and boiling. Lane 2 used a 3% SDS buffer instead of RIPA buffer. **B**. Testing rabbit antiserum for WT NS2 detection. Putative NS2 bands are boxed and approximately 15kDa. Lane 1 contains HIV-1 infected MT-2 cell lysate. Lane 2 contains uninfected MT-2 cells control lysate. Lane 3 contains 293T cell control lysate. Lane 4 contains lysate from NS2-V5 transfected 293T cells. **C.** Lanes 1 and 2 contain non-transfected and transfected 293T lysate, respectively and were blotted with anti-V5 antibodies.

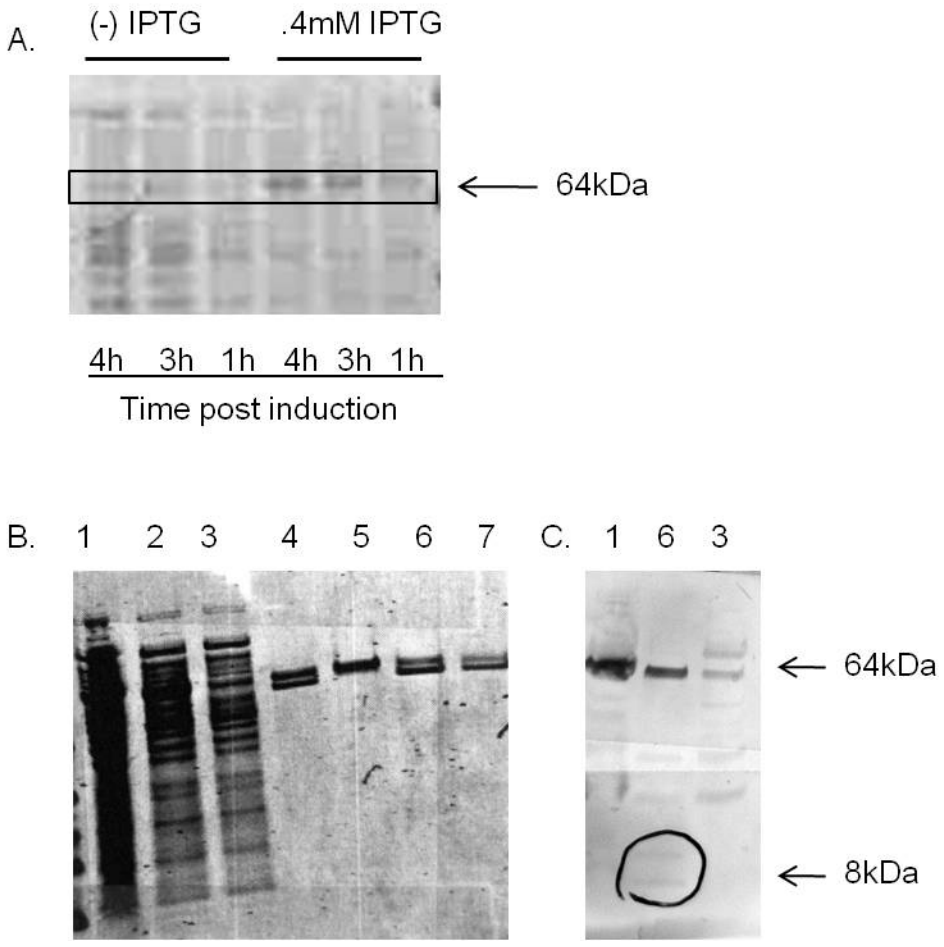


Figure 4.7. Expression and isolation of NS2 from IMPACT kit. A. *E. coli* transformed with pTYB11-NS2 was induced to express CBP-intein-NS2 fusion protein with .4mM IPTG. A band at the predicted molecular weight of 64kDa is apparent by coomasie stain from cell lysate in as little as one hour post induction. **B.** CBP-intein-NS2 readily binds to chitin beads, but it does not cleave. Image show a coomasie gel. Lane 1: Induced E. coli cells, Lane 2: clarified lysate, Lane 3: Column Eluant. Columns 4-7 show protein stripped from chitin beads using SDS after trying several cleavage induction conditions containing 50nM DTT. Lane 4: 1M urea, Lane 5: 4M urea, Lane 6: pH 7.3, Lane 7: pH 9.2. **C.** Western blot using rabbit NS2 antiserum. The sample numbering corresponds to the lanes in 4.7B. Sample one shows that NS2 was expressed in the induced cells. Sample 6 demonstrates the poor cleavage efficiency, potential NS2 bands are circled. Sample 3 shows that little NS2 was lost in the elution of unbound protein prior to cleavage.

