Analysis of Viral Entry Factors and Discovery of Anti-filoviral Entry Inhibitors

BY

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DISSERTATION

Submitted as partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Microbiology and Immunology
in the Graduate College of the
University of Illinois at Chicago, 2016

Chicago, Illinois

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This thesis is dedicated to my husband, Aron Varhegyi, without whom it would not have been accomplished. Thank you for always supporting me and encouraging me to do my best.
ACKNOWLEDGEMENTS

Throughout the duration of my time at UIC, my advisor Dr. Lijun Rong has been a wonderful mentor. He has helped me develop both scientifically and personally, always encouraging me to do my best. I truly appreciate all of the support he has given me and the opportunity to study under his guidance. I would also like to thank my committee members, Dr. Nancy Freitag, Dr. Bin He, Dr. Deepak Shukla, and Dr. Michael Caffrey. They have been a great source of help in my research and have been very supportive. I would also like to thank all of the members of the Rong Lab who have been instrumental in helping me improve as a scientist.

I would also like to thank my family and friends for all of their support and love throughout this whole process. I especially want to thank my parents for always believing in me, not just for these past five years, but throughout my entire life. I also want to thank my husband, Aron Varhegyi, for his unwavering support and love, and our son Finley, who accompanied me on the last leg of this journey.
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<td>Description</td>
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<tr>
<td>293T</td>
<td>Human Embryonic Kidney Cell Line</td>
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<tr>
<td>A549</td>
<td>Human Lung Adenocarcinoma Cell Line</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
</tr>
<tr>
<td>ASPGR-1</td>
<td>Asialoglycoprotein Receptor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<tr>
<td>AZT</td>
<td>Azidothymidine</td>
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<td>BDBV</td>
<td>Ebola Virus Bundibugyo</td>
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<td>BDNF</td>
<td>Brain-Derived Neurotrophic Factor</td>
</tr>
<tr>
<td>CatB</td>
<td>Cathepsin B</td>
</tr>
<tr>
<td>CatL</td>
<td>Cathepsin L</td>
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<tr>
<td>CC50</td>
<td>50% Cytotoxicity Concentration</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control</td>
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<tr>
<td>CFR</td>
<td>Case Fatality Rate</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CV-N</td>
<td>Cyanovirin-N</td>
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<tr>
<td>DC-SIGN</td>
<td>Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin</td>
</tr>
<tr>
<td>DC-SIGNR</td>
<td>DC-SIGN homolog</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DRC</td>
<td>Democratic Republic of Congo</td>
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<tr>
<td>dsRNA</td>
<td>Double Stranded RNA</td>
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<td>EBOV</td>
<td>Ebola Virus Zaire</td>
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<tr>
<td>EXT1</td>
<td>Exostosin Glycosyltransferase 1</td>
</tr>
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<td>Full Form</td>
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<tr>
<td>FDA</td>
<td>Federal Department of Agriculture</td>
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<tr>
<td>GP</td>
<td>Glycoprotein</td>
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<td>GPCR</td>
<td>G-Protein Coupled Receptor</td>
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<td>HA</td>
<td>Hemagglutinin</td>
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<td>HeLa</td>
<td>Human Cervical Carcinoma Cell Line</td>
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<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>hMGL</td>
<td>N-acetylgalactosamine-specific C-type lectin HRP</td>
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<td>HSV</td>
<td>Herpes Simplex Virus</td>
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<tr>
<td>HTS</td>
<td>High-throughput Screen</td>
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<tr>
<td>IAV</td>
<td>Influenza A Virus</td>
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<tr>
<td>IC$_{50}$</td>
<td>50% Inhibitory Concentration</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IIV</td>
<td>Inactivated Influenza Vaccine</td>
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<td>IV</td>
<td>Intravenous</td>
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<td>KSHV</td>
<td>Kaposi's Sarcoma-associated Herpesvirus</td>
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<tr>
<td>LAIV</td>
<td>Live Attenuated Influenza Vaccine</td>
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<td>LASV</td>
<td>Lassa Virus</td>
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<td>LINGO-1</td>
<td>LRR and Ig domain containing, nogo receptor-interacting protein</td>
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<td>LLOV</td>
<td>Lloviu Virus</td>
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<tr>
<td>LSECtin</td>
<td>Liver and lymph node sinusoidal endothelial cell C-type lectin</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal Antibody</td>
</tr>
<tr>
<td>MARV</td>
<td>Marburg Virus</td>
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<td>MERS</td>
<td>Middle East Respiratory Virus</td>
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<td>MOI</td>
<td>Multiplicity of Infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NA</td>
<td>Neuraminidase</td>
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<td>NGF</td>
<td>Nerve Growth Factor</td>
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<tr>
<td>NP</td>
<td>Nucleoprotein</td>
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<td>NPC1</td>
<td>Niemann-Pick C1</td>
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<tr>
<td>NT</td>
<td>Non-Targeting</td>
</tr>
<tr>
<td>NT3</td>
<td>Neurotrophin 3</td>
</tr>
<tr>
<td>NT4</td>
<td>Neurotrophin 4</td>
</tr>
<tr>
<td>p75</td>
<td>Pan-neurotrophin Receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethyleneimine</td>
</tr>
<tr>
<td>PMOplus</td>
<td>Positively Charged Phosphorodiamidate Morpholino Oligomers</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
</tr>
<tr>
<td>RAVV</td>
<td>Ravn Virus</td>
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<tr>
<td>RBD</td>
<td>Receptor Binding Domain</td>
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<tr>
<td>RESTV</td>
<td>Ebola Virus Reston</td>
</tr>
<tr>
<td>rhAPC</td>
<td>Recombinant Human Activated Protein C</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RNAP</td>
<td>RNA Dependent Polymerase</td>
</tr>
<tr>
<td>rNAPc2</td>
<td>Recombinant Nematode Anticoagulant Protein c2</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>ROCK</td>
<td>Rho-associated Protein Kinase</td>
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<td>RSV</td>
<td>Respiratory Syncytial Virus</td>
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<tr>
<td>RTN4R</td>
<td>Reticulon 4 Receptor</td>
</tr>
<tr>
<td>SA</td>
<td>Sialic Acid</td>
</tr>
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<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SI</td>
<td>Selectivity Index</td>
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<tr>
<td>siRNA</td>
<td>Small Interfering RNA</td>
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<tr>
<td>SUDV</td>
<td>Ebola Virus Sudan</td>
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<td>TAFV</td>
<td>Ebola Virus Tai Forest</td>
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<td>TAM</td>
<td>Tyro3/Axl/Mer</td>
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<td>TIM-1</td>
<td>Human T-cell Ig Mucin 1</td>
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<tr>
<td>TrkA</td>
<td>Tropomyosin Receptor Kinase A</td>
</tr>
<tr>
<td>TrkB</td>
<td>Tropomyosin Receptor Kinase B</td>
</tr>
<tr>
<td>TrkC</td>
<td>Tropomyosin Receptor Kinase C</td>
</tr>
<tr>
<td>TROY</td>
<td>TNF-Receptor Super family member 19</td>
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<tr>
<td>USAMRIID</td>
<td>United States Army Medical Research Institute of Infectious Diseases</td>
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<tr>
<td>V-ATPase</td>
<td>Vacuolar (H⁺)-ATPase</td>
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<td>African Green Monkey Kidney Cell Line</td>
</tr>
<tr>
<td>VP</td>
<td>Viral Protein</td>
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<tr>
<td>vRNA</td>
<td>Viral RNA</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular Stomatitis Virus</td>
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<td>WHO</td>
<td>World Health Organization</td>
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SUMMARY

Emerging infectious diseases have burdened humans for ages. The viruses which cause these diseases are unpredictable and often difficult to treat. Two such emerging viruses are H5N1 avian influenza and the filoviruses, Ebola and Marburg virus. Influenza virus entry is mediated by one of its glycoproteins, hemagglutinin (HA), which is known to bind to sialic acid (SA) moieties on the cell surface. Filoviral entry is mediated by its glycoprotein (GP). While some host factors have been implicated in the entry process for these viruses, there is still a lot about their entry mechanisms that is not understood. For this thesis we focused on two main parts: 1) identifying host factors that are involved in both influenza and filoviral infection, and 2) discovering new anti-filoviral therapeutics. First, we explored the role of the reticulon 4 receptor (RTN4R) in influenza viral entry. We determined through siRNA knockdown that RTN4R is utilized during influenza infection, but not through the traditional ROCK signaling pathway activated by the RTN4R signaling complex. Next, we identified the tropomyosin receptor kinase B (TrkB) as a host factor in filoviral infection via siRNA knockdown. We determined that a natural ligand for TrkB, the brain-derived neurotrophic factor (BDNF), blocks Marburg pseudovirus infection. For the second part of this thesis we focused on discovering anti-filoviral therapies. We screened a small molecule library and identified nine compounds that reduced filovirus infection by greater than 90%. We also identified 6 plants with anti-filoviral properties, which have been fractionated to further isolate the active compounds. These findings contribute to our knowledge of these emerging pathogens as well as our ability to combat them.
1. INTRODUCTION
1.1 **Emerging infectious diseases**

Infectious diseases caused by pathogenic microorganisms have plagued humans for centuries. From the bubonic plague and smallpox in ancient history to HIV/AIDS and Ebola virus in recent history, we humans have been embattled with an assortment of diseases. Emerging and re-emerging infectious diseases are of particular concern because they are often difficult to predict and control. An emerging infectious disease is one that is novel or has drastically increased in incidence in the past two decades, and a re-emerging infectious disease is one that has come back after a considerable decline (NIAID 2010; CDC 2014b). Emergence and re-emergence of infectious diseases can arise from several factors including genetic mutations or reassortments within the pathogen, a change in geographic location or ecosystem, and human behavior (NIAID 2010; CDC 2014b; Morens et al. 2004). Some of the most notable emerging and re-emerging infectious viral diseases of recent history include the emergence of the filoviruses - Marburg virus in 1967 and Ebola virus in 1976, HIV and hepatitis C virus (HCV) in the 1980s, H5N1 avian influenza, Hendra and Nipah viruses in the 1990s, the SARS corona virus, West Nile virus, and H1N1 swine flu in the 2000s, as well as MERS corona virus and a re-emergence of Ebola virus in the past few years. When a new pathogen emerges, it is often difficult to know how to handle it initially. We cannot know how to best treat the disease caused by a new virus if we know nothing about it. Thus, it is important to study the basic mechanism of viral replication and infection, and to develop effective antiviral therapeutics.

1.2 **Influenza virus**

Influenza A virus belongs to the Orthomyxoviridae family, which consists of three genera of influenza; influenza A, B, and C virus. The natural host for influenza A virus (IAV) are wild aquatic birds, but it is able to also infect other wild and domesticated fowl as well as humans, swine, horses, and canines (Wang et al. 2012). Influenza B virus mainly infects humans, but has also been shown to infect
seals, and influenza C virus can infect humans and pigs (Osterhaus et al. 2000). Influenza A virus is the most concerning of the three due to its ability to efficiently infect humans leading to severe illness and economic losses due to seasonal flu, and leading to occasional but devastating pandemics.

Influenza A virus is further classified based on the subtype of the glycoproteins upon its surface. It has two major surface glycoproteins; hemagglutinin (HA) and neuraminidase (NA). Hemagglutinin is responsible for viral binding to the cell surface and fusion with the cell membrane and will be discussed further in subsequent sections, while neuraminidase is responsible for viral release from the host cell. There are currently 18 known HA subtypes and 11 NA subtypes (CDC, 2014).

Influenza A virus is an enveloped virus that is usually either spherical in shape or filamentous, with a diameter of ~100nm for the spherical shape or a length greater than 300nm for the filamentous form. It has a negative-sense, single-stranded, segmented RNA genome that is 13.1kb. It contains eight RNA segments encoding eleven proteins: the polymerase subunits PB1, PB2, and PA, PB1-F2, hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix proteins M1 and M2, and two nonstructural proteins NS1 and NEP (Figure 1). The glycoproteins HA and NA are found in the virus envelope in a ratio of about four to one. Another protein, the ion channel matrix protein M2, is also located in the viral envelope. Beneath the lipid envelope is a layer of matrix protein M1 which surrounds the viral core. Inside the viral core the ribonucleoprotein complex (RNP) can be found. The RNP is made of the RNA segments which are encapsulated by nucleoprotein. Also located within the core is the nuclear export protein (NEP) as well as the RNA dependent RNA polymerase (Shaw & Palese 2013; Guo et al. 2009; Bouvier & Palese 2008).
Figure 1. Influenza A viral particle structure. (a) Schematic representation of an influenza A viral particle indicating the viral RNA segments and the structural proteins.
1.3 Influenza epidemics and pandemics

Influenza virus is responsible for millions of human cases of severe illness each year resulting in the deaths of between 250,000 to 500,000 worldwide (Fauci 2006; CDC 2015c). The CDC estimates that over 200,000 people are hospitalized and around 36,000 people die from the seasonal flu every year in the United States alone. Infection with influenza virus can lead to serious complications including pneumonia, ear and sinus infections, and may exacerbate certain medical conditions such as asthma, congestive heart failure, and diabetes (CDC 2015a). It primarily infects the lungs and is not only the cause of seasonal influenza epidemics, but is responsible for several pandemics that occurred in recent history. The three major pandemics that occurred in the twentieth century were the Spanish Flu in 1918, the Asian Influenza in 1957, and the Hong Kong Influenza in 1968; all of which resulted in an immense number of deaths and hospitalizations as well as considerable economic losses worldwide (Fauci 2006; Ekiert et al. 2011; Ekiert et al. 2009; Palese 2004). In 2009 the H1N1 Swine flu pandemic occurred, and reports estimate the death toll to be between 151,700 and 575,400; fifteen times higher than originally thought (CDC 2012). There is a constant concern of the threat of new influenza outbreaks and potential pandemics, especially of highly pathogenic avian influenza (H5N1), which has a 60 percent mortality rate among those infected, as well as H7N9 which has recently experienced outbreaks in China (CDC 2015a; WHO 2014).

Epidemics and pandemics occur when humans are exposed to a previously unseen virus strain, which arise primarily via two routes; antigenic shift and antigenic drift. Antigenic shift occurs when two or more strains of a virus co-infect a host and their genetic segments reassort during viral replication to create an antigenically new virus. The H2N2 “Asian Influenza” pandemic of 1957 occurred because of a genetic reassortment between an avian and human virus. The HA, NA and PB1 genes from an avian virus combined with the other five gene segments from a human virus to create this new strain. The
H3N2 “Hong Kong Influenza” pandemic of 1968 was also the result of antigenic shift between avian and human viruses; the HA and PB1 segments from an avian virus recombined with the remaining segments in the human virus circulating at the time (Neumann & Kawaoka 2015). The most recent pandemic, the H1N1 “Swine Influenza” in 2009 was the result of a triple reassortment of gene segments from an avian virus, a human virus, and swine virus.

Antigenic drift results from an accumulation of small changes in the genome that occur over time, such as point mutations, which causes the virus to no longer be recognized by the host immune system. When this change happens, people who have been previously infected with the virus may no longer be immune, and vaccines against the former strain are not effective. Antigenic drift is thought to be responsible for the most lethal influenza pandemic, the 1918 H1N1 Spanish Flu which resulted in an estimated 50 million deaths. Genetic analysis of the virus shows that all of the viral gene segments originated from an avian virus which adapted to infect humans (Gamblin et al. 2004; Belshe 2005; Reid et al. 1999; Rumschlag-Booms & Rong 2013). Antigenic drift is also a concern in regards to the current avian influenza viruses which can mutate to be able to infect humans. If a highly pathogenic avian influenza virus were to acquire mutations that allow it to be able to efficiently infect humans then a new pandemic could arise. This was the fear in 1997 when human cases of the avian H5N1 virus were discovered in Hong Kong. Fortunately, while the virus has been able to jump the host barrier from birds to humans, spread amongst humans has been limited.

1.4 Influenza viral entry and replication

It has long been known that influenza virus recognizes and binds to N-acetylneuraminic acid (sialic acid, SA) that is attached to glycoproteins and glycolipids present on the host cell surface. Sialic acids are connected to these glycoconjugates through two main linkages; α2,3 or α2,6. Sialic acids of the
α2,3 linkage are mainly found in the gastrointestinal tract of birds, hence this is the preferred linkage for avian influenza viruses. Sialic acids with α2,6 linkage are mainly found on cells located in the human trachea, and viruses preferring this linkage are considered human flu viruses. While most of the sialic acids within the upper human lung have the α2,6 linkage, there are also sialic acids of the α2,3 variety located within the lower portions of the lung, which means that infection by an avian virus is possible. Swine epithelial cells contain both α2,3 and α2,6 linked sialic acids and can therefore be infected by both avian and human influenza viruses, which makes them an ideal vessel for genetic reassortment.

Hemagglutinin (HA), the viral glycoprotein responsible for binding to sialic acid, is present on the viral cell surface as a trimer. It is comprised of two subunits, HA₁ and HA₂, which are linked together by a disulfide bond. HA is a highly N-glycosylated protein that is initially synthesized as HA₀, which is then proteolytically cleaved into the two subunits. HA₁ contains the receptor binding domain (RBD) that is responsible for viral binding to the sialic acids on the host cell as well as the antigenic sites that are recognized by the host immune system. HA₂ is responsible for fusion of the viral envelope with the host cell membrane. If the HA₀ precursor is not cleaved into these subunits, the virus is not able to infect cells.

There are two classes of proteases that cleave HA₀. The first class recognizes a single arginine present at the cleavage site. These proteases, known as trypsin-like proteases, are only found in certain cells and organs within the body, which limits the replication and spread of viruses containing this monobasic cleavage site. Viruses with a monobasic cleavage site are generally considered low pathogenic strains. The second class of proteases recognizes a polybasic amino acid sequence at the cleavage site, R-X-K/R-R. These proteases are the subtilisin-like proteases furin and proprotein
convertase 6 (PC6) and are expressed throughout the body, which allows for more efficient spread of the virus. Viruses with a polybasic cleavage site are considered highly pathogenic strains, like H5 and H7.

This proteolytic cleavage is not the only alteration to HA that is required for entry to be successful however. Once the virus binds to the receptor it is endocytosed. Upon this endocytosis, the vacuole pH drops to around 5 and 5.5 and this triggers conformational changes which exposes the fusion peptide in HA₂ and allows fusion to occur. While this fusion process is taking place, the M2 ion channel pumps hydrogen ions into the viral capsid from the endosome. This results in a disruption of the viral capsid protein interactions which allows the viral contents to escape into the host cell cytoplasm.

Once the viral proteins are in the cytoplasm, they are transported to the host nucleus where viral RNA (vRNA) synthesis occurs. vRNA synthesis is performed by the RNA-dependent RNA polymerase (RNAP) which is comprised of PB2, PB1, and PA. This RNAP turns the negative-sense vRNA into positive-sense viral mRNA which is then exported out of the nucleus and translated into viral protein. This export process is mediated by the viral proteins M1 and NEP. The proteins then accumulate at the cell membrane where budding occurs. An infectious virion must contain all eight of the genomic segments. Once the virion buds from the cell membrane, the HA molecules on the surface bind to the sialic acids on the host membrane. In order for the virus to be released from the host cell, NA must cleave this attachment via its sialidase activity (Bouvier & Palese 2008; Matrosovich et al. 2004; Rumschlag-Booms & Rong 2013; Couceiro et al. 1993; Suzuki et al. 2000; Shaw & Palese 2013).

1.5 Treatment and therapeutics for influenza

To combat influenza infection, there are annual flu vaccines available to prevent infection as well as drugs to treat individuals that are already infected with the influenza virus (Palese 2004). The
two main types of seasonal vaccines recommended by the CDC are either the inactivated influenza vaccine (IIV) or a live attenuated influenza vaccine (LAIV). These vaccines target the head region of HA which contains the receptor binding domain, aiming to block viral attachment to the cell (Rumschlag-Booms & Rong 2013). The IIV is either a trivalent or quadrivalent vaccine that combines two strains of influenza A and one strain of influenza B, or two strains of influenza A with two strains of influenza B, respectively. The LAIV is a quadrivalent vaccine in the form of a nasal spray (CDC 2014e). The strains to be included in the vaccines are chosen each year based on data of circulating virus patterns. While these vaccines are effective against the particular strains selected for that year, it is possible that a different strain will circulate and cause illness even in those who received the vaccine.

For patients already infected with influenza there are two main classes of drugs available: those that target the uncoating step of viral infection and those that target viral release from the cell (Palese 2004). The first class, the adamantanes, target the ion channel activity of the M2 protein of influenza which blocks the uncoating step of entry. The two FDA approved drugs in the adamantane class are amantadine and rimantadine, however both the CDC and WHO acknowledge that more than 99% of circulating strains are now resistant to these drugs, thus these drugs are not recommended anymore for treatment. The second class of anti-influenza drugs is the NA inhibitors that interfere with the viral budding process and release of viral progeny from host cells. There are three types of NA inhibitors approved by the FDA; zanamivir (Relenza), oseltamivir phosphate (Tamiflu), peramivir (Rapivab)(Rumschlag-Booms & Rong 2013; Govorkova & McCullers 2013; Memoli et al. 2008; CDC 2014e). Peramivir was approved in December of 2014 for use in the United States, and was the first influenza anti-viral drug to be approved in the U.S. since 1999 (FDA 2014). While most influenza strains are susceptible to the effects of NA inhibitors, resistant strains have been reported against both Relenza and Tamiflu.
Due to these resistant strains, there is always a need for new antivirals. There are currently a few antivirals being developed against various targets for influenza. Cyanovirin-N (CV-N) targets HA by binding to oligosaccharides present on the protein, thereby rendering the viral particle inactive (Rumschlag-Booms & Rong 2013; Govorkova & McCullers 2013; O’Keefe et al. 2003; Boyd et al. 1997). Another anti-HA set of drugs is the thiazolides. These work against HA by blocking its maturation and intracellular transport (Rumschlag-Booms & Rong 2013; Rossignol et al. 2009). T-705 (Favipiravir) is a drug which targets the viral RNA-dependent RNA-polymerase (Baranovich et al. 2013; Govorkova & McCullers 2013). Ribavirin is a broad spectrum nucleoside inhibitor that inhibits RNA synthesis and mRNA capping. Ribavirin is used against many viruses, most commonly hepatitis C virus and respiratory syncitial virus, but has been shown to be effective against influenza infection as well (Govorkova & McCullers 2013; De Clercq 2006; Sidwell et al. 2005; Memoli et al. 2008). One drug that acts on host factors is DAS181 (Fludase) which acts as a sialidase and cleaves both α2,3 and α2,6 linked sialic acids (Govorkova & McCullers 2013; Belser et al. 2007; Marjuki et al. 2014). The strategy of targeting host factors is desirable because resistant flu strains are less likely to develop.

1.6 Filoviruses

The family Filoviridae is comprised of three genera; Ebolavirus, Marburgvirus, and the more recently discovered Cuevavirus. The five species of Ebolavirus are Zaire (EBOV), Sudan (SUDV), Bundibugyo (BDBV), Tai Forest (TAFV), and Reston (RESTV). There is one species of Marburgvirus (Lake Victoria Marburgvirus) which consists of two viruses, Marburg virus (MARV) and Ravn virus (RAVV), and one species of Cuevavirus, Lloviu virus (LLOV). The suspected natural reservoir for Ebola- and Marburgvirus is the fruit bat, but they are able to infect and cause disease in nonhuman primates and humans as well. LLOV has only been found in bats up to this point (Feldmann et al. 2013; Hofmann-
Filoviruses are enveloped viruses that are pleomorphic in shape and appear as either long filaments, 6-shaped, U-shaped, or circular in form. They have a diameter of 80nm and the length can reach up to 14,000nm. Filoviruses have a negative-sense, single-stranded, non-segmented RNA genome that is 19kb. It contains seven genes which encode for seven proteins in Marburg and two additional secreted proteins in Ebola: the nucleoprotein (NP), VP35, VP40, glycoprotein (GP), VP30, VP24, and the polymerase protein (L) (Figure 2). As mentioned, Ebola virus also encodes for two soluble GPs, sGP and ssGP, expressed from the GP transcript that have unknown function. The glycoprotein GP is located in the virus envelope as a trimer. Beneath the lipid envelope are VP40 and VP24, which are the major and minor matrix proteins, respectively. NP and VP30 are the major and minor nucleoproteins which form part of the nucleocapsid along with the polymerase complex consisting of VP35 and L (CDC 2014f; Feldmann et al. 2013).
Figure 2. Filoviral particle structure and genome organization. (a) Schematic representation of a filoviral particle indicating the structural proteins. (b) Schematic of the filoviral genome organization.
1.7  **Filoviral epidemics**

Marburg and Ebola viruses have been responsible for sporadic outbreaks since their discovery in 1967 and 1976, respectively. Infection by these viruses results in the most severe forms of viral hemorrhagic fever, and the case fatality rate (CFR) has reached 90% in some outbreaks. Due to this high mortality rate and lack of treatment, it is classified as a biosafety level 4 (BSL-4) pathogen. It has an incubation period of 2 to 21 days before symptoms set in and is spread via bodily fluids. The initial symptoms are flu-like in nature (fever, chills, aches, etc.) followed by more severe symptoms such as nausea, vomiting, chest pain, cough, postural hypotension, edema, headache, confusion, and even coma. There can also be petechiae, ecchymosis, and a macropapular rash visible on the skin. In late stages of infection the patient may experience coagulopathy, shock, and convulsions (Feldmann et al. 2013).

The initial outbreak and discovery of Marburg virus occurred in 1967 in Marburg, Germany, originating from a lab working with African green monkeys with a CFR of 23%. From then until 1998 there were a few small outbreaks, but in 1998 in the Democratic Republic of Congo (DRC) there was a larger outbreak infecting 154 individuals (83% CFR). In Angola in 2004, 252 people were infected (90% CFR). Since this outbreak there have been several small outbreaks of Marburg (CDC 2014d).

The discovery of Ebola virus in 1976 occurred during two concurrent outbreaks in the DRC and Sudan. There were 318 cases in the DRC (EBOV) with a CFR of 88% and 284 cases in Sudan (SUDV) with a CFR of 53%. There were numerous small outbreaks up until 1995 when 315 people were infected in the DRC (EBOV) (81% CFR), followed by many more small outbreaks. In Uganda (SUDV) in 2000, 425 people were infected (53% CFR) and in 2002-2003 in the Republic of the Congo (EBOV) 143 people were infected (89% CFR). In 2007 there were two outbreaks, one in DRC (EBOV) and one in Uganda (BDBV). In
the DRC, 264 people were infected (71% CFR) and in Uganda there were 149 infected (25% CFR).

Following these outbreaks there were many more small occurrences. The largest and most deadly outbreak of filoviruses since their discovery began in late 2013 (EBOV) in Western Africa and is still ongoing today. This outbreak has spanned several countries, with the main countries affected being Liberia, Guinea, and Sierra Leone. The other countries that have had/handled cases are Nigeria, Senegal, Mali, Italy, the United States, Spain, and the United Kingdom. Thus far, 27,550 people have been reportedly infected with a CFR of ~41% (CDC 2015b).

1.8 Filoviral entry and replication

The first step in filoviral infection is attachment of the virus to the host cell. This is mediated by the viral glycoprotein, GP. GP, like HA of influenza viruses, is a type 1 transmembrane glycoprotein that is responsible for attachment as well as fusion of the viral and host membranes. It is present on the viral surface as a trimer. GP is initially synthesized as the precursor protein GP$_0$ and is transported through the Endoplasmic Reticulum and the Golgi apparatus, where it gets glycosylated with both N-linked and O-linked glycans. GP$_0$ is then proteolytically cleaved by furin into the GP$_{1,2}$ heterodimer which is linked by a disulfide bond. GP$_1$ is responsible for viral binding to cells and contains the receptor binding domain (RBD) as well as a glycan cap at the C-terminus which is important for regulating entry. There is a mucin-like region present in the GP$_1$ portion of the glycoprotein, which is not required for entry into cell lines, and its function in natural infection is unknown. GP$_2$ is the portion responsible for membrane fusion and contains the fusion loop. GP$_2$ must undergo conformational changes in order for this fusion to occur.

Upon viral binding to the cell, the filovirus is brought into the cell via either endocytosis or macropinocytosis. Once the virus is in the endosome the pH drops which activates the cysteine proteases Cathepsin B (CatB) and Cathepsin L (CatL). These proteases cleave at the mucin-like region in
the GP₁ portion of GP₁₂, removing the glycan cap. This primes the protein for membrane fusion by exposing the fusion loop in GP₂, and causing a conformational change that triggers the fusion process. Once fusion has occurred between the viral and endosomal membranes, the nucleocapsid is released into the cytoplasm.

Once the nucleocapsid is in the cytoplasm, transcription of monocistronic mRNAs occurs from the encapsidated viral RNA. When there are high enough levels of mRNA, the process switches over to replication of the genome for packaging. This genomic transcription and replication is completed by the viral proteins L and VP35 which make up the polymerase complex. L has the RNA-dependent RNA polymerase activity of the complex while VP35 controls whether the genome is transcribed or replicated. After the mRNAs are produced, translation begins from these transcripts. The viral proteins then accumulate and assemble at the host cell membrane, followed by release of the completed viral particle (Feldmann et al. 2013; Hofmann-Winkler et al. 2012; Miller & Chandran 2012).

Many host factors have been implicated in the different stages of filoviral entry. Some of these include: C-type lectins, the human T-cell Ig Mucin 1 (TIM-1), the TAM (Tyro3/Axl/Mer) family of tyrosine kinase receptors, α5β1-Integrin, CatB and CatL (mentioned above), Niemann-Pick C1 (NPC1), Exostosin Glycosyltransferase 1 (Ext1), and G protein-coupled receptors (GPCRs). The C-type lectins, including dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), a DC-SIGN homolog (DC-SIGNR), asialoglycoprotein receptor (ASPGR-1), the liver and lymph node sinusoidal endothelial cell C-type lectin (LSECtin), and N-acetylgalactosamine-specific C-type lectin (hMGL) have been shown to mediate viral attachment and entry into host cells. TIM-1 was also shown to be involved in filovirus entry and a physical interaction between GP and TIM-1 was shown, however, macrophages and dendritic cells do not have this molecule but are still permissive to the virus. The TAM family of
tyrosine kinase receptors have been shown to affect entry by enhancing macropinocytosis. α5β1-Integrin has been shown to be necessary for CatL activity which is needed for GP priming. NPC1 is an endosomal protein that interacts with GP after it has been primed by CatB and CatL. Ext1 is important for heparin sulfate production, which is utilized by filoviruses for cellular attachment. GPCR receptors also play a role in filoviral infection as shown recently by our group. We found that antagonists against several different classes of GPCRs, including histamine receptors, adrenergic receptor, 5-HT (serotonin) receptors, and muscarinic acetylcholine receptor, were able to inhibit the entry of Marburg and Ebola viruses. While all of these factors have been implicated in filoviral entry, none of them (with the exception of NPC1) have been shown to be absolutely required for infection (Hofmann-Winkler et al. 2012; Miller & Chandran 2012; O’Hearn et al. 2015; Cheng et al. 2015).

1.9 Treatment and therapeutics for filoviruses

Due to the high pathogenicity of filoviruses and their relatively small outbreak sizes (up until the most recent outbreak), research on therapeutics for filoviruses has previously not been as big of a priority compared to influenza drug research. There are currently no FDA approved vaccines or anti-viral therapies for filoviruses, however since the latest outbreak began there has been a push to find treatments. According to the CDC, the present treatment for someone infected with filovirus is to quarantine them and manage their symptoms by using IV fluids, maintaining oxygen levels and blood pressure as well as treating any secondary infections that occur (CDC 2014a).

The antiviral treatments currently being developed for filoviral infection fall into several categories: those that treat clinical symptoms, those that inhibit viral processes and viral proteins, those that limit viremia and virus spread, and those that boost the host immune response. The therapeutics under development for treating clinical symptoms include the recombinant nematode anticoagulant
protein c2 (rNAPc2) and the recombinant human activated protein C (rhAPC). rNAPc2 inhibits coagulation seen in infected patients, while rhAPC treats sepsis. The issues with these drugs are that they need many doses (between nine-15 doses) and have experienced low efficacy in human trials (Wong et al. 2014; De Clercq 2015; WHO 2015).