Chapter 5: General Discussion

Despite the incredible amount of research that has already been conducted on HIV-1, we see that there are still fundamental aspects of the viral lifecycle that are very poorly understood. The goal of this research has been to approach the problem of HIV-1 by simultaneously investigating drug candidates utilizing the current state of knowledge while characterizing a novel protein that might be targeted in the next generation of therapeutics.

The interaction between RhoA and the fusion protein of RSV (Pastey, et al., 1999) combined with the recognition of structural homology to the HIV-1 fusion protein gp41 (Colman & Lawrence, 2003) provided an opportunity for rationale drug design by using a RhoA derived peptide to interfere with viral entry. We were able to successfully demonstrate that this peptide can inhibit HIV-1 infection by poly-anionic mechanism. From a therapeutic perspective, it raises the possibility of screening endogenous host proteins for functions outside of their normal biological roles. The lack of predicted immune response against such proteins would allow them to avoid allergic reactions or clearance by circulating antibodies.

Any serious attempt to eliminate HIV-1 must be primarily targeted towards the resource limited setting of the developing world where the majority of HIV-1 infected people are found (UNAIDS. & World Health Organization., 2010). Beyond financial and infrastructural constraints, cultural issues must also be considered. In particular, the power of women to effectively control their ability to protect themselves is paramount. Our work on Basant has advanced a topical microbicide candidate that has been found to be acceptable to both women and men, requires no refrigeration is made of compounds that are traditionally acceptable and is effective against a broad spectrum of sexually transmitted infections. The next step of testing Basant in a novel mouse model of HIV-1 infection will allow us to further evaluate Basant without exposing humans to undue risk (Joseph, et al., 2010).

The research on RhoA and Basant demonstrate that there is some forward movement towards the treatment and prevention of HIV-1. However, there is no doubt that we are a long way from being able to provide the prophylaxis and treatment to all of those who need it. New approaches are critically needed and translational research must also be balanced with studies into the basic biology of HIV-1 pathogenesis. Our work on NS2 is an important first step in understanding the function of this novel HIV-1 protein. Difficulties in purification hampered progress, but our results suggest that understanding

NS2 may provide insight into multiple processes that are important to HIV-1 biology that have not been previously described.

The importance of HIV-1 research is difficult to overstate. The human, social and economic costs in the course of the 30 years since it was first described have been incredible. The response of the scientific community has also been remarkable. Scientific advances have allowed for those with sufficient resources to escape what amounted to a death sentence in the early days of the HIV-1 epidemic. However, huge challenges still remain for those in the developing world to gain access to treatment (t Hoen, Berger, Calmy, & Moon, 2011). As scientific advances are made on both the basic and translational fronts against HIV-1, the realities that face most of those suffering with the disease must be not be overlooked.

APPENDIX

Appendix A: MCL-1 blocks RSV replication in mouse embryonic fibroblasts.

Maciej Maselko, Meagan Prescott, and Manoj Pastey

ABSTRACT

Respiratory Syncytial Virus (RSV) is a leading cause of respiratory infection. Here we describe that contrary to expectation, the apoptosis suppressing protein MCL-1 blocks RSV replication in mouse embryonic fibroblasts. Although both wild type and knockout cells appear to be infected at the same level, infection of MCL-1 knockout cells exhibited greater viral titers, syncytia formation and apoptosis. The timing of syncytia formation preceding an increase in apoptosis in MCL-1 knockout cells may provide evidence for a novel MCL-1 function outside of apoptosis regulation.