The antiviral drugs being developed to inhibit viral processes and viral proteins include siRNAs targeting viral protein mRNAs, positively charged phosphorodiamidate morpholino oligomers (PMOplus), BCX4430, Favipiravir (T-705), CMLDBU3402, C-peptides, Cyanovirin-N (CV-N), adamantanes, benzodiazepine derivatives, and LJ001. siRNAs targeting the VP24, VP35, and L genes were made into a cocktail (TKM-Ebola) to inhibit viral replication. PMOplus is a treatment similar to siRNAs, but they have been optimized to resist degradation. PMOplus binds to the viral mRNA and prevents translation of these mRNAs via steric hindrance. The benefit of both siRNAs and PMOplus is that they can be synthesized quickly and changed if the virus mutates. The downside is that many doses are needed (between 7-15 doses) and they are not as stable as other options like proteins or other small molecules. Several of these drugs target the viral RNA polymerase and transcription. BCX4430 is an adenosine analog that results in RNA chain termination. Favipiravir (T-705) and CMLDBU3402 also inhibit the polymerase. These compounds are advantageous because they are broad spectrum, acting against other viruses that utilize an RNA-dependent RNA polymerase. The disadvantage is that they need a high number of doses which could potentially lead to toxicity issues. Several of these drugs work on the viral entry process, such as Cyanovirin-N (CV-N), C-peptides, benzodiazepine derivatives, adamantanes, and LJ001. CV-N works against filoviruses like it does against influenza by binding oligosaccharides present on the viral GP. This blocks the interaction between the GP and the host cell. One caveat is that when tested in mice it required several high doses to be effective. C-peptides, benzodiazepine derivatives, adamantanes, and LJ001 block the fusion step of viral entry. Both C-peptides and benzodiazepine
derivatives do so by preventing the conformational changes necessary for fusion. Adamantanes block
the fusion step by blocking the interaction between GP and NPC1. LJ001 blocks fusion by disrupting the
viral membrane which viruses are unable to repair (Wong et al. 2014; De Clercq 2015; Nyakatura et al.
2015; WHO 2015; Shurtleff et al. 2015).

The treatments meant to limit viremia and virus spread are monoclonal antibody (mAb)
cocktails such as ZMapp and the antibodies of convalescent patients. These antibody treatments work
mainly by either neutralizing the virus or activating the host complement system. ZMapp is a mAb
cocktail that consists of three different mAbs; c13C6, c2G4, and c4G7. c13C6 activates the complement
pathway while c2G4 and c4G7 work to neutralize the virus. This cocktail was able to protect non-human
primates from death due to Ebola infection and reduce the severe symptoms of the infection. mAb
therapy has previously shown a low rate of adverse reactions, however this particular cocktail has not
been thoroughly tested in humans. There is also an issue of efficacy should mutations in the virus arise.
Another tactic for treating Ebola is the use of either whole blood or plasma from convalescent Ebola
patients containing antibodies against the virus (Wong et al. 2014; Nyakatura et al. 2015; De Clercq

The last set of therapies is designed to boost the host immune response, such as vaccines.
According to the WHO, the number of vaccines being developed against Ebola is at least 15. There are 4
top candidates of these vaccines; rVSV-EBOV, cAd3-ZEBOV, a prime boost regimen of Ad26-EBOV and
MVA-EBOV, and a recombinant particle of EBOV-GP. The rVSV-EBOV candidate utilizes an attenuated
VSV that has one of its genes replaced with an Ebola GP gene. The cAd-ZEBOV vaccine uses an
adenovirus vector from chimpanzees which expresses two Ebola GPs, one from EBOV and one from
SUDV. Both of these vaccines began clinical trials in fall of 2014 and are now in phase III trials. The Ad26-
EBOV and MVA-EBOV is a two part vaccine, where patients receive two doses and each dose is a different vaccine. This vaccine has undergone phase I trials, and the recombinant EBOV-GP particle is set to begin phase I trials this year. The WHO is hoping to have an approved vaccine by the end of 2015 (Wong et al. 2014; De Clercq 2015; WHO 2015; Shurtleff et al. 2015).

1.10 siRNA screening

Genomic screening using RNA interference (RNAi) is a powerful tool that has been developed and utilized recently for various applications, from searching for genes involved in cancers and mitochondrial diseases to investigating host-pathogen interactions. RNAi is a gene silencing process dependent upon RNA which results in the destruction of an mRNA molecule via the RNA-induced silencing complex (RISC). RNAi is initiated when a short, double stranded RNA (dsRNA) molecule is processed by the Dicer enzyme into a small interfering RNA (siRNA). This siRNA is then split into two strands, the guide strand and the passenger strand. The guide strand becomes integrated into the RISC while the passenger strand is degraded. From there, the guide strand and RNAi complex find an mRNA molecule with a complementary sequence and degrade the mRNA molecule, thus silencing (knocking down) that gene. This technique has been utilized for large scale screens by many groups (Mohr et al. 2010; Iorns et al. 2007).

There are two main types of siRNA screens; a pooled format screen and an arrayed format screen. For a pooled format screen, the siRNA library is introduced into cells at random and then a selection is performed on the cells to determine which cells successfully incorporated the siRNAs. The researcher then must determine which genes were knocked down via PCR. This format is not conducive for a large scale high-throughput screen (HTS) because there are many steps involved and it is somewhat time consuming. The arrayed format screen is better suited for performing a HTS. In this
method, siRNAs against single genes are placed in particular wells of a microtiter plate, thus creating a specifically designed library of siRNAs. This method is advantageous because the investigator knows exactly which gene is in which well and the assay readout can be done with a plate reader to speed the process along (Mohr et al. 2010; Iorns et al. 2007).

There are several important factors to consider when designing an siRNA HTS. It is essential to include the proper controls, both negative and positive. One should also devise a way to determine if the results are reliable and reproducible. Data normalization is also essential when performing a large screen as well as deciding cut offs for ‘hits’. It is also important to consider that false positives might occur in the screen, and how to determine which hits are real. This has been an issue among previous host-pathogen screens, because there is very little overlap between the hits from various screens. With HIV-1 screens there is only 3 to 6% overlap, and a 1 to 12% overlap between the influenza screens (Friedel & Haas 2011; Bushman et al. 2009). This could be due to differences in the experiments such as cell types, virus strains, and libraries used. For these reasons, it is extremely important to consider all the variables and possible issues when designing a HTS with siRNAs.

1.11 Objectives of this study

The main objectives of this study can be divided into two parts: 1) basic research on both influenza and filoviruses that aims to elucidate host factors involved in viral entry and 2) translational research to identify new anti-viral therapies that target filoviruses. For part 1 we specifically looked at: 1) the role of the reticulon 4 receptor (RTN4R) in influenza viral entry and 2) the role of the tropomyosin receptor kinase B (TrkB) in filoviral entry. For part 2 we focused on 3) searching for anti-viral compounds in the form of both small compounds and plant extracts.
2. MATERIALS AND METHODS
2.1 **Cell lines, plasmids, and antibodies**

Human 293T embryonic kidney cells, A549 human lung epithelial cells, and HeLa cell lines were obtained from the ATCC. They were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum (GIBCO, Carlsbad, CA), 100 µg/mL streptomycin, 100 U penicillin (Invitrogen, Carlsbad, CA) and kept at 37°C with 5% CO₂. For infectious filovirus work, A549 cells (also obtained from the ATCC) were cultured for 3 days in T175 or T225 flasks (Corning, Tewksbury, MA) in culture media containing F-12K media (Kaighn’s modified Ham’s F-12 media, ATCC 30-2004) supplemented with 10% Fetal Bovine Serum (Hyclone, Logan, UT), 1% of penicillin/streptomycin (Sigma, St. Louis, MO).

The pseudovirions were generated using the following plasmids: hemagglutinin (HA), isolated from a A/Viet Nam/1203/2004 (H5N1) strain; neuraminidase (NA), isolated from a A/Puerto Rico/8/1934 (H1N1) strain; Marburg virus glycoprotein (GP); Ebola virus Zaire glycoprotein (GP); Vesicular stomatitis virus glycoprotein (VSV-G); Lassa virus glycoprotein (LASV-GP); and the HIV-1 proviral vector pNL4-3.Luc.R°E°, which was obtained through the National Institutes of Health (NIH) AIDS Research and Reference Reagent program. Cellular transfection of reticulon 4 receptor (RTN4R) was done using the RTN4R gene (Sino Biological Inc., Beijing, China) cloned into the pcDNA3.1+ plasmid. Transduction of the NTRK2/TrkB gene was performed using NTRK2 transcript variant A in pEZ-LV105 plasmid (GeneCopoeia, Rockville, MD) and the Δ8.2 HIV packaging construct plasmid, which was kindly provided by Tom Hope.

The rabbit polyclonal antibody AP12238c, which recognizes the central region (amino acids 305-334) of the RTN4R protein, was purchased from Abgent (San Diego, CA). The rabbit polyclonal antibody sc-8316, which recognizes amino acids 160-340 of the TrkB protein, was purchased from Santa
Cruz Biotechnology, Inc. (Dallas, TX). The mouse monoclonal anti-β actin antibody A5316, goat anti-mouse IgG HRP, and the goat anti-rabbit IgG HRP were purchased from Sigma (St. Louis, MO).

2.2 Production of pseudovirus and lentivirus

Human 293T cells were transiently co-transfected using a polyethyleneimine (PEI)-based protocol. For pseudovirus, cells were transfected with a pNL4.Luc.R-E- HIV reporter core plasmid and either the HA and NA expression plasmids, MARV GP, EBOV GP, VSV-G, or LASV GP expression plasmids. For lentivirus, cells were transfected with the Δ8.2 HIV packaging construct plasmid, the NTRK2/TrkB plasmid, and the VSV-G plasmid. Sixteen hours post transfection, media was removed and fresh phenol red–free DMEM with 10%FBS, 100µg/ml streptomycin, 100 U penicillin, and 4mM L-Glutamine was added. Forty-eight hours post transfection, the supernatant was collected and passed through a 0.45 µM pore size filter (Nalgene, Rochester, NY) and stored at 4°C prior to use.

2.3 Infectious filovirus

All work with infectious filoviruses was performed at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) at Fort Detrick, Frederick, MD, USA within maximum (biosafety level 4) containment. All filoviruses were propagated in Vero cells. Viral infectivity was titrated by plaque assays as previously described (Mudhasani et al. 2013).

2.4 siRNA screen and assay

The Silencer Select Human Druggable Genome siRNA Library V4, Human Druggable Genome siRNA Library V4 Extension Set, and Human Genome siRNA Library V4 Extension Set libraries were purchased from Ambion (Austin, TX). A549 cells were reverse transfected with 10 nM siRNAs and 0.1µl Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) in 384 well plates (CulturPlate; PerkinElmer,
Waltham, MA) according to the RNAiMAX recommended protocol with a Perkin Elmer Janus liquid handling system. Forty-eight hours post transfection, transfection media was removed and 30 μl of either MARV or Influenza pseudotyped virus was added. Media was changed 24 hours post infection, and 48 hours post infection 15 μl of NeoLite luciferase substrate (Perkin Elmer) was mixed in, incubated for 5 minutes, and luciferase was detected with a Perkin Elmer Envision plate reader (O’Hearn et al. 2015).

siRNAs to RTN4R, TrkB, ATP6VOC, NPC1, and the Non-targeting (NT) and Firefly Luciferase control siRNAs were obtained from Ambion. Reverse transfection of A549 cells was carried out in 96 well plates with 10 nM siRNA and 0.3 μl Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to recommended protocol. Forty-eight hours post transfection, transfection media was removed and 100 μl of pseudotyped virus was added. Media was changed 24 hours post infection, and 48 hours post infection luciferase was read using 50 μl of NeoLite substrate. Cell viability was read at 48 and 96 hours post transfection with 50 μl CellTiter-Glo Substrate (Promega, WI) according to the CellTiter-Glo protocol (O’Hearn et al. 2015).

2.5 Co-transfection/transduction and western blotting

For RTN4R blotting:

Human 293T cells were transfected with 10 nM siRNA using Lipofectamine RNAiMAX in 6 well plates. Twenty-four hours post transfection, 1 μg RTN4R plasmid was transfected in each well with PEI according to manufacturer’s instructions. Media was changed 16 hours post plasmid transfection to complete media. Forty-eight hours post plasmid transfection, cells were washed with PBS and then lysed with 0.2 ml Triton X-100 lysis buffer containing 50mM Tris-HCL (pH 7.5), 150 mM NaCl, 5mM EDTA, 1% Triton-X and a protease inhibitor cocktail. Samples were then subjected to SDS-PAGE and transferred to
a PVDF membrane. RTN4R was detected with polyclonal antibody AP12238c (Abgent, San Diego, CA) and β-actin with monoclonal antibody A5316 (Sigma, St. Louis, MO). The membrane was then probed with peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody, respectively. The protein bands were visualized by chemiluminescence (Thermo Scientific).

For TrkB blotting:

Human 293T cells were transduced with 200 ul of TrkB lentivirus in 6 well plates. Twenty-four hours post transduction, the cells were transfected with 10 nM siRNA using Lipofectamine RNAiMAX. Forty-eight hours post transfection the cells were washed with PBS and then lysed with 0.2 ml Triton X-100 lysis buffer containing 50mM Tris-HCL (pH 7.5), 150 mM NaCl, 5mM EDTA, 1% Triton-X and a protease inhibitor cocktail. Samples were then subjected to SDS-PAGE and transferred to a PVDF membrane. TrkB was detected with polyclonal antibody sc-8316 (Santa Cruz, Dallas, TX) and β-actin with monoclonal antibody A5316 (Sigma, St. Louis, MO). The membrane was then probed with peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody, respectively. The protein bands were visualized by chemiluminescence (Thermo Scientific).

2.6 Infectious influenza virus siRNA assay

Influenza A virus subtype A/PR/8/34 was used in this study as previously described (Basu et al. 2014). A549 cells were reverse transfected with siRNA as described in the previous section. Forty-eight hours post-transfection the cells were infected with influenza virus at an MOI of 1. The cells were incubated with virus for two hours at 37°C. After adsorption, virus was removed and the cells were washed and fresh Modified Eagles Medium (MEM) with 0.125% BSA, 1 mM HEPES, and 1 µg/ml TPCK-treated trypsin was added. Virus titer was measured 48 hours post-infection using the Influenza A NCP ELISA (Photometric) kit (Virusys, Taneytown, MD).
2.7 Y-27632 infection assay

A549 cells were seeded in a 96 well plate (5000 cells/well) 24 hours prior to experiment. Various concentrations of Y-27632 (Tocris, Bristol, UK) (1µM-150µM) were added to the wells for one hour at 37°C. Media was then removed and 100 µl of pseudovirus was added to the wells. Twenty-four hours post infection drug was re-applied to wells for one hour at 37°C, then the drug/media was removed and fresh media was added. Forty-eight hours post infection luciferase was read using 50 µl of NeoLite substrate. Cell viability was read at 48 hours post infection with 50 µl CellTiter-Glo Substrate.

2.8 RTN4R overexpression infection assay

Human 293T cells were seeded in a 96 well plate 6 hours prior to the experiment. Cells were then transfected with either 0.1 µg, 0.2 µg, or 0.4 µg of RTN4R plasmid in each well with PEI according to manufacturer’s instructions. Media was changed 16 hours post plasmid transfection to complete media. At 24 hours post transfection, media was removed and 100 µl of pseudovirus was added to the wells. Forty-eight hours post infection luciferase was read using 50 µl of NeoLite substrate. Cell viability was read at 48 hours post infection with 50 µl CellTiter-Glo Substrate.

2.9 Infectious filovirus siRNA Assay

siRNAs were transiently reverse-transfected into HeLa cells (10,000 cells per well) in six replicates at a 30 nM final concentration, using HiPerfect (Qiagen, Valencia, CA) in a 96-well format (Greiner, Monroe, NC). Media were removed the next day, cells were washed once with PBS, and then supplemented with fresh growth media. Cells were infected after 24 h with MARV Ci67 or EBOV Kikwit at a multiplicity of infection (MOI) of 0.5 or 5 in 50 µl of complete media for 1 h. Media were removed
48 h later, and cells were fixed with 10% formalin and stained for high-content quantitative image-based analysis. In 6 wells on each plate, cells were transfected with a negative control siRNA (non-targeting, NT).

2.10 **High-content quantitative image-based analysis for infectious filovirus**

Virus-infected cells were fixed in 10% buffered formalin for 72 h and blocked in 3% bovine serum albumin-PBS for 1 h. For analysis of the siRNA screen, Cells were then stained with murine monoclonal antibodies against EBOV or MARV GP1,2 (6D8 or 9G4 antibodies, respectively, 1:1,000 dilution in blocking solution), followed by Alexa Fluor 488-conjugated goat anti-mouse IgG (ThermoFisher) (1:1,000 dilution in blocking solution). All infected cells were also stained with Hoechst 33342 and HCS CellMask Red (ThermoFisher) for nuclei and cytoplasm detection, respectively.