INTRODUCTION

Human Respiratory Syncytial Virus (RSV) is a major cause of respiratory infections with an estimated worldwide annual incidence of 33.8 million cases and up to nearly 200,000 deaths in children under 5 years old (Nair, et al., 2010). Serious cases of RSV in early childhood may also play a role in the development of asthma later in life (Jackson & Lemanske, 2010). There are currently no vaccines available for RSV and a humanized monoclonal mouse antibody is the only prophylactic treatment available to high risk children (Wang, Bayliss, & Meads, 2011).

RSV primarily infects respiratory epithelial cells as well as alveolar macrophages (Openshaw, 2002). Pathogenesis, though poorly understood, is mediated via both viral replication and immune response (Tregoning & Schwarze, 2010). The immunological bias towards type-2 cytokines (ie. IL-4, IL-5, IL-9 and IL-13) in the infant immune system is thought to enhance the severity of disease (Lee, et al., 2007) which includes granulocyte infiltration, increased mucous production and difficulty in breathing (McNamara, et al., 2004).

Apoptosis is a process which leads to an orderly cell death without damage to neighboring cells or immune activation. It is a highly conserved pathway in metazoans and is important both for normal organismal development and immune function (Taylor, Cullen, & Martin, 2008). Cells can be stimulated to undergo apoptosis by detecting intracellular stress as well as from extracellular signals such as those provided by immune cells that may have detected a viral infection (Benedict, Norris, & Ware, 2002). It is intuitive to consider apoptosis during viral infections exclusively as an antiviral strategy since viruses depend on a living host to complete their lifecycles. It has, however, been found to have both pro and antiviral effects that viruses have evolved strategies to exploit and avoid (Teodoro & Branton, 1997). For example, the tumor suppressor protein p53 is an important positive regulator of apoptosis that is targeted for destruction by adenovirus and papillomavirus proteins (Scheffner, Werness, Huibregtse, Levine, & Howley, 1990; Steegenga, Riteco, Jochemsen, Fallaux, & Bos, 1998). Many viruses encode orthologs of antiapoptotic proteins (Cuconati & White, 2002). On the other hand, Chikungunya virus exploits apoptosis by entering apoptotic membrane bodies, allowing it to gain access into macrophages during phagocytosis (Krejbich-Trotot, et al., 2011). During HIV-1 infection, many uninfected bystander cells undergo apoptosis, contributing to loss of $CD4^+$ T-cells and immune dysfunction (Doitsh, et al., 2010) and multiple HIV-1 proteins contribute to the apoptotic death of the infected cell (Shedlock, et al., 2008).

The role of apoptosis during RSV infection is still poorly understood. The overall picture that is emerging is that early during infection the RSV proteins NS1 and NS2 block apoptosis and premature cell death is further averted by the degradation of p53, the upregulation of antiapoptotic host proteins and the activation of the epidermal growth factor receptor (Bitko, et al., 2007; Groskreutz, et al., 2007; Lindemans, et al., 2006; Monick, et al., 2005). During later stages of infection, cells are more susceptible to TRAIL and the fusion protein promotes apoptosis which may aid in viral exit (Eckardt-Michel, et al., 2008; Kotelkin, Prikhod'ko, Cohen, Collins, & Bukreyev, 2003). However, the same study that found RSV delayed cell death via degradation of p53 could boost RSV titers by stabilizing p53 (Groskreutz, et al., 2007). Conversely, blocking apoptosis using Z-VAD-FMK has been found to boost viral replication (Bitko, et al., 2007). It is therefore not entirely clear when modulation of the apoptotic machinery is part of the viral strategy or host defense.

Myeloid Cell Leukemia -1 (MCL-1) is a negative regulator of apoptosis whose expression has been found to be rapidly induced during RSV infection which is considered to be part of RSV's strategy to avoid cell death (Lindemans, et al., 2006). MCL-1 is an antiapoptotic Bcl-2 family protein localized to the outer mitochondrial membrane (OMM) where it heterodimerizes with proapoptotic

Bcl-2 family proteins (ie BAX & BAD). In the absence of sufficient MCL-1, proapoptotic Bcl-2 proteins homodimerize and form pores in the OMM which release cytochrome c and induce apoptosis (Thomas, Lam, & Edwards, 2010).