High-content quantitative imaging data were acquired and analyzed on an Opera confocal reader (model 3842 and 5025; quadruple excitation high sensitivity; Perkin-Elmer), at two exposures using a ×10 air objective lens as described in (Radoshitzky et al. 2010). Analysis of the images was accomplished within the Opera environment using standard Acapella scripts. Nuclei and cytoplasm staining were used to determine total cell number and cell borders, respectively. Mock-infected cells were used to establish a threshold for virus-specific staining. Quantification of virus positive cells was subsequently performed based on mean fluorescent intensities in the virus-specific staining channel. Infection rates were then determined by dividing the number of virus positive cells by the total number of cells measured.
2.11 **Lentiviral transduction**

A549 cells were seeded into a 6 well plate. Cells were then transduced with 500 µl TrkB lentivirus and 1.25 µl Polybrene transduction reagent (Santa Cruz, Dallas, TX) per well. Forty-eight hours post transduction, media was removed and a 1µg/ml puromycin selection media (1µg/ml puromycin in complete DMEM) was added to the wells. Cells were kept and split with fresh selection media until a stable cell line was established. TrkB expression was verified via western blot.

2.12 **TrkB overexpression infection assay**

A549 cells which stably expressed TrkB (see lentiviral transduction) and non-transduced A549 cells were seeded in a 96 well plate (5000 cells/well) 24 hours prior to the experiment. Wells were infected with 100 µl of pseudovirus. Forty-eight hours post infection, luciferase was read using 50 µl of NeoLite substrate. Cell viability was read at 48 hours post infection with 50 µl CellTiter-Glo Substrate.

2.13 **Neurotrophin blocking assay**

A549 cells were seeded in a 384 well plate (1000 cells/well) 24 hours prior to the experiment. Soluble TrkB (Novoprotein, Summit, NJ), NGF (Sino Biological Inc., Beijing, China), BDNF, NT3, and NT4 (Prospec Bio, Rehovot, Israel) were added in various amounts to wells along with pseudovirus. Six hours post infection, virus and drug were removed and replaced with fresh media. Forty-eight hours post infection, luciferase was read using 20 µl of NeoLite substrate. Cell viability was read at 48 hours post infection with 20 µl CellTiter-Glo Substrate.

2.14 **TrkB inhibitor assay**

A549 cells were seeded in a 96 well plate (5000 cells/well) 24 hours prior to the experiment. Cells were then incubated with various concentrations of either GNF 5837 or ANA-12 (Sigma Aldrich, St.
Louis, MO) for 1 hour at 37°C. Media was then removed and pseudovirus with drug was then added to the wells. Twenty-four hours later virus was removed and fresh media was added to the wells. Forty-eight hours post infection, luciferase was read using 50 µl of NeoLite substrate. Cell viability was read at 48 hours post infection with 50 µl CellTiter-Glo Substrate.

2.15 **Compound screen and assay**

**Compound Screen:**

Nineteen thousand and two hundred compounds from the Chembridge DIVERSet Chemical Library were screened in this HTS. Three hundred twenty unique compounds were arrayed in a 384-well plate at a final concentration of 12.5 µM in DMSO. The positive control drug for this assay, azidothymidine (AZT; Sigma, St. Louis, MO), was at a final concentration of 5 µM. A549 cells were seeded into 384 well plates (1000 cells/well) 24 hours prior to infection. Cells were infected with either Marburg, Influenza H5N1, or Lassa pseudovirus along with compounds. Forty-eight hours post infection, luciferase was read using 15 µl of NeoLite substrate(Wang et al. 2013).

**Assay:**

A549 cells were seeded in 96 well plates (5000 cells/well) 24 hours prior to the experiment. 100 µl pseudovirus along with compound (10µM) were added to the wells. Forty-eight hours post infection, luciferase was read using 50 µl of NeoLite substrate. Cell viability was read at 48 post infection with 50 µl CellTiter-Glo substrate.
2.16 **Half maximal inhibitory concentration (IC\textsubscript{50}) and half maximal cytotoxicity concentration (CC\textsubscript{50}) assay**

Compounds and plant extracts were serially diluted for IC\textsubscript{50} and CC\textsubscript{50} evaluation. A549 cells were seeded in a 96 well plate (5000 cells/well) 24 hours prior to the experiment. For the IC\textsubscript{50}, cells were infected with either Marburg or Ebola pseudovirus along with compound or extract. Forty-eight hours post infection, luciferase was read using 50 µl of NeoLite substrate. For the CC\textsubscript{50}, cells received fresh media with compound or extract. Forty-eight hours later, viability was read using 50 µl CellTiter-Glo Substrate. IC\textsubscript{50} and CC\textsubscript{50} were determined by fitting the dose-response curves against infection of Marburg or Ebola pseudovirus or control DMSO wells, respectively, with four-parameter logistic regression in GraphPad.

2.17 **Compound and extract IC\textsubscript{50}/CC\textsubscript{50} assay with infectious filovirus**

To determine the IC\textsubscript{50} and CC\textsubscript{50} scores, compounds and plant extracts were serially diluted. A549 cells were seeded in a 384 well plate (3000 cells/well) 20 hours prior to the experiment. Cells were then treated with compounds or extracts and controls 2 hours before infection. For the compound assay, cells were then infected with EBOV Kikwit at a multiplicity of infection (MOI) of 2. For the plant extract assay, cells were infected with either EBOV Kikwit at an MOI of 0.5 or MARV Ci67 at an MOI of 15. After 48 hours, infection is terminated by fixing the samples in formalin and stained for high-content quantitative image-based analysis (see above). Analysis of the dose response curve in order to determine EC\textsubscript{50} was performed using GeneData Condoseo software by applying the Levenberg-Marquardt algorithm (LMA) for curve fitting strategy.
2.18 **Time of addition assay**

A549 cells were seeded in a 96 well plate (5000 cells/well) 24 hours prior to the experiment. Cells were spinoculated for one hour at 4°C at 1000 rpm to allow virus attachment to the cells. Virus was then removed and cells were washed twice with PBS, then fresh media was added to the wells, followed by a temperature shift to 37 °C to trigger virus entry. At various time points (-1hr, 0hr, 1hr, 2hr, 3hr, 4hr, 5hr, 6hr), compound (20 μM), heparin (10 μg/mL), or AZT (5 μM) was added to the wells to determine their role in viral entry in triplicate wells. The drug vehicle, DMSO, was used as a negative control. Forty-eight hours post infection, luciferase levels were determined.

2.19 **Sampling and preparation of plant materials and extracts**

Plants of the genus *Agrimonia* were collected from the South-Eastern region of China. The dried plant materials were processed via methanol extraction. These extracts were then fractionated over a silica gel column eluting with CHCl₃/Me₂CO/MeOH gradient solutions. Fraction 26 of the HPARB sample was further fractionated using a silica gel column. Fractions were then resuspended in DMSO to make a 4 mg/mL stock.

2.20 **Plant extract assay**

A549 cells were seeded in a 96 well plate (5000 cells/well) 24 hours prior to the experiment. Cells were infected with pseudovirus along with either extract (10 μg/mL), DMSO (0.25% final conc.), or AZT (5 μM). Forty-eight hours post infection, luciferase levels were determined.
3. THE RETICULON 4 RECEPTOR IS INVOLVED IN INFLUENZA VIRAL INFECTION
3.1 Introduction

Influenza A viruses, of the family Orthomyxoviridae, are enveloped viruses with a negative-sense, single-stranded, segmented RNA genome. Influenza virus infections result in the hospitalization of over 200,000 people each year in the United States and result in between 250,000 to 500,000 deaths worldwide (Fauci 2006; CDC 2015c). Aside from seasonal outbreaks of influenza, there have been several large pandemics throughout the past century that have caused widespread economic devastation and death. There were four major pandemics in the last 100 years, the 1918 Spanish Flu, the 1957 Asian influenza, the 1968 Hong Kong flu, and the 2009 H1N1 Swine flu. While there are vaccines produced seasonally and drugs available to combat influenza infection, the ease of influenza transmission and the emergence of drug-resistant strains limit their impact (Ekiert et al. 2009; Fauci 2006; Palese 2004).

The influenza virus encodes for eleven proteins: PB1, PB1-F2, PB2, PA, hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), M1, M2, NS1, and NEP. Influenza’s two major surface glycoproteins are NA and HA. NA is involved in viral egress from the cell by releasing viral progeny from the cell surface. HA is initially synthesized as a single polypeptide precursor, HA0. HA0 is then proteolytically cleaved into two subunits, HA1 and HA2, which are linked via disulfide bonding. The HA1 subunit is responsible for viral binding to sialic acid (SA) moieties as entry receptors while the HA2 subunit is responsible for viral fusion with the host membrane in the endosome. SA is attached to host membrane proteins via two main linkages, α2,3 or α2,6. The α2,3 linkage SA is predominantly found in the gut of avian species, while the α2,6 linkage is mainly found in cells in the human respiratory tract. Different HA subtypes prefer binding to either α2,3 or α2,6, and this is a major factor in species tropism. Upon HA1 binding to SA the virion is endocytosed via either clathrin-coated pits or nonclathrin, noncaveolae pathways. Once the pH drops in the endosome, viral proteins are then released into the
host cell cytoplasm and brought into the nucleus, where viral replication continues (Bouvier & Palese 2008; Shaw & Palese 2013; Rumschlag-Booms & Rong 2013).

Although SA has been identified as the major host receptor for the influenza virus, the proteins to which the SA is linked that trigger internalization are still largely unknown. To search for potential host factors involved in influenza viral entry, our lab recently completed a high-throughput screen (HTS) of the human genome using siRNA knockdown and pseudotyped H5N1 virus. From this screen we identified the reticulon 4 receptor (RTN4R) as a candidate host factor involved in influenza entry.

### 3.2 Results

#### 3.2.1 Knockdown of RTN4R using siRNA results in a reduction of influenza pseudovirus infection levels

To identify host factors involved in influenza and filovirus entry, our laboratory recently completed a high-throughput screen of the human genome. We screened 64,755 unique siRNAs which targeted 21,566 human genes using pseudotyped H5N1 influenza virus and Marburg virus in parallel. Based on the results of this primary screen, we identified the reticulon 4 receptor (RTN4R) as a factor involved specifically in influenza viral entry. There were 3 unique siRNAs targeting RTN4R used in the primary screen. While knockdown from siRNA-2 had no effect on the infection of either pseudotyped H5N1 influenza virus or Marburg virus, both siRNA-1 and -3 knockdown caused a reduction in influenza infection levels by more than half of that of the non-targeting (NT) siRNA (Figure 3a). Similar results were seen in the confirmation screen.

To validate the results from the primary and confirmation screens, we obtained new batches of RTN4R siRNAs -1 and -3 which were separately transfected into A549 cells. These cells were then infected with either influenza or Marburg pseudovirus and infection levels were assessed. Consistent
with the screening results, siRNAs targeting RTN4R had a greater effect on reducing influenza infection versus that of Marburg infection (not shown).

To confirm that the screen was valid for identifying host factors, we used a gene known to be involved in influenza and Marburg viral entry as a positive control. ATP6V0C encodes the 16 kDa subunit of the vacuolar (H+)\-ATPase (V-ATPase) that has been shown to play an important role in the acidification of the endosome which is a required step for the fusion processes of both influenza and Marburg virus during entry (Cotter et al. 2015; Forgac 2007; Hinton et al. 2009; König et al. 2010). Knockdown of ATP6V0C by both siRNA-1 and -2 resulted in more than 80% reduction in infection for H5N1 and Marburg pseudovirus, and while siRNA-3 gave similar results for influenza infection it had a reduced effect on Marburg infection (Figure 3b). As an example of the robustness of the siRNA screen, we assessed the results for Niemann-Pick disease, type C1 (NPC1), which was recently discovered to be required for filovirus infection (Carette et al. 2011; Côté et al. 2011). As seen in Figure 3c, knockdown of NPC1 via siRNA resulted in a specific reduction in Marburg pseudovirus infection for all three siRNAs.

Together, these results demonstrate that the results from the HTS are valid, and that knockdown of RTN4R via siRNA results in a specific reduction of influenza infection.
Figure 3. siRNA knockdown of RTN4R, ATP6VOC, and NPC1 in primary high-throughput screen results in a reduction in pseudotyped virus infection. (a) Introduction of siRNAs targeting RTN4R resulted in reduced infection for influenza H5N1 pseudovirus. The siRNAs of RTN4R (labeled as 1, 2, and 3) were transfected into A549 cells, and their effects on influenza H5N1 or Marburg pseudovirus were assessed as described in Materials and Methods. Data were normalized to non-targeting (NT) siRNA. (b) Same as in (a), except with ATP6VOC siRNAs. (c) Same as in (a), except with NPC1 siRNAs.
3.2.2 Reduction in influenza infection levels is not due to siRNA toxicity

To ensure that this reduction in infection was not due to toxicity from the siRNAs, we performed a cell viability assay at 48 and 96 hours post transfection. Cell viability was minimally affected at both time points, showing that the siRNAs are not toxic to the cells (Figure 4). This suggests that the decrease in infection was likely due to the siRNAs knocking down the RTN4R gene, not from cellular toxicity.
Figure 4. RTN4R siRNAs are not toxic to A549 cells. Introduction of siRNAs targeting RTN4R (1 and 3) did not result in a reduction in cell viability at 48 and 96 hours post transfection. The siRNAs against RTN4R, and non-targeting (NT) were transfected into A549 cells, and their effects on A549 cell viability were assessed as described in Materials and Methods. Data were normalized to non-targeting (NT) siRNA. Error bars represent standard deviations.
3.2.3 **RTN4R siRNA knockdown specifically results in a reduction of RTN4R protein levels**

To confirm that the siRNA knockdown leads to a reduction in RTN4R protein levels, a western blot was performed. Due to initial difficulties detecting any level of the protein in either A549 or 293T cells, we utilized a co-transfection system as described in the Materials and Methods section. Briefly, 293T cells were transfected with 10nM of either RTN4R or NT siRNA followed by 1µg of RTN4R plasmid DNA 48 hours later. Knockdown with RTN4R siRNA-1 and -3, shown in lanes two and three of Figure 5, both resulted in a clear reduction of RTN4R protein as compared to NT siRNA, shown in lane one. This shows that the RTN4R siRNAs are specifically targeting the RTN4R mRNA, leading to a reduction in protein levels.
Figure 5. RTN4R siRNA knockdown results in a reduction of RTN4R protein levels. The siRNAs of RTN4R resulted in a reduction of the protein level of RTN4R in A549 cells. An RTN4R plasmid and RTN4R (siRNAs 1 and 3) or non-targeting (NT) siRNAs were cotransfected into A549 cells, and the RTN4R level was evaluated by Western blot analysis. In this experiment 1 µg of RTN4R plasmid DNA was used. β-actin was used as a control.
3.2.4 Knockdown of RTN4R using siRNA results in a reduction in infectious H1N1 influenza infection

To validate that RTN4R is involved in viral entry with infectious influenza virus, siRNA treated cells were infected with infectious influenza virus subtype A/PR/8/34 (H1N1) and infection was assessed. Knockdown of RTN4R via siRNA-1 and -3 resulted in an 80% reduction in infection, similar to the level of reduction by the ATP6V0C siRNA (Figure 6). This reduction in infection agrees with our results from the pseudovirus assays and confirms the involvement of RTN4R in influenza infection.
Figure 6. siRNA knockdown of RTN4R results in a reduction in infectious H1N1 influenza infection. siRNAs against RTN4R, ATP6VOC, and non-targeting (NT) were transfected into A549 cells, and their effects on influenza H5N1 or Marburg pseudovirus were assessed as described in Materials and Methods. ATP6VOC and NT were used as controls. Data were normalized to non-targeting (NT) siRNA. Error bars represent standard deviations.
3.2.5 ROCK inhibitor Y-27632 does not reduce influenza infection levels

RTN4R is a member of a signaling complex located on the host cell membrane. When a ligand binds to the receptor complex, signaling is triggered through RhoA, which then activates Rho-associated protein kinase (ROCK), which then signals through different pathways to alter the cell morphology in various ways (Figure 7) (Schwab 2010; Blöchl & Blöchl 2007; Ren et al. 1999). To determine if signaling from the RTN4R complex was playing a role in the entry of influenza virus, we utilized an inhibitor of ROCK. This inhibitor, Y-27632, selectively inhibits ROCK by competing with ATP binding in the catalytic site of the protein. For this assay, A549 cells were treated for one hour pre-infection with various concentrations (1µM-150µM) of Y-27632. Drug was removed and virus was added to the wells. Twenty-four hours post infection drug was re-applied to the cells for one hour and then removed. Luciferase levels were read 48 hours post infection as well as viability levels.

At all of the concentrations tested except the highest concentration, viral infection was not reduced but was slightly enhanced to varying degrees. At the 150 µM concentration viral infection was slightly reduced, but this correlated with the reduction in cell viability at that concentration (Figure 8a, b). These results suggest that signaling from the RTN4R complex through ROCK is not involved in influenza infection.
Figure 7. Schematic of the RTN4R signaling pathway.
Figure 8. Inhibition of ROCK signaling does not affect influenza infection. (a) The ROCK inhibitor Y-27632 was evaluated against influenza H5N1 pseudovirus infection at concentrations ranging from 1 µM to 150 µM as described in Materials and Methods. Data were normalized to untreated infection wells. Error bars represent standard deviations. (b) Y-27632 was evaluated for toxicity in A549 cells at various concentrations as described in Materials and Methods. Data were normalized to untreated cells. Error bars represent standard deviations.
3.2.6 RTN4R overexpression enhances influenza infection levels in a dose sensitive manner

To determine if increasing the amount of RTN4R protein on the cell surface would increase infection levels, we overexpressed RTN4R via a plasmid followed by infection. Briefly, 293T cells were transfected with either 0.1, 0.2, or 0.4 µg of RTN4R expression plasmid using the standard PEI protocol. Twenty-four hours later cells were infected with virus. Forty-eight hours post infection, luciferase levels were measured.

At the lowest level (0.1 µg) of plasmid used, infection was increased by 100% over that of cells treated with vector alone. At 0.2 µg, infection levels were increased by 50% over cells with vector alone. At the highest concentration (0.4 µg), infection was only increased by about 25% (Figure 9). Marburg infection levels were not affected at any concentration. This reduction in influenza infection as the RTN4R levels increased suggests that there is a delicate balance required between the levels of RTN4R present on the cell surface and the ability for influenza to utilize these proteins for enhanced entry.
Figure 9. RTN4R overexpression enhances influenza infection in a dose dependent manner. An RTN4R plasmid was transfected into A549 cells in various concentrations (0.1 µg to 0.4 µg), and pseudovirus infection levels were assessed as described in Materials and Methods. Data were normalized to empty vector control. Error bars represent standard deviations.
3.3 Discussion

The role of the reticulon 4 receptor in influenza viral entry was explored in this study. Our results show that knockdown of RTN4R via siRNA leads to a reduction in influenza infection levels while Marburg infection remains steady. This reduction in influenza infection is not due to toxicity from the siRNA. We also showed that the knockdown with RTN4R siRNA results in a reduction at the protein level as well, confirming the specificity of the siRNAs. When tested against infectious influenza virus, the siRNA knockdown also caused a reduction in infection levels. Overexpression of RTN4R leads to an enhancement in influenza infection. Based on these results, we believe that RTN4R plays a role in influenza infection, and our study supports this hypothesis.

It has been long known that the influenza glycoprotein hemagglutinin binds to sialic acid to initiate infection. What is not known is which host proteins these sialic acids are linked to, or what other factors are involved in the entry of influenza. We recently screened an siRNA library of the human genome to determine which host factors are important for influenza entry into host cells. Based on the results from this screen, we identified RTN4R as a putative host factor.

RTN4R, also known as NgR1 or NogoR, is a glycosylphosphatidylinositol (GPI)-anchored protein that is highly expressed on cells in the central nervous system (CNS) but is also expressed in other areas of the body such as the lung (Mi et al. 2004; Yamashita et al. 2005; Schwab 2010). RTN4R is a part of a receptor complex on the cell surface, and initiates cell signaling once it binds one of its ligands. In addition, parts of this receptor complex have been shown to be internalized upon ligand binding (Bronfman et al. 2003; Joset et al. 2010). The most studied and well known function of RTN4R is its ability to inhibit neuronal growth and cause growth cone collapse upon ligand binding after injury. It is part of a receptor/signaling complex localized in the cell membrane (McDonald et al. 2011). This is
significant because influenza viruses have been shown to associate with lipid rafts (Rossman & Lamb 2011). The interactions between the proteins in this complex are mediated by gangliosides - specifically the sialic acid terminal residues on the gangliosides (Saha et al. 2011). The receptor complex consists of the LRR and Ig domain containing, nogo receptor-interacting protein (LINGO-1), the p75 neurotrophin receptor (p75), and the TNF-Receptor Super family member 19 (TROY) (Saha et al. 2011; Joset et al. 2010). LINGO-1 is a transmembrane glycoprotein with a cytoplasmic tail that contains an epidermal growth factor receptor-like tyrosine phosphorylation site (Mi et al. 2004). p75 is also a transmembrane glycoprotein and is the signal-transducing member of the complex (Wang et al. 2002). TROY has similar function to p75 and has been shown to be interchangeable with p75 in the receptor complex (McDonald et al. 2011). RTN4R has recently been identified as potential receptor for mammalian reovirus, a neurotropic virus (Konopka-Anstadt et al. 2014). p75 has been identified as a host factor involved in rabies virus infection (Gluska et al. 2014).

Our results demonstrate that siRNA knockdown of RTN4R specifically reduces influenza infection while showing no effects of cytotoxicity. These results were confirmed with infectious influenza virus subtype A/PR/8/34 (H1N1). While overexpression of RTN4R via plasmid led to an increase in viral infection, this was only seen with lower levels of overexpression, with a decrease in enhancement being seen as more RTN4R is introduced. There are several plausible reasons as to why this is occurring. It is possible that higher levels of RTN4R cause an increase in endocytosis of the entire complex, actually reducing the amount of available RTN4R on the surface. It is also possible that even though there is more RTN4R on the surface, the other members of the complex are not being overexpressed to similar levels, causing the number of complete signaling complexes to plateau at a certain point. It would be of interest to determine if overexpression of the other members of the complex in addition to RTN4R results in an increase in infection.
Upon ligand binding, the RTN4R receptor complex initiates signaling through p75/TROY, activating RhoA. RhoA then activates ROCK which then activates various different pathways that have an effect on cell morphology. RhoA and ROCK signaling has been implicated in several different viral life cycles, including respiratory syncytial virus (RSV), Kaposi’s sarcoma-associated herpesvirus (KSHV), HIV, rotavirus, and herpes simplex virus (HSV). (Gower et al. 2001; Pastey et al. 1999; Shepard et al. 2001; Sharma-walia et al. 2004; Krogh et al. 2015; Zambrano et al. 2012; Zheng et al. 2014). Based on this information, we initially thought signaling from the receptor complex was involved, however, the experiments which inhibited ROCK showed no reduction in infection. These results suggest that RTN4R plays a role in influenza viral entry, but not through the RhoA/ROCK signaling pathway. It is quite possible that there is another signaling pathway other than the ROCK pathway that is being utilized for entry, or influenza could be binding and inducing endocytosis through the complex in a more transient manner.

Notably, when we looked at the screening data for the rest of the members of the complex to determine if they were involved in influenza viral entry, we discovered that they do seem to play a role. While our screen did not indicate LINGO-1 had an effect on infection, it was identified as a host factor in influenza infection in a separate screen performed by Karlas et al in 2010. Also, knockdown of p75 and TROY significantly reduced infection of both influenza virus and Marburg virus in our siRNA screen (Figure 10)(Karlas et al. 2010). This suggests that the whole receptor complex is important for viral entry and infection, with RTN4R being specific to influenza virus.
Figure 10. siRNA knockdown of members of the RTN4R receptor complex in primary high-throughput screen results in a reduction in pseudotyped virus infection. Introduction of siRNAs targeting p75 (siRNAs 1 and 2) and TROY (siRNAs 2 and 3) resulted in reduced infection for influenza pseudovirus, while LINGO-1 had no effect on either pseudovirus. The siRNAs against RTN4R, p75, LINGO-1, and TROY (labeled as 1, 2, and 3) were transfected into A549 cells, and their effects on influenza H5N1 or Marburg pseudovirus were assessed as described in Materials and Methods. Data were normalized to non-targeting (NT) siRNA.
These findings suggest that RTN4R is involved in influenza viral entry. Hopefully this knowledge will allow us to further understand the complicated life cycle of influenza and better help us understand how to combat this virus.

_Infectious influenza virus work in this chapter of the thesis was done by Dr. Arnab Basu, our collaborator at Microbiotix, Inc., Worcester, MA._
4. THE TROPOMYOSIN RECEPTOR KINASE B IS INVOLVED IN FILOVIRAL INFECTION
4.1 Introduction

Ebola and Marburg viruses are members of the family Filoviridae. They are enveloped viruses with a single-stranded, negative-sense, non-segmented, 19kb genome. Filovirus outbreaks occur intermittently causing very severe viral hemorrhagic fever, and can have a case fatality rate of up to 90%. These viruses are classified as biosafety level 4 pathogens due to their high fatality rate and the fact that there are no FDA approved treatments available (Feldmann et al. 2013). The current Ebola virus epidemic in Western Africa is by far the most severe since the discovery of the virus in 1976, having infected over 28,000 people with a fatality rate of 41% (CDC 2015b).

The filoviral genome encodes for seven proteins in both Ebola and Marburg, as well as two other secreted proteins in Ebola. These seven genes are: the nucleoprotein (NP), VP35, VP40, glycoprotein (GP), VP30, VP24, and the polymerase protein (L). The secreted proteins in Ebola are soluble forms of the GP, sGP and ssGP (CDC 2014f; Feldmann et al. 2013). GP is a transmembrane glycoprotein present on the viral envelope. It is the viral protein that is responsible for both binding to the host cell as well as fusion with the host cell membrane. GP is synthesized as a precursor protein, GP₀. It is glycosylated by cellular machinery, then gets cleaved by furin into two subunits, GP₁ and GP₂. The GP₁ subunit is the portion that contains the receptor binding domain and therefore is responsible for binding to host cells. The GP₂ subunit is the part which controls viral fusion with the host cell (Feldmann et al. 2013; Hofmann-Winkler et al. 2012; Miller & Chandran 2012).

Though numerous host proteins have been implicated in various steps of filovirus entry and infection, including: the TAM family of tyrosine kinase receptors, C-type lectins (DC-SIGN, ASPGR-1, LSECtin, hMGL), α5β1-Integrin, Cathepsin B, Cathepsin L, TIM-1, NPC1, and EXT1, the entry mechanism of filoviruses is still poorly understood. In an effort to identify host factors involved in filoviral entry, we
conducted a high-throughput siRNA screen of the human genome. We executed this high-throughput screen (HTS) using pseudotyped Marburg virus along with influenza H5N1 pseudovirus. From this screen we identified the tropomyosin receptor kinase B (TrkB) as a candidate host factor in filovirus entry.

4.2 Results

4.2.1 Knockdown of TrkB using siRNA results in a reduction of filoviral infection levels

To discover host factors that play a role in filovirus entry, our laboratory completed a high-throughput screen of the human genome. We screened over 64,000 unique siRNAs which targeted over 21,000 human genes using pseudotyped H5N1 influenza virus and Marburg virus in parallel. Based on the results of this primary screen, we identified the tropomyosin receptor kinase B (TrkB) as a factor involved specifically in filovirus entry. The library included 3 unique siRNAs which targeted TrkB in the primary screen. Knockdown from all three of the siRNAs in the primary screen resulted in a reduction in Marburg pseudovirus infection by at least 60% compared to that of the non-targeting (NT) siRNA. siRNA-2 reduced influenza infection to similar levels of Marburg, but siRNAs -1 and -3 had less effect on influenza infection (Figure 11a). We observed similar results in the confirmation screen (not shown).

To confirm the results from the primary and confirmation screens, we ordered new sets of siRNAs -1, -2, and -3 and individually transfected them into A549 cells. Influenza or Marburg pseudovirus was then used to infect these cells and after 48 hours, infection levels were assessed. These siRNAs reduced Marburg infection greater than that of influenza infection, which was consistent with the results of the screen (Figure 11b).

To examine if TrkB was involved in Ebola virus infection as well as other viruses such as Lassa virus (LASV) and vesicular stomatitis virus (VSV). We performed another siRNA knockdown assay and
infected with these pseudoviruses. We found that Ebola virus infection was reduced by at least 65% for all three siRNAs, similar to the results seen with Marburg virus. LASV infection was reduced by at least 60% for siRNAs -1 and -2, and by about 40% for siRNA-3. VSV infection was not reduced with siRNA-3 and was only reduced by about 30% for siRNAs -1 and -2 (Figure 11c).
**Figure 11.** siRNA knockdown of TrkB results in a reduction in pseudotyped Marburg, Ebola, and Lassa virus infection. (a) Introduction of siRNAs targeting TrkB resulted in reduced infection for Marburg pseudovirus in our primary siRNA screen. The siRNAs of TrkB (labeled as 1, 2, and 3) were transfected into A549 cells, and their effects on influenza H5N1 or Marburg pseudovirus were assessed as described in Materials and Methods. Data were normalized to non-targeting (NT) siRNA. (b) New siRNAs targeting TrkB were synthesized and tested with similar results to the primary screen. Same method as in (a). Error bars represent standard deviations. (c) TrkB siRNAs caused a reduction in infection for Ebola and Lassa pseudovirus as well. Same method as in (a). Error bars represent standard deviations.
It is important to note that siRNAs against TrkA showed no reduction in infection, while knockdown of TrkC actually resulted in an increase in infection for two siRNAs and a greater than 50% reduction for both influenza and Marburg for siRNA-3 (Figure 12). As mentioned in the previous chapter, siRNA knockdown of p75 reduces infection for both Marburg and influenza pseudovirus.
**Figure 12.** siRNA knockdown of Trk family receptors. TrkB is the only family member whose siRNA knockdown resulted in reduced infection for Marburg pseudovirus in our primary siRNA screen. The siRNAs were transfected into A549 cells, and their effects on influenza H5N1 or Marburg pseudovirus were assessed as described in Materials and Methods. Data were normalized to non-targeting (NT) siRNA.
4.2.2  **Reduction in filoviral infection levels is not due to siRNA toxicity**

To exclude the possibility that the reduction in infection seen from siRNA knockdown was caused by siRNA toxicity, we evaluate the effect of these siRNAs on cell viability 96 hours post siRNA transfection. We found that cell viability was not affected by the siRNA transfection (Figure 13). This suggests that the reduction in infection that we saw was not due to siRNA toxicity, but was likely a result of the siRNAs specifically knocking down the TrkB gene.
TrkB siRNAs are not toxic to A549 cells. Introduction of siRNAs targeting TrkB (1, 2, and 3) did not result in a reduction in cell viability at 96 hours post transfection. The siRNAs against TrkB, and non-targeting (NT) were transfected into A549 cells, and their effects on A549 cell viability were assessed as described in Materials and Methods. Data were normalized to either non-targeting (NT) siRNA or transfection reagent (RNAiMAX) alone. Error bars represent standard deviations.