The rapid upregulation of MCL-1 during RSV infection and the observation that MCL-1 can be upregulated by IL-6 to inhibit apoptosis even by heat inactivated virus (Lindemans, et al., 2006) caused us to speculate that MCL-1 may in fact play a role in antiviral defense. In this study we find that the elimination of MCL-1 causes mouse embryonic fibroblasts (MEF) to become highly permissive to RSV infection. We also find that MCL-1 may have an antiviral effect that is distinct from its role in the regulation of apoptosis.

RESULTS

RSV replication is more robust in MCL-1 knockout cells

We first investigated the role of MCL-1 by infecting both ΔMcl-1 knockout and WT MEFs with RSV at an MOI of 2pfu/cell. The cells were observed daily for three days. After 48hr the knockout cells exhibited significantly more cytopathicity and syncytia than WT cells which did not show signs of infection until 72 hour post infection (Fig A-1). 72hr post infection media and cells were collected and virus was tittered by plaque assay. RSV replicated to a far

higher titer in knockout cells (Fig A-2). These results were very surprising since our initial expectation was that much of the cytopathicity in the knockout cells was due to cells undergoing apoptosis as an antiviral mechanism and demonstrate that MCL-1 upregulation during RSV infection is not to the benefit of the virus.

Apoptosis is not induced in knockout cells until late in infection

A previous study found that MCL-1 is upregulated two hours of infection (Lindemans, et al., 2006). Since the only known function for MCL-1 is to inhibit apoptosis we wanted to determine if apoptosis is indeed upregulated early in RSV infection when MCL-1 is absent. WT and ΔMcl-1 cells were infected with RSV at an MOI of 2pfu/cell or mock treated. Apoptotic activity was then measured every 12hr by caspase 3/7 assay for 48hr. We controlled for differences in the number of cells and background caspase activity by performing a permutation test of the ratios of caspase activity of infected cells divided by the caspase activity of mock treatment for each cell type. We found no substantial increase in caspase activity seen in the knockout cells until 36hr post infection (Figure A-3) indicating that MCL-1 does not block apoptosis early in RSV infection.

MCL-1 inhibits late stage events in RSV infection

The only known function of MCL-1 is to regulate apoptosis, however, the BH3 domains responsible for this activity account for about half of the protein. The function of the N-terminal half of MCL-1 has not been characterized (Thomas, et al., 2010). It is therefore possible that the N-terminal portion of MCL-1 plays some role in the inhibition of RSV. If, on the other hand, the principal function of MCL-1 is to block late stage apoptosis then earlier stages of RSV infection should be no different between knockout and WT cells.

WT and knockout cells were infected with RSV at an MOI of 2pfu/cell. The cells were examined by immunofluorescent microscopy for differences in RSV replication phenotype using a polyclonal anti-RSV antibody at 12hr, 24hr and 48hr post infection (Figure A-4). At 12hr post infection, we did not notice any differences between cell types. Notably, the proportion of cells infected appeared roughly equivalent indicating that the multiple-log differences in viral titer are not the result of differences in number of infected cells. After 24hr, syncytia were present in both cell types, however, ΔMcl-1 cell syncytia were much larger and composed of many more cells. After 48hr, knockout cell syncytia continued to grow. WT infection did not appear to progress indicating a possible block in late stage infection.

MATERIALS AND METHODS

Cells and Virus

Mouse embryonic fibroblasts were a kind gift from Dr. Joseph Opferman. The generation of the ΔMcl-1 mutation is previously described (Opferman, et al., 2003). Cells were cultured in DMEM (Invitrogen Carlsbad, CA) supplemented with 10% fetal bovine serum, non-essential amino acids and beta-mercaptoethanol. Cells were incubated at 37ºC with 5% CO_2 atmosphere.

Respiratory Syncytial Virus VR-1540 HRSV-A2 (ATCC, Manassas,

VA) stock was generated by infecting HEp-2 cells at an MOI of .05. Two days later 5ml of cells and media were transferred to a new T75 flask of HEp-2 cells. After subsequent two day incubation, the cells and media were collected by scrapping, vortexed and centrifuged at 1,000rcf for 10min. The supernatant was overlayed onto a 30% sucrose gradient (30% sucrose w/v in 1M $MgSO_4$ •7H_2O, 50mM HEPES, 150mM NaCl) and centrifuged for 30min at ~60,000rcf. Virus was combined from multiple tubes and centrifuged again to concentrate. Aliquots were frozen in an ethanol dry-ice bath and stored at -80°C.