**Figure 13.** TrkB siRNAs are not toxic to A549 cells. Introduction of siRNAs targeting TrkB (1, 2, and 3) did not result in a reduction in cell viability at 96 hours post transfection. The siRNAs against TrkB, and non-targeting (NT) were transfected into A549 cells, and their effects on A549 cell viability were assessed as described in Materials and Methods. Data were normalized to either non-targeting (NT) siRNA or transfection reagent (RNAiMAX) alone. Error bars represent standard deviations.
4.2.3 **TrkB siRNA specifically results in a reduction of TrkB protein levels**

To verify that the siRNA knockdown is resulting in a reduction of TrkB protein levels, western blot analysis was used to detect TrkB protein. We initially had trouble detecting any level of TrkB protein in either A549 or 293T cells, so we introduced a TrkB-encoding plasmid into the cells via transduction with a lentivirus vector as described in the materials and methods section. Briefly, TrkB lentivirus was produced and collected from 293T producer cells. New 293T cells were then transduced with TrkB lentivirus. Twenty-four hours post transduction, the 293T cells were transfected with 10nM of either TrkB or NT siRNA. Cell lysates were collected forty-eight hours post transfection and a western blot was performed. Knockdown with TrkB siRNAs -1, -2, and -3 showed a reduction in TrkB protein levels as compared to NT siRNA, however the knockdown was not complete as there is still faint evidence of TrkB protein seen (Figure 14). This shows that the siRNAs are specifically targeting TrkB, resulting in a reduction of the TrkB protein levels.
TrkB siRNA knockdown results in a reduction of TrkB protein levels. The siRNAs of TrkB resulted in a reduction of the protein level of TrkB in the target cells. A TrkB plasmid was used to create a lentivirus which was transduced into 293T cells. TrkB (#1, #2, and #3) or non-targeting (NT) siRNAs were the transfected into the cells, and the TrkB level was evaluated by Western blot analysis. β-actin was used as a control.
4.2.4 **Knockdown of TrkB using siRNA results in a reduction in infectious filovirus infection**

To validate the siRNA knockdown results with pseudovirus infection, our collaborators at USAMRIID tested our siRNAs against infectious Marburg (Ci67 strain) in their BSL-4 facility. We found that knockdown with siRNA-1 resulted in a 50% reduction in infection, and siRNA-3 reduced infection by about 60% at an MOI of 0.5. Surprisingly, siRNA-2 showed no effect at an MOI of 0.5. The siRNAs were also tested against infection at an MOI of 5, and showed less of a reduction in infection. Both siRNAs-1 and -3 resulted in about 40% reduction in infection and siRNA-2 resulted in a 20% reduction in infection (Figure 15). While the results with infectious virus are not completely consistent with the pseudovirus results, the trend is similar in that siRNAs-1 and -3 reduced Marburg virus infection by at least 50%. We are unsure why siRNA-2 reduced pseudotyped virus infection yet had little effect on infectious virus.
siRNA knockdown of TrkB results in a reduction in infectious Marburg (Ci67) virus infection. siRNAs against TrkB were transfected into HeLa cells, and their effects on infectious Ci67 Marburg virus were assessed as described in Materials and Methods. NT was used as a control. Data were normalized to non-targeting (NT) siRNA. Error bars represent standard deviations.

Figure 15. siRNA knockdown of TrkB results in a reduction in infectious Marburg (Ci67) virus infection. siRNAs against TrkB were transfected into HeLa cells, and their effects on infectious Ci67 Marburg virus were assessed as described in Materials and Methods. NT was used as a control. Data were normalized to non-targeting (NT) siRNA. Error bars represent standard deviations.
4.2.5  **TrkB overexpression does not enhance filoviral infection**

To determine if increasing the amount of TrkB protein on the cell surface would increase infection levels, we overexpressed TrkB in A549 cells. To do this, a stable A549 cell line expressing TrkB was created. To create this cell line, A549 cells were transduced with TrkB lentivirus vector using the Polybrene transduction reagent. Forty-eight hours later media was replaced with puromycin selection media to select for cells that had been successfully transduced with the lentivirus. Cells were maintained in this selection media until a stable line was established and confirmed via western blot. These transformed cells were then infected with either influenza, Marburg, Ebola, LASV, or VSV pseudovirus in parallel with untransformed A549 cells and forty-eight hours later infection levels were assessed. Interestingly, none of the viral infection levels were enhanced by the increase in TrkB protein in the cells (Figure 16).
Figure 16. TrkB overexpression does not affect pseudovirus infection. A stable cell line expressing TrkB was created using lentivirus in A549 cells. Cells were then infected with pseudovirus and infection levels were assessed as described in Materials and Methods. Data were normalized to A549 cells that were not transduced with the lentivirus. Error bars represent standard deviations.
4.2.6 Brain-derived neurotrophic factor (BDNF) blocks Marburg pseudovirus infection

It is possible that infection by Marburg pseudovirus could be blocked through competition from the ligands that naturally bind to TrkB (Figure 17a). To assess this, a competition assay was performed. Briefly, either Marburg or influenza pseudovirus was combined with varying amounts of the different neurotrophins (NGF, BDNF, NT3, and NT4) as well as soluble TrkB and then added to A549 cells for infection. Six hours later, virus and protein were removed from the cells and replaced with fresh media. Forty-eight hours post infection we assessed infection and viability levels. We found that BDNF ligand did block Marburg pseudovirus infection in a dose dependent manner while having no effect on influenza infection (Figure 17b). While BDNF was able to compete with Marburg pseudovirus and affect entry and infection, the other neurotrophins as well as soluble TrkB resulted in no reduction in Marburg infection (not shown). These results demonstrate that BDNF, the ligand which has the greatest affinity for TrkB, is able to compete with Marburg GP for binding to the protein, while the other ligands with less affinity cannot. This suggests that Marburg GP has a higher affinity for TrkB binding than its other ligands, NT3 and NT4.
Figure 17. Soluble BDNF is able to inhibit Marburg pseudovirus infection in a dose-dependent manner. (a) Schematic of Trk family ligands. (b) Pseudotyped H5N1 influenza and Marburg virus were mixed with various concentrations of BDNF; the mixtures were then used to challenge A549 cells and the effects of BDNF was assessed as described in Materials and Methods. Error bars represent standard deviations.
4.3 Discussion

In this study, the role of the tropomyosin receptor kinase B in filovirus entry was investigated. Our results show that siRNA knockdown of TrkB leads to a reduction in both Marburg and Ebola pseudovirus, with little toxicity to the cells. When tested against infectious Marburg virus, knockdown also resulted in a reduction in infection. We also show that knockdown with TrkB siRNAs is causing a reduction at the protein level. The major ligand for TrkB, BDNF, was able to compete with Marburg pseudovirus for binding to TrkB, resulting in a dose-dependent reduction in infection. These results strongly suggest that TrkB is involved in the viral entry step of filoviral infection.

There have been many host factors implicated in filoviral entry throughout the years, including C-type lectins, integrins, NPC1, EXT1, and GPCRs. Despite the knowledge of these factors, we still have a poor understanding of the filovirus entry process. Our laboratory recently completed a high-throughput screen of the human genome to elucidate previously unknown host factors involved in filovirus entry. From this screen, we identified TrkB as a potential host factor.

TrkB is a transmembrane protein that is a member of a family of three receptor tyrosine kinases; TrkA, TrkB, and TrkC, that are prominent in neurons but are also expressed in other cells throughout the body. The ligands for these receptors are the neurotrophins. These include the nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins 3 and 4 (NT-3, NT-4). Signaling from these ligand-receptor interactions regulate many processes throughout the cell. These processes include cell survival, proliferation, cell differentiation, remodeling of the cytoskeleton, membrane trafficking and fusion, as well as exo- and endocytosis of vesicles. These three receptors are also able to interact and signal with another transmembrane receptor, the pan-neurotrophin receptor p75 (p75) which was mentioned in the previous chapter as a signaling partner for RTN4R as well. p75 can bind to the
neurotrophins which activates a different set of pathways, and it can also bind to and signal with the Trk receptors as heterodimers (Huang & Reichardt 2003; Kaplan et al. 2000; Bucci et al. 2014). TrkA involvement has been implicated in herpes simplex virus (HSV) infection, and while TrkB was identified in our screen as a putative filoviral host factor, TrkA and TrkC were not (Cabrera et al. 2015).

The TrkB ligands bind to the dimerized receptors, which results in a phosphorylation of cytoplasmic tyrosine residues on the receptors. The ligand-receptor complexes are then internalized via either clathrin-coated pits or macropinocytosis and are then able to signal from within the endosome. This signaling activates one of three pathways; the PI3-Kinase pathway, the MAP Kinase pathways, or the PLC-γ pathway. This is significant because filovirus infection has been shown to utilize both the PI3K and PLC-γ pathways. Signaling through these pathways requires many different adaptor molecules. Some of these adaptors, such as Cdc42, Rac-1, Rho, and Rab GTPases, have been found to be utilized during filoviral infection as well (Huang & Reichardt 2003; Kaplan et al. 2000; Bucci et al. 2014; Nanbo et al. 2010; Saeed et al. 2010; Saeed et al. 2008). From this information we hypothesized that TrkB plays a role in filoviral infection, and this study supports this.

We have shown that knockdown of TrkB via siRNA causes a reduction in pseudotyped filovirus infection, as well as a reduction in Lassa pseudovirus infection. While siRNA-2 did cause a reduction in influenza virus similar to that of Marburg and Ebola, the other two siRNAs were less effective against it, and there was even less of an effect on VSV pseudovirus infection for all three siRNAs. Two of the siRNAs also reduced infection when tested against infectious Marburg virus, confirming that TrkB is involved in infection. Overexpression of TrkB does not result in an enhancement in filovirus infection, nor an increase in any other pseudovirus tested. There are a few possible reasons for this. It is plausible that the level of TrkB present of the cell surface is adequate and optimal for infection. Also, we are not
increasing the amount of downstream effectors, which could create a possible bottleneck. Another plausible reason is that Marburg is binding to a TrkB/p75 heterodimer, and since we are not increasing the level of p75, the rate of infection remains the same.

To determine if Marburg virus is competing with the natural ligands of TrkB for binding, we performed a competition assay. We found that BDNF protein does inhibit Marburg infection in a dose-dependent manner, while having no effect on influenza. When tested, none of the other neurotrophins tested nor soluble TrkB had an effect on Marburg infection, which could be due to a few different factors. It was not expected that NGF would compete with Marburg since it is not a ligand of TrkB, but is a ligand of TrkA. While NT4 is able to bind TrkB as a ligand, it has a much lower affinity for TrkB than BDNF. Perhaps this lower affinity makes it less able to compete with Marburg for binding to the TrkB receptor. As far as NT3 and soluble TrkB go, they both actually seemed to enhance Marburg infection in a dose-dependent manner. It is possible that NT3 and Marburg bind to the receptors at different places, and are able to bind at the same time and get endocytosed together with the receptor. It is unclear why soluble TrkB would result in an increase in infection.

We were interested to see if signaling from TrkB was required for Marburg infection. To determine this, we used two inhibitors, ANA-12 and GNF 5837. TrkB can signal through three different pathways upon activation by BDNF; the Ras/MAPK pathway, the PI3K/Akt pathway, or the PLCγ pathway (Figure 18) (Huang & Reichardt 2003). ANA-12 specifically inhibits signaling from TrkB, while GNF 5837 is a pan Trk inhibitor, inhibiting TrkA, B, and C (Cazorla et al. 2011; Albaugh et al. 2012). At the lowest concentration we saw no reduction in Marburg infection, and as we increased the dose, the toxicity increased as well (Figure 19). Due to this increase in toxicity from both drugs, we cannot conclude whether signaling from TrkB is involved in Marburg infection.
Figure 18. Schematic of the TrkB signaling pathways.
Figure 19. Inhibitors of TrkB exhibit toxicity to cells at higher doses. (a) A549 cells were treated with various concentrations of ANA-12 and infected with pseudotyped H5N1 influenza or Marburg virus and the effects of ANA-12 were assessed as described in Materials and Methods. Data were normalized to vehicle (DMSO). Error bars represent standard deviations. (b) A549 cells were treated with various concentrations of ANA-12 and cell viability was assessed as described in Materials and Methods. Data were normalized to vehicle (DMSO) or untreated cells. Error bars represent standard deviations. (c) Same as in (a) but using GNF 5837. (d) Same as in (b) but using GNF 5837.
Interestingly, it has been shown that TrkB is able to signal with at least two different G-protein coupled receptor (GPCR) ligands. The ligands for the A2a receptor and PAC1 receptor, adenosine and pituitary adenylate cyclase-activating polypeptide (PACAP), respectively, were shown to be able to activate TrkB in the absence of its natural ligands (Rajagopal et al. 2004; Lee & Chao 2001; Francis S Lee et al. 2002; Francis S. Lee et al. 2002). This is significant because our lab recently showed that GPCR antagonists are able to inhibit filovirus infection. Perhaps there is a link between the ability of TrkB to signal with GPCR ligands and our findings that GPCR antagonists significantly inhibit infection.

The findings presented in this chapter suggest that TrkB is involved in filovirus entry. Hopefully this work will enable us to further understand the complicated life cycle of Marburg and Ebola virus and better help us understand how to combat these devastating viruses.

*Infectious filovirus work in this chapter of the thesis was done by the Sina Bavari group at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID).*
5. DISCOVERY OF ANTI-FILOVIRAL SMALL MOLECULE ENTRY INHIBITORS
5.1 Introduction

The filoviruses, consisting of Ebola and Marburg virus, are the causative agents of severe hemorrhagic fever in humans. There is one species of Marburg virus (Lake Victoria Marburgvirus) and five species of Ebola virus; Zaire (EBOV), Sudan (SUDV), Bundibugyo (BDBV), Tai Forest (TAFV), and Reston (RESTV). These viruses are enveloped viruses with a filamentous shape. They have a genome of roughly 19 kbs that is non-segmented, single-stranded, and negative-sense (Feldmann et al. 2013; Hofmann-Winkler et al. 2012; Wong et al. 2014).

Filovirus entry is mediated by a single surface glycoprotein, GP. GP is a type 1 transmembrane protein located on the cell surface of the virion. It is responsible for both binding to the host cell and fusion of the host and viral membranes. GP is synthesized as a single polypeptide, GP0, which is then cleaved by furin into a GP1,2 heterodimer. GP1 is the portion of the protein which contains the receptor binding domain (RBD) and mediates attachment to the host cell surface. The GP2 portion contains the fusion peptide, and is responsible for fusion of the membranes (Feldmann et al. 2013; Hofmann-Winkler et al. 2012; Miller & Chandran 2012).

Since Marburg and Ebola viruses were discovered in 1967 and 1976, respectively, they have caused many outbreaks with case fatality rates reaching up to 90%. The symptoms of hemorrhagic fever caused by these viruses begin as flu-like at the onset and progress to more serious symptoms like vomiting, edema, confusion, coma, rash, coagulopathy, and shock (Feldmann et al. 2013). Currently, there are no FDA approved treatments or vaccines for filoviral infection. The latest Ebola outbreak in Western Africa is the most severe to date since the discovery of the virus. This outbreak has infected over 28,000 individuals, causing death in over 11,000 of those infected, underscoring the urgency for anti-filoviral drug discovery (CDC 2015b).
We have been working to find effective treatments for filoviral infection, specifically at the step of viral entry. We are searching for anti-filoviral treatments in the form of both small compounds and natural plant extracts. We recently performed a high-throughput screen of a small molecule library and identified several potential anti-filoviral compounds. This chapter will discuss our efforts to discover new small molecule anti-viral entry inhibitors for Marburg and Ebola viruses.

5.2 Results

5.2.1 High throughput screen of a small molecule library and characterization of hit compounds

To discover potential anti-viral compounds that inhibit filovirus entry, our laboratory completed a high-throughput screen of the Chembridge DIVERSet Chemical Library of small molecules. We screened 19,200 compounds from this library against Marburg, influenza H5N1, and Lassa pseudoviruses in parallel (see Table 1) following a protocol established by us (Wang et al. 2013). The advantage of this parallel screening protocol is that it allowed us to quickly identify inhibitors specific to one particular virus. In total, we identified 118 putative Marburg specific antiviral compounds from our primary screen.