Plaque Assay

Viral titer was determined by plaque assay using HEp-2 or HeLa cells. Cells > 90% confluent in a 12-well plate were infected with serial dilutions of sample

and overlayed with 1% low melting point agarose in DMEM (Cambrex, Rockland, ME). Cells were incubated inverted and after three days, cells were fixed with 4% paraformaldehyde and the agarose plugs were removed. Wells were blocked with 5% milk in PBS and subsequently probed with goat polyclonal anti-RSV antibodies (# AB1128 Millipore, Billerica, MA) and then HRP-conjugated donkey anti-goat secondary (Rockland, Gilberstville, PA). Viral plaques are then visualized and counted after incubation with TMB substrate (BioFX, Owings Mills, MD).

Apoptosis Assay

A clear bottom 96-well plate (Perkin Elmer, Waltham, MA) was seeded with 25,000 wild type or ΔMcl-1 mouse embryonic fibroblasts in 100μL of culture media. The next day, cells were infected with RSV at a multiplicity of infection of 2 or mock treated. At twelve hour intervals thereafter, three wells for each cell type and condition were assayed for caspase activity using Apo-one Caspase 3/7 assay (Promega, Madison, WI) according to manufacturer instructions.

Assessment of Cytopathicity

A twelve well plate was seeded with 200,000 wild type or ΔMcl-1 mouse embryonic fibroblasts in 1ml of media. The next day, cells were infected with RSV at a multiplicity of infection of 2 or mock infected. The cells were observed for cytopathicity by light microscopy at 24hr intervals and photographed.

Fluorescent Microscopy

Cells grown on poly-L-lysine coated glass coverslips were washed with cold PBS and then fixed with 4% Paraformaldehyde then permeabilized with .2% Triton X-100 in PBS. Next, the cells were washed again with PBS and blocked with 2% bovine serum albumin solution in PBS. Goat polyclonal anti-RSV (# AB1128, Millipore, Billerica, MA) was used as a primary and donkey anti-goat secondary (#605-732-125, Rockland, Gilbertsville, PA). Coverlslips were then mounted onto glass slides using Vectasheild mounting media with DAPI nuclear stain (Vector Labs) prior to imaging.

DISCUSSION

This preliminary study has demonstrated that the upregulation of the host protein MCL-1 during RSV infection found by others (Lindemans, et al., 2006) in not likely to benefit the virus. Rather, our results indicate that MCL-1 has a protective role during infection. Apoptosis increased by 36hr post-infection in knockout cells (Figure A-3), the infection was already noticeably advanced 12hr earlier when compared to WT cells (Figure A-4). This suggests a role for MCL-1 that may be distinct from modulation of apoptosis during RSV infection, possibly via its uncharacterized N-terminal half. If the observed phenotype is due to the suppression of apoptosis, then it is conceivable that RSV may exploit a mechanism similar to Chikungunya virus to enter apoptotic bodies that facilitate viral spread (Krejbich-Trotot, et al., 2011).

The possibility that MCL-1 could act in a defensive capacity helps to explain previous observations that it is rapidly upregulated during infection and that the anti-apoptotic effect of RSV can be induced by heat inactivated virus in manner that is dependent on PI3K signaling and endosomal acidification (Kotelkin, et al., 2003; Lindemans, et al., 2006). Since RSV fusion is pH independent (Morrison, 2003), entry into an acidified endosome is not part of the normal viral lifecycle and seems unlikely to be part of a viral strategy. Furthermore, the RSV fusion protein, is needed for syncytia formation (Harris

& Werling, 2003) and induces late stage apoptosis (Eckardt-Michel, et al., 2008) both of which were increased in the absence of MCL-1. Since cells were not observed to undergo apoptosis in the absence of the fusion protein, it seems unlikely that induction of apoptosis is part of the host response. Critically, blocking cellular anti-apoptotic responses during *in vitro* infection leads to increased viral replication (Groskreutz, et al., 2007).

In summary, the results presented in this study challenge the concept that MCL-1 upregulation is part of the viral strategy to subvert the apoptotic response to infection. Ongoing research in our group is characterizing which domains of MCL-1 can complement the knockout phenotype and whether the anti-apoptotic functions of MCL-1 alone account for the relative suppression of RSV replication in WT cells. We are also exploring how MCL-1 modulates the pathogenesis of other viral pathogens. Finally, understanding the role of MCL-1 *in vivo* during RSV infection is important not only for understanding RSV pathogenesis but may also be critical for the development of safe anti-cancer therapies that target MCL-1 over-expression in tumors.

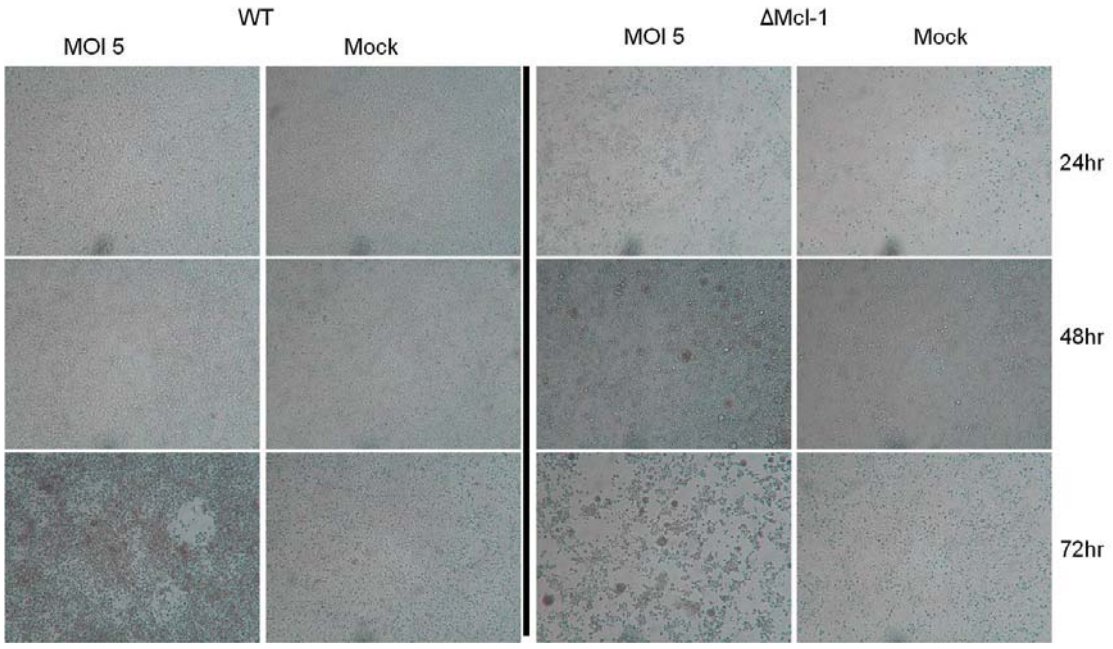


Figure A-1. Cytopathic effect of RSV replication in WT and ΔMcl-1 MEFs (200X magnification). WT and ΔMcl-1 MEFs were infected with RSV at an MOI of 2pfu/cell or mock treated then observed daily for 72hr post infection. Cytopathicity was observed in the infected knockout cells 48hr post infection. Infected WT cells were indistinguishable from mock until 72hr post infection.