A hit compound was selected based on the following criteria: the compound reduced viral infection for Marburg virus by 80% or more, while reducing infection of influenza and Lassa viruses by less than 30% in both the primary and confirmation screens. One-hundred-and-one of these hits were found to have less than 25% toxicity to the cells at a concentration of 12.5µM. A confirmation screen was performed to verify these hits, and based on the infection results from that screen 64 hits were confirmed. To further evaluate the hit compounds, we determined the IC$_{50}$ and CC$_{50}$ values (data not shown). Based on these results, 18 compounds were chosen with IC$_{50}$s below 10µM and CC$_{50}$s greater than 50µM (Table 1). We then selected the the 9 most potent compounds with the lowest IC$_{50}$s for further analysis (Figure 20).
<table>
<thead>
<tr>
<th></th>
<th>MARV</th>
<th>LASV</th>
<th>FLU</th>
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<td>265</td>
<td>55</td>
<td>54</td>
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<tr>
<td>Non-toxic</td>
<td>101</td>
<td>230</td>
<td>48</td>
<td>19</td>
</tr>
<tr>
<td>&gt;80% inhibition for target virus</td>
<td>67</td>
<td>48</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>No effect against control viruses</td>
<td>64</td>
<td>44</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Best hit for follow-up</td>
<td>18</td>
<td>9</td>
<td>9</td>
<td>14</td>
</tr>
</tbody>
</table>
Figure 20. Compounds specifically inhibit Marburg pseudovirus infection in A549 cells. Compounds (12.5 µM) and pseudovirus were added to A549 cells and infection was assessed as described in Materials and Methods. Vehicle (DMSO) and AZT were used as controls. Data were normalized to the plate median.
To further evaluate these compounds, another serial dilution was performed to determine the IC\textsubscript{50} and CC\textsubscript{50} values using both Marburg and Ebola pseudovirus. As shown in Table 2, all nine compounds displayed potent anti-filoviral activity, with IC\textsubscript{50}s ranging from 0.41 µM to 5.7 µM for Marburg pseudovirus and from 0.35 µM to 9.07 µM for Ebola pseudovirus. The CC\textsubscript{50}s for the compounds ranged from 13.1 µM to 126 µM. Based on this data we were able to determine the selectivity index (SI), which is calculated by dividing the CC\textsubscript{50} by the IC\textsubscript{50} (CC\textsubscript{50}/IC\textsubscript{50}). For Marburg, compounds 5, 8, 21, 22, 23, 24, and 32 had SIs above 10, and compounds 5, 8, 10, and 24 had SIs greater than 10 for Ebola pseudovirus (Table 2).
<table>
<thead>
<tr>
<th>Compound No.</th>
<th>Marburg</th>
<th>Ebola</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
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<td>5.7</td>
<td>12.3</td>
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</tr>
<tr>
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<td>0.35</td>
</tr>
<tr>
<td>C21</td>
<td>1.1</td>
<td>12.26</td>
<td>4.3</td>
</tr>
<tr>
<td>C22</td>
<td>1.3</td>
<td>33.07</td>
<td>4.4</td>
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<tr>
<td>C23</td>
<td>0.41</td>
<td>131.7</td>
<td>9.07</td>
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<tr>
<td>C24</td>
<td>1.8</td>
<td>70</td>
<td>8.58</td>
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<tr>
<td>C32</td>
<td>0.55</td>
<td>78.2</td>
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</table>
5.2.2 Validation of the hit compounds with infectious filovirus

To confirm that these hit compounds reduce filovirus infection, all of the compounds were tested against infectious Ebola virus (Kikwit strain) and four of the compounds were tested against infectious Marburg Ci67 in the BSL-4 facility at USAMRIID. To test the compounds, serial dilutions were performed to determine the IC$_{50}$ and CC$_{50}$, and infections were performed using an MOI of 2. To assess infected cells, immuno-staining was performed using an anti-GP antibody for high-content quantitative image-based analysis (see Materials and Methods). Of the four compounds tested against infectious Marburg virus, the IC$_{50}$s ranged from 11.26 to 13.89, and none had SIs greater than 10. The IC$_{50}$s for Ebola virus ranged from 2.83 µM to 15.40 µM and the CC$_{50}$s ranged from 66 µM to greater than 100 µM (there was no toxicity seen at 100 µM, which was the highest concentration tested). Based on this data, 6 compounds (compounds 10, 22, 23, 24, and 32) have SI scores above 10 for infectious Ebola virus (Table 3). These results demonstrate the effectiveness of the compounds in reducing infectious filovirus infection.
<table>
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<tr>
<th>Compound No.</th>
<th>Ebola</th>
<th>Marburg</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
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<td>SI</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</td>
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<tr>
<td>C5</td>
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<td>5</td>
<td>11.26</td>
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<tr>
<td>C6</td>
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<td>5</td>
<td>13.89</td>
</tr>
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<td>C8</td>
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<td>5.27</td>
<td>&gt;19</td>
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<tr>
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<td>&gt;11</td>
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<tr>
<td>C32</td>
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<td>35.38</td>
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</table>
5.2.3 **Time of addition experiments give insight into the mechanism of action**

To determine the step(s) during viral entry these hit compounds are acting, a time-of-addition experiment was performed. For this experiment, each of the compounds was tested at various time points along with heparin, azidothymidine (AZT), and DMSO as controls (Cheng et al. 2015). Briefly, cells were spinoculated with Marburg pseudovirus for one hour at 4°C to allow viral attachment to cells. Virus was washed from cells and the temperature was shifted 37°C to trigger virus entry. Compounds and controls were added to the wells at various time points (-1hr, 0hr, 1hr, 2hr, 3hr, 4hr, 5hr, 6hr).

Heparin has been shown by us and others to block the attachment step of viral entry, therefore as expected, it has inhibitory effects at the earliest time points (-1hr, 0hr, 1hr)(O’Hearn et al. 2015; Salvador et al. 2013). AZT works post-fusion on the reverse transcriptase of HIV, it still has inhibitory effects at the later time points (>6hr). As shown in Figure 21, one of the compounds, C23, showed inhibition during early time points (-1hr, 0hr, 1hr) similar to that of heparin. This suggests that this compound is working at the early attachment step of filoviral entry. The remainder of the compounds exhibited their inhibitory effects at a later time point than heparin, but earlier than AZT. This suggests that these compounds are working at a post-attachment step, but prior to the membrane fusion step.
Figure 21. Time of addition experiments give insight into compound mechanism. Marburg pseudovirus was incubated with A549 cells at 4°C for 1 hour. After 1 hour of incubation, virus was removed and the temperature was shifted to 37°C to trigger internalization. Compound (20µM), Heparin (10 µg/ml), AZT (5µM), or drug vehicle (DMSO) was introduced at different time points during infection and the compounds effects were assessed as described in Materials and Methods. Data were normalized to vehicle (DMSO). Error bars represent standard deviations.
5.2.4  **Structure-activity relationship analysis**

To investigate the structure-activity relationship (SAR) of the hit compounds, we purchased 76 compounds from a vendor which are derivatives of the 9 hit compounds, and tested these compounds for their antiviral activity using the HIV pseudovirions bearing either Ebola or Marburg GP. The compounds can be classified into four different groups: those that inhibit both Ebola and Marburg pseudovirus infection, those that inhibit Ebola but not Marburg, those that inhibit Marburg but not Ebola, and those that lost activity against both. Among the 76 compounds tested, 8 of the compounds inhibited infection of both Ebola and Marburg pseudovirus by at least 70% when compared to DMSO controls. Three of the compounds, 9114905, 9122646, and 76567953, specifically inhibited Marburg infection by 70% or more while reducing Ebola infection by less than 50%. Two of the compounds, 9133657 and 9210865, inhibited Ebola by greater than 70% but only reduced Marburg infection by less than 40% (Figure 22). The remaining 63 compounds lost activity against Ebola and Marburg pseudovirus (not shown).
Figure 22. Structure-activity relationship analysis of compounds alters their anti-filoviral activity. SAR analysis was performed on the nine compounds and these derivatives were tested against Marburg and Ebola pseudovirus. Compounds (12.5 µM) and pseudovirus were added to A549 cells and infection was assessed as described in Materials and Methods. Vehicle (DMSO) and AZT were used as controls. Data were normalized to the vehicle (DMSO).
5.3 Discussion

In this report, we explored several novel anti-filoviral small molecule compounds. These antivirals are potent entry inhibitors for Marburg and Ebola viruses, as demonstrated by their IC₅₀S and CC₅₀S for both pseudotyped and infectious filovirus.

Due to the large Ebola virus outbreak in Western Africa, a great deal of research is being performed to identify safe and effective anti-filoviral treatments. Some of the antiviral treatments being developed include those that inhibit viral replication steps and proteins, those that limit viremia and spread of the virus, those that treat the symptoms, and those that boost the immune response (De Clercq 2015; Nyakatura et al. 2015; Shurtleff et al. 2015; WHO 2015; Wong et al. 2014). While there has been a lot of progress on this front, none of them have been approved for use in humans. In order to discover reliable treatments for filoviral infection, our laboratory has performed a high-throughput screen of a compound library to identify new anti-filoviral compounds to be used either independently or in combination with other therapies.

Through the small molecule HTS we identified nine potential anti-filoviral compounds. To further understand the antiviral properties of these compounds, we performed titrations to find the IC₅₀ and CC₅₀ values using both Marburg and Ebola pseudovirus. Based on these values, we were then able to determine the selectivity index for these compounds, which demonstrates the potential therapeutic window. These results were confirmed with infectious Ebola Kikwit virus. There are differences between the SIs of pseudotyped virus and infectious virus, highlighting the fact that while pseudotyping is a safe and effective method for studying viral entry, there are still differences that need to be taken into account (Table 4).
<table>
<thead>
<tr>
<th>Compound</th>
<th>Ebola SI</th>
<th>Marburg SI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pseudovirus</td>
<td>Infectious Virus</td>
</tr>
<tr>
<td>C5</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>C6</td>
<td>4.67</td>
<td>5</td>
</tr>
<tr>
<td>C8</td>
<td>18.9</td>
<td>11.83</td>
</tr>
<tr>
<td>C10</td>
<td>108.5</td>
<td>27.50</td>
</tr>
<tr>
<td>C21</td>
<td>3.05</td>
<td>6</td>
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<tr>
<td>C22</td>
<td>9.08</td>
<td>&gt;10</td>
</tr>
<tr>
<td>C23</td>
<td>5.95</td>
<td>&gt;19</td>
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<tr>
<td>C24</td>
<td>14.6</td>
<td>&gt;11</td>
</tr>
<tr>
<td>C32</td>
<td>6.32</td>
<td>35.38</td>
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</table>
To gain further insight into the possible mechanisms being utilized by these compounds to inhibit filoviral entry, we performed a time-of-addition experiment using controls known to inhibit different stages of entry (Cheng et al. 2015). Of all nine compounds, only one compound, compound 23, showed an infection profile similar to that of heparin. It exerted its inhibitory effect early on, at hours -1, 0, and 1, suggesting that this compound works at the attachment/binding stage. This compound could possibly be binding to either the filoviral glycoprotein (GP) or a host protein on the cell surface to block entry as its mechanism. The remainder of the compounds all showed their inhibitory effect at later time points, falling between the action of Heparin and AZT. This suggests that these compounds work at a post-attachment, but pre-fusion step in viral entry. It is possible that they are binding to the viral GP and preventing fusion once in the endosome. It is also possible that they are binding to an internal host protein and preventing an interaction which is essential for viral fusion. This is the case with two other small molecules which block the interaction between GP and NPC1; a benzylpiperazine adamantane diamide, 3.0, and the benzodiazepine derivatives MBX2254 and MBX2770 (Côté et al. 2011; Basu et al. 2015). Another possibility is that they are blocking the cleavage of GP by Cathepsin L, which is the method of inhibition for the small molecules 5705213 and 7402683 (Elshabrawy et al. 2014). One small molecule that blocks viral fusion, LJ001, does so by intercalating into and disrupting the viral membrane (Wolf et al. 2010). This is not likely how the compounds tested here work, though, because LJ001 is a broad spectrum antiviral, working against all enveloped viruses, while ours specifically inhibited filovirus infection but not influenza or Lassa virus which are both enveloped. It would be interesting to see how our hit compounds work in combination with these other entry inhibitors.

SAR analysis was performed on the chemical structures of these compounds to better understand which portion(s) of the compound are required for activity and which are dispensable. Of
the 76 derivatives, 13 inhibited filovirus infection of either Ebola, Marburg, or both by greater than 70%, with the remainder losing activity. The effects of these derivatives varied depending on whether they were challenged with Ebola or Marburg pseudovirus. None of the new compounds were able to inhibit Marburg infection better than the original compounds. Three of the compounds were able to inhibit Ebola virus by at least 90%, so more work needs to be done on those derivatives to assess their effectiveness. Through medicinal chemistry evaluation, we will be better able to discern which side groups are important for inhibiting filoviral infection, and how to improve these compounds.

The results presented in this chapter show that we have identified several potential anti-filoviral compounds. Further studies need to be performed to confirm the mechanism of action for these therapies, but the results thus far are promising. The need for anti-filoviral therapies is great, and hopefully this work will be the building blocks to enable us to fight these destructive viruses.

*Infectious filovirus work in this chapter of the thesis was done by the Sina Bavari group at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID).*
6. DISCOVERY OF ANTI-FILOVIRAL PLANT EXTRACTS
6.1 Introduction

Filoviral hemorrhagic fever is a deadly disease caused by either Ebola or Marburg virus, with fatality rates of up to 90% (CDC 2014f). Its symptoms begin as flu-like (chills, fever, etc.), and develop into more severe symptoms like chest pain, vomiting, edema, rash, shock, and coma (Feldmann et al. 2013). The most severe Ebola outbreak in history began in March 2014 and is still ongoing. This outbreak has affected over 28,000 people, causing over 11,000 deaths (CDC 2015b).

Ebola and Marburg virus have a single surface glycoprotein, GP, which is responsible for viral entry. GP is located on the viral membrane as a trimer of heterodimers. It is initially produced as the precursor protein GP0, which is cleaved into two subunits, GP1,2, which are linked by a disulfide bond. Of the two subunits, GP1 is responsible for viral attachment to the host cell, while GP2 mediates fusion of the virus and host cell membranes (Feldmann et al. 2013; Hofmann-Winkler et al. 2012; Miller & Chandran 2012).

There are currently no FDA approved treatments or vaccines available to combat these viruses, which are classified as biosafety level 4 (BSL-4) pathogens. Due to this high pathogenicity, research to identify potential therapeutics for filovirus infection is difficult. To perform filoviral research in a BSL-2 facility, we have employed a surrogate pseudotyping system. This system utilizes the filoviral GP in combination with a replication deficient HIV core, allowing us to study Ebola and Marburg virus entry in a safer environment (Rumschlag-Booms et al. 2011). Using this method, we investigated 6 plants whose extracts displayed specific anti-filoviral activity.

While many drug therapies come in the form of synthetically derived small molecule compounds, natural plants are a great source of previously undiscovered antiviral molecules. Many of
today’s medicines were derived from plant materials. Some examples include the painkillers morphine and codeine, the anti-malarial quinine, and the cardiac glycoside digitoxin (Fridlender et al. 2015). Plant derivatives have also been shown to be effective in treating a wide variety of diseases, such as various cancers, Alzheimer’s disease, diabetes, and hypertension (Fridlender et al. 2015; Atanasov et al. 2015; Cragg & Newman 2013; Taghizadeh et al. 2015). In this study, we identified native plant extracts which have potent and specific anti-filoviral activity.

6.2 Results

6.2.1 Identification of potential antiviral extract fractions

Based on previous work by us, we identified 6 plants that exhibit specific anti-filoviral activity, which were collected in China (Figure 23). To determine which extracts have potential anti-filoviral molecules, we used a bioassay directed fractionation approach. Briefly, the plants were collected, dried, and extracts were produced, which were then resuspended in DMSO. A549 cells were infected with either Marburg, Ebola, or influenza pseudovirus along with individual plant fractionations, DMSO, or AZT. Based on these results, many of the fractions contain potential anti-filoviral molecules. As shown in Figure 24, plant SHA5 exhibits activity in fractions 74-82, 83-90, and 91-98, and fraction 12 from plant SHA7 showed anti-filoviral activity (Figure 24a). Plant SHB5 has an effect on filovirus infection in fractions 55, 64, and 66 as seen in Figure 24b. Plant SHB7 shows activity in fractions 9, 10, and 11 (Figure 24c). Plant HPARB had anti-filoviral activity in fractions 26 and 29. Fraction 26 was further fractionated and most of the activity is in fractions AESP 9, 11, and 13 (Figure 24d). Finally, plant AEGZ had activity through many of its fractions; fractions 0-12, 14, 16, 17, and 18 all had an anti-filoviral effect (Figure 24e). We are still currently working on further fractionation of these active extracts to further narrow down the active components, however the results thus far show potent anti-filoviral activity in many of the fractions.
Figure 23. Plant extract fractionation chart.
Figure 24. Effect of plant extract fractions on pseudovirus infection. A549 cells were infected with H5N1 influenza, Marburg, or Ebola pseudovirus along with either extract (10 μg/mL), DMSO (0.25% final conc.), or AZT (5 μM) and the effects were assessed as described in Materials and Methods. (a) Extracts from SHA5 and SHA7. (b) Extracts from SHB5. (c) Extracts from SHB7. (d) Extracts from HPARB. (e) Extracts from AEGZ. Data were normalized to vehicle (DMSO). Error bars represent standard deviations.
6.2.2 **Characterization of hit extract fractions**

Based on the results from the bioassay directed fractionation, a few of the active fractions were selected for further characterization. A titration was performed to determine the IC50 and CC50 values for eighteen of the fractions against both Marburg and Ebola pseudovirus (see Table 4). The IC50s for pseudotyped Marburg virus were promising, ranging from 0.22 µg/ml for fractions SHA7-F12 and AESP 9, to 1.55 µg/ml for fraction SHB5 F55. The IC50s for Ebola pseudovirus varied from 0.36 µg/ml for fraction SHB5 F66, to 31.54 µg/ml for fraction SHB5 F55. We also tested the CC50 values for these fractions, and all of the fractions except for two showed no toxicity at the highest concentration tested, 20 µg/ml. Based on these IC50s and CC50s we were then able to calculate the SIs for these fractions. For Marburg, all eighteen of the fractions had SIs above 10. For Ebola, twelve of the fractions had SIs above 10 (Table 4). It is important to note that since the CC50s for most of the fractions was above the maximum dose tested, the actual SIs are probably much higher than what was calculated. These values demonstrate the effectiveness of these fractions against filovirus infection.
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6.2.3 Extract fractions inhibit infectious filovirus infection