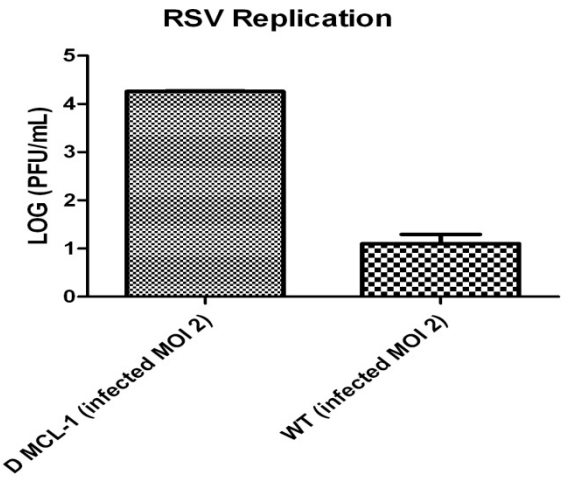


Figure A-2. RSV replication in WT & ΔMcl-1 cells. WT and ΔMcl-1 cells were infected with RSV at an MOI of 2pfu/cell. After 72hr media and cells were collected and viral titer was quantified by plaque assay. RSV replicates to far higher titers in ΔMcl-1 cells than WT. n=3.

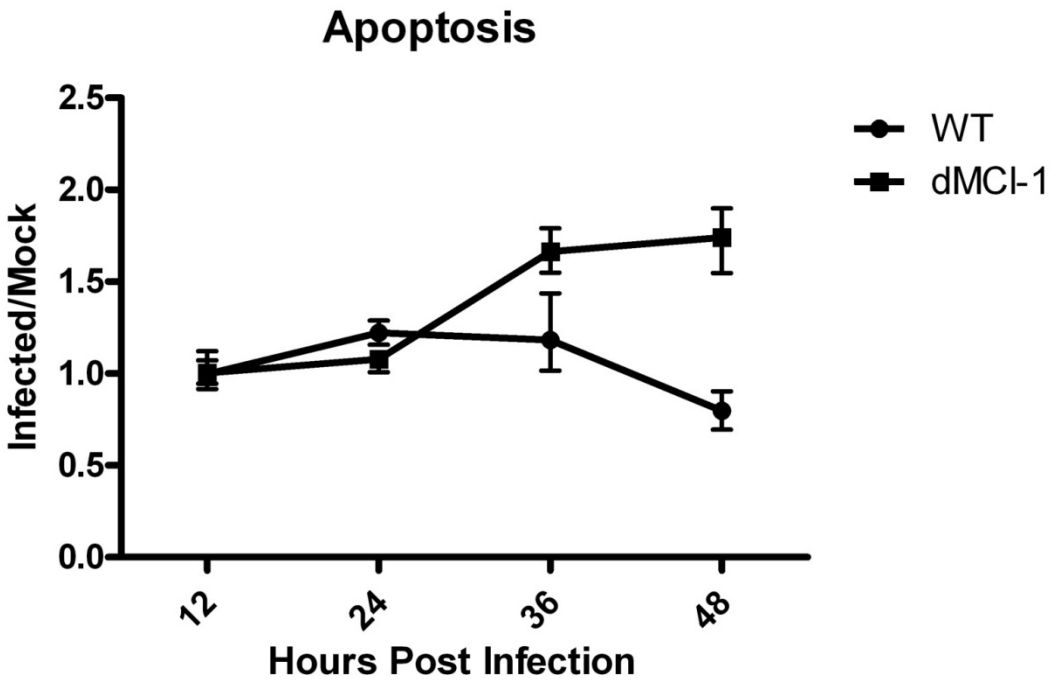
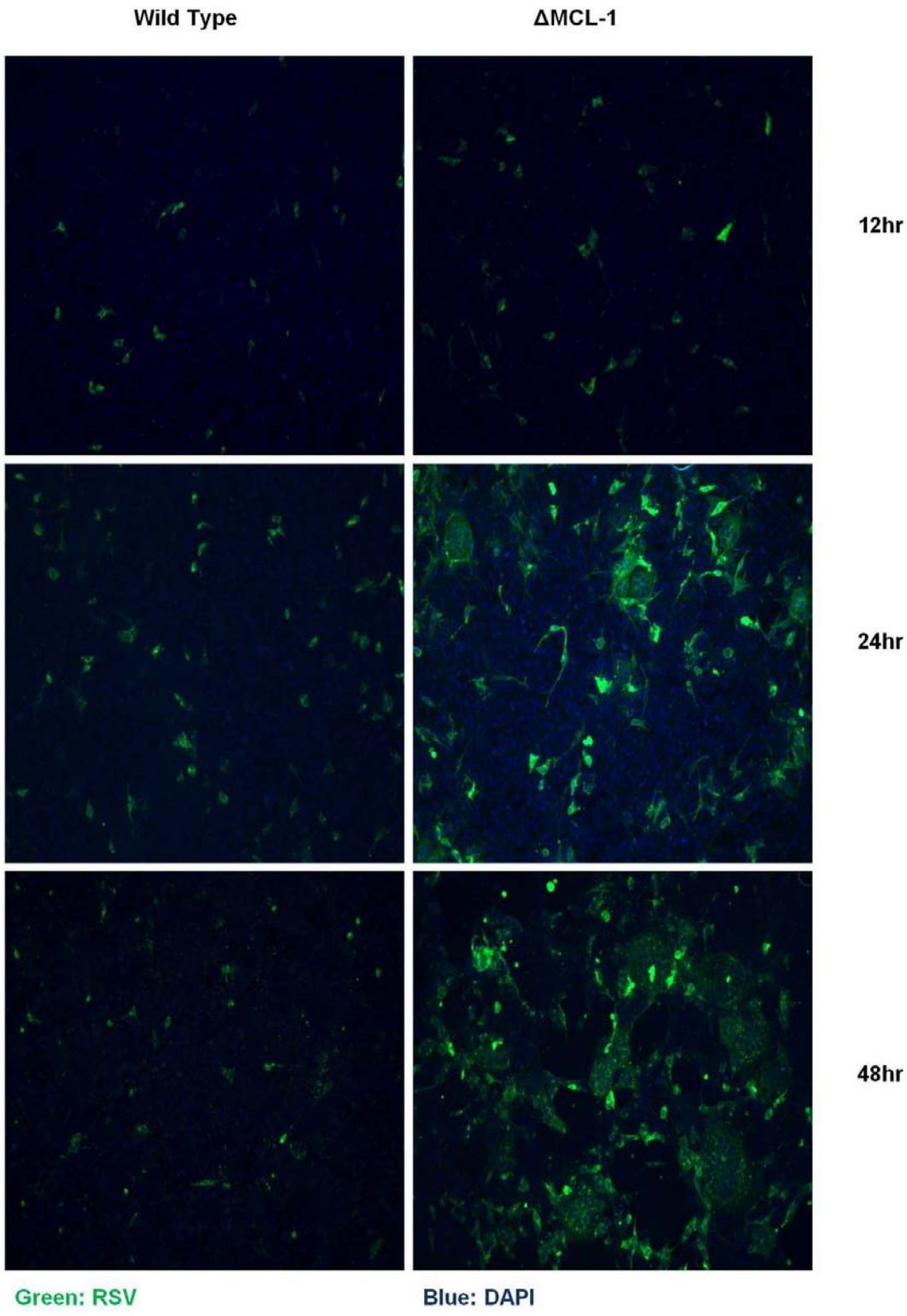


Figure A-3. Apoptosis time series. WT and ΔMcl-1 cells were infected with RSV at an MOI of 2pfu/cell or mock treated. Caspase 3/7 activity was assayed every 12hr for 48hr. Knockout cells did not exhibit an increase in caspase activity until 36hr post infection. Apoptosis is expressed as a ratio of the caspase 3/7 activity of the infected cells to the uninfected cells to control for background differences in caspase activity between cell types. n=3.

Figure A-4. Immunofluorescent microscopy of infected MEFs. Cells were infected with RSV at an MOI of 2pfu/cell. At 12, 24 and 48hr post infection the cells were fixed and probed with polyclonal anti-RSV antibodies (green) and stained with DAPI (blue). **Top**: There are no noticeable differences in the viral replication phenotype 12hr post infection. Cell morphology and proportion of infected cells appears the same. **Middle**: After 24hr, ΔMcl-1 cells contain far larger syncytia than WT. **Bottom:** 48hr post infection there are no noticeable changes to the WT cells. The ΔMcl-1 cell syncytia continue to expand and dark areas indicate locations where many cells have become detached from growth surface.


Wild Type
ΔMCL-1
12hr
24hr
48hr
Green: RSV
Blue: DAPI

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