To confirm the effectiveness of these extracts against infectious filovirus, we tested four of the samples with both infectious Marburg (CI67) and Ebola (Kikwit) virus in the BSL-4 facility. To determine the IC50 and CC50, the extracts were serially diluted and infections were performed using an MOI of 15 for Marburg and an MOI of 0.5 for Ebola. Infected cells were then immuno-stained with an anti-GP antibody and infection was assessed via a high-content quantitative image-based analysis (see Materials and Methods). The IC50s for Marburg ranged from 1.8 µg/ml to 17.1 µg/ml. The IC50s for Ebola virus varied from 1.8 µg/ml to 20 µg/ml. CC50 values for all five of the samples were greater than the highest concentration tested, which was 20 µg/ml. From these scores we calculated the SIs. Only one of the samples, SHA7-F12, had an SI above 10, however since all of the fractions had CC50s above the maximum dose tested, the SIs are probably much higher and it is possible that these other fractions will have SIs above 10 (Table 5). These results confirm that the plant extracts can inhibit infectious Marburg and Ebola virus, although at higher concentrations than seen with pseudovirus.
TABLE VI. SUMMARY OF IC₅₀ AND CC₅₀ WITH INFECTIOUS FILOVIRUS

<table>
<thead>
<tr>
<th>Extract</th>
<th>IC₅₀ (µg/ml)</th>
<th>SI</th>
<th>IC₅₀ (µg/ml)</th>
<th>SI</th>
<th>CC₅₀ (µg/ml)</th>
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</thead>
<tbody>
<tr>
<td>SHA7-F12</td>
<td>1.8</td>
<td>&gt;11</td>
<td>1.8</td>
<td>&gt;14</td>
<td>&gt;20</td>
</tr>
<tr>
<td>SHA7-Whole Extract</td>
<td>3.6</td>
<td>&gt;6</td>
<td>4.2</td>
<td>&gt;4</td>
<td>&gt;20</td>
</tr>
<tr>
<td>HPARB-F26</td>
<td>3.4</td>
<td>&gt;6</td>
<td>7.3</td>
<td>&gt;3</td>
<td>&gt;20</td>
</tr>
<tr>
<td>SHA5-F72-82</td>
<td>6.8</td>
<td>&gt;3</td>
<td>10.2</td>
<td>&gt;2</td>
<td>&gt;20</td>
</tr>
</tbody>
</table>
6.3 Discussion

In this study, we utilized an HIV-1 pseudotyping system to identify novel anti-filoviral plant extracts that inhibit both pseudotyped and infectious filovirus entry. The antiviral activity of these plants was determined based on their ability to inhibit Marburg and Ebola virus infection, as well as their IC$_{50}$ and CC$_{50}$ values.

Plants have long been used for medicinal purposes. Aside from treating diseases such as cancer and diabetes as mentioned above, active compounds from plant extracts are also being sought out for treatment of infectious diseases as well. Some recent discoveries include the use of *Pedilanthus tithymaloides*, against herpes simplex virus type 2 (HSV-2), *Melia azedarach* against both flaviviruses (Dengue, West Nile, and Yellow Fever viruses) and *Mycobacterium tuberculosis*, *Aloe arborescens* against influenza A and B viruses, as well as an anti-HIV molecule from plant extracts discovered in our laboratory (Ojha et al. 2015; Sanna et al. 2015; Glatthaar-Saalmüller et al. 2015; Rumschlag-Booms et al. 2011).

We were interested in identifying plant extracts with anti-filoviral properties. Working with our collaborators in China, we identified 6 plants with anti-filoviral activity. To test these plants and determine what the active components are in the plants, we utilized a bioassay directed fractionation approach. We began by fractionating the plants and testing each fraction for activity against either Marburg, Ebola, or influenza pseudovirus. The fraction activity fell into one of four categories regarding the filoviruses: fractions that inhibited both Marburg and Ebola pseudovirus, fractions that inhibited Marburg but not Ebola, fractions that inhibited Ebola but not Marburg, and fractions which had no antiviral activity. Many of the fractions affected both Marburg and Ebola pseudovirus as seen in Figure 24. Some of the fractions that inhibited Marburg but not Ebola include SHB5-F55, SHB7-F9, and AESP 1.
One fraction that inhibited Ebola but not Marburg was SHA5 F62-73. It will be interesting to understand why we see this difference in effect between Marburg and Ebola infection for these fractions. This confirms that while the viruses are very similar, there are still some differences between them that we have yet to understand. Some of the fractions also had an effect on influenza pseudovirus, suggesting the molecules present in those fractions have a broad spectrum effect and require more follow up. Based on these results from the bioassay, we chose various fractions to characterize by performing a titration to determine the IC50 and CC50 scores.

We only tested four of these plant extract fractions against infectious filovirus because there is still a lot of further fractionation that needs to be performed, however we wanted to get a general sense of whether these extracts were effective against infectious virus as well. We found that while they were effective in inhibiting both infectious Marburg and Ebola infection, the IC50s were slightly higher than those of the pseudovirus. It is important to note that fraction 12 of SHA7 had a lower IC50 than the whole SHA7 extract that was tested against infectious virus. This suggests that we are getting a purer fraction that has an antiviral molecule enriched in it, however further fractionation still needs to be done.

These results show that we have identified several plants with anti-filoviral activity. Further fractionations need to be performed based on the results presented here in order to isolate the potent anti-filoviral compounds. Additionally, further studies need to be done to elucidate the mechanism of action for these plants and extracts. There is a great need for anti-filoviral therapeutics, and this research is the first step towards accomplishing this goal.
Infectious filovirus work in this chapter of the thesis was done by the Sina Bavari group at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID). Plant extract and purification was performed by Dr. Hongjie Zhang at the Hong Kong Baptist University.
7. CONCLUSIONS AND DISCUSSION
Emerging infectious viral diseases have burdened humans for thousands of years. They are difficult to predict and even more difficult to control once they appear. We must continually be learning about the viruses that cause them so that we can better understand how to fight them. The aims of this thesis are two-fold: 1) identifying host factors involved in viral entry of influenza A virus and filoviruses, and 2) discovering new anti-filoviral therapies.

The first focus of this study involved basic research to identify potential host factors that are involved in influenza and filovirus entry. There is currently a basic understanding about both influenza and filovirus infection, but there is still a lot of unknown variables in the process for each virus that need to be determined. Identifying host factors for these viruses would not only help us gain a better understanding of these viruses, but would provide us with new targets for antiviral therapies. To identify such host factors, our lab performed a high-throughput screen (HTS) of a library containing ~65,000 unique siRNAs that targeted about 21,500 human gene sequences using both influenza and Marburg pseudovirus. From this screen, we identified two potential host factors discussed in this work, the reticulon 4 receptor (RTN4R) for influenza infection and the tropomyosin receptor kinase B (TrkB) for filovirus infection.

The first host factor discussed was RTN4R and its role in influenza infection. Influenza A virus infection results in serious illness for millions of people each year (CDC 2015c; Fauci 2006). This infection can lead to severe complications including death, and the threat of pandemic influenza is always present. Sialic acid (SA) has long been accepted as the host receptor involved in influenza attachment and entry. The influenza glycoprotein hemagglutinin (HA) binds directly to sialic acids found on host cells that have either an α2,3 or α2,6 linkage. It is also known that this linkage plays a role in host tropism, with avian influenza viruses preferring the α2,3 linkage and human influenza viruses preferring the α2,6
linkage. While HA may be binding to SA for initial attachment to host cells, there are implications of additional host factor(s) involved in influenza virus entry based on recent studies. For example, it was shown that MDCK cells which were desialylated were able to be infected by influenza, suggesting there is an additional factor being used in entry (Stray et al. 2000; Rapoport et al. 2006).

To better understand the influenza entry process we looked into RTN4R based on the results from our HTS. Here we determined that siRNA knockdown of RTN4R results in a specific reduction in influenza pseudovirus infection levels, which was validated with infectious H1N1 influenza virus.

RTN4R is a protein that is most notably expressed on cells found in the central nervous system (CNS) but can also be found on cells located in other areas such as the lung (Mi et al. 2004; Yamashita et al. 2005; Schwab 2010). RTN4R is a member of a receptor signaling complex, which initiates signaling upon ligand binding. The members of the signaling complex are RTN4R, the LRR and Ig domain containing, nogo receptor-interacting protein (LINGO-1), the pan-neurotrophin receptor p75 (p75), and the TNF-Receptor Super family member 19 (TROY) (Saha et al. 2011; Joset et al. 2010). Interestingly, when we looked into the members of the receptor complex to determine if they possibly play a role in influenza infection, we discovered that they do seem to play a role based on our results from our HTS. siRNA knockdown of both p75 and TROY caused a reduction in infection for both influenza and Marburg pseudovirus in the screen, and another group identified LINGO-1 as a potential host factor in their screen (Karlas et al. 2010). This suggests that not only is RTN4R involved in viral entry, but that the whole complex plays a role. Based on our findings, our model of infection is that influenza is binding to RTN4R and the complex, possibly utilizing the sialic acid residues present on the members of the complex. The complex then signals within the cell, possibly through an unknown pathway, inducing endocytosis of the complex and the virus (Figure 25).
Figure 25. Model of influenza viral entry including RTN4R and its receptor complex.
Identifying RTN4R as a host factor involved in influenza infection has several implications. The first is that anti-influenza therapies can be developed which target RTN4R. Currently there are no drugs which act directly on RTN4R. If a drug targeting RTN4R was synthesized or identified via a HTS it would provide a useful therapy in addition to the current influenza therapies available. Targeting RTN4R is an attractive method for an antiviral because the chance of viral escape mutants developing against the drug is reduced. Also, it will be interesting to determine if RTN4R is involved in infection by all HA subtypes. We attempted to produce influenza pseudovirions with different HAs, but were unsuccessful. If RTN4R is utilized by all HA subtypes then a therapy targeting it would be even more powerful.

These findings suggest that RTN4R plays a role in influenza viral entry, however there are still several questions that need to be addressed. The main question is: What is the exact mechanism that is used for viral entry? While we attempted to block RTN4R signaling by inhibiting ROCK, we saw no effect on infection. This suggests that while RTN4R plays a role in flu entry, signaling through the ROCK pathway is not involved. It is possible that the RTN4R complex is able to signal through an unidentified pathway not involving ROCK. One way to determine this is by inhibiting RhoA, the protein that activates ROCK. Another possibility is to mutate the residues present on either p75 or TROY that are responsible for activating RhoA to see if this inhibits influenza infection. Also, several failed attempts at co-immunoprecipitation (Co-IP) leave us wondering if there is direct binding occurring between influenza HA and RTN4R. While our Co-IPs were not successful, it is possible that an ELISA would shed some light on whether they bind/interact. It would also be interesting to mutate the residues on RTN4R while are glycosylated to determine if they play a role in attachment. Co-localization studies would also provide great insight into the role of the complex in influenza infection. Microscopy would be an ideal method to determine if influenza is interacting with any of the members of this complex, and would also allow us to identify where infection is blocked when RTN4R is knocked out by siRNA. Finally, testing infection in vivo
in an RTN4R knockout mouse system would be ideal for determining the effect of RTN4R in infection. RTN4R knockout mice are available and show only minor defects such as impaired coordination and have a greater ability for nerve regeneration upon spinal cord injury (MGI 2015b). Answering these questions will give us greater insight into the viral entry mechanism for influenza virus, and provide us with a host factor to target when designing new therapeutics for influenza infection.

The second host factor I examined in this thesis is TrkB and its role in filovirus infection. While many host proteins have been implicated in filovirus infection, such as Cathepsin B, Cathepsin L, the TAM family of tyrosine kinase receptors, α5β1-Integrin, C-type lectins (DC-SIGN, ASPGR-1, LSECtin, hMGL), TIM-1, NPC1, and EXT1, most of these factors are not unconditionally required for infection (Hofmann-Winkler et al. 2012; Miller & Chandran 2012; O’Hearn et al. 2015). Because of this, our knowledge on the actual filoviral entry process is still incomplete.

In order to gain a more thorough understanding of the filoviral infection process, more specifically filoviral entry, we investigated TrkB based on our findings through the HTS. Through this work we discovered that knockdown of TrkB using siRNA resulted in a decrease in Marburg pseudovirus infection levels, which was confirmed with infectious Marburg Ci67 virus. These siRNAs also reduced infection levels for Ebola and Lassa pseudovirus. It was also determined via a competition assay that Marburg virus competes with the main ligand for TrkB, the brain derived neurotrophic factor (BDNF), for binding in a dose dependent manner.

TrkB is a member of the Trk family of receptor tyrosine kinases. There are three members of this family; TrkA, TrkB, and TrkC. The members of this family are also able to bind and signal with another protein, the pan-neurotrophin receptor p75 (p75), which was mentioned above as a signaling partner
with RTN4R as well. While TrkB siRNAs resulted in a reduction in infection for filoviruses, it is important to note that siRNAs targeting TrkA exhibited no reduction in infection, and siRNAs targeting TrkC interestingly caused an increase in infection for two of the siRNAs while causing a reduction in both flu and Marburg infection for the third siRNA. As mentioned above, both influenza and Marburg infection levels were reduced by p75 siRNA. This suggests that of the three Trk receptors, only TrkB is involved in filovirus infection, while p75 plays a role in filoviral infection, it may also play a role in influenza virus infection. From these findings, our model of infection is that filovirus binds to TrkB either in a homodimer or a heterodimer with p75, inducing cell signaling through one of the three pathways activated by TrkB and is then endocytosed into the cell (Figure 26).
Figure 26. Model of filoviral entry including TrkB and p75.
The discovery of TrkB as a filoviral host factor is significant for several reasons. First, since there are no FDA–approved drugs to treat filoviral infection, TrkB may be an attractive target for drug development. There are currently two drugs available which target TrkB: GNF5837 and ANA-12. GNF5837 is a pan Trk receptor inhibitor, working on all three Trk receptors, while ANA-12 inhibits signaling from TrkB only (Cazorla et al. 2011; Albaugh et al. 2012). We tested these drugs and saw no inhibitory effects at the lowest concentrations used for each drug, however, as the dosage increased, so did the toxicity. Because of this, it would be beneficial to identify other drugs which target TrkB with less toxicity. Also, as discussed previously, targeting TrkB would be a good option for preventing escape mutants against potential drugs.

It is important to note that TrkB has been linked to signaling with two different G-protein coupled receptor (GPCR) ligands. Adenosine and pituitary adenylate cyclase-activating polypeptide (PACAP) are the ligands for the A2a receptor and PAC1 receptor, respectively, and are able to activate TrkB independently of its natural ligands (Rajagopal et al. 2004; Lee & Chao 2001; Francis S Lee et al. 2002; Francis S. Lee et al. 2002). This is significant because it has been shown by us that GPCR antagonists inhibit filoviral entry. It is possible that there is a connection between our findings of the GPCR antagonists ability to inhibit filoviral infection and the GPCR ligands ability to activate TrkB signaling, but more work is needed to verify this.

In this portion of the study, our work suggests that TrkB is involved in filovirus infection. While our work gives some insight into its role during entry, there are still some issues that need to be addressed.
Firstly, while we know that Marburg seems to be competing with BDNF for binding to TrkB, we cannot conclusively state that it is binding to the protein. We attempted to complete Co-IPs to address this issue, but these attempts were unsuccessful. Again, an ELISA could perhaps solidify the answer to whether they are binding or not. Another way to address this would be microscopy to determine if TrkB and Marburg co-localize with one another. Another mechanistic aspect we were unsuccessful in addressing was whether signaling was involved in the entry process. One possible way to get around the toxicity of the inhibitors would be to directly mutate the tyrosine sites on the cytoplasmic portion of the TrkB protein that are responsible for signaling. Finally, studying filovirus infection in a TrkB knockout mouse would be ideal for studying the involvement of this gene in infection. TrkB knockout mice, however, have various abnormalities including neural defects and a reduced lifespan, therefore using these mice for an infection might prove difficult (MGI 2015a). Investigating these questions will further our knowledge on how TrkB is involved in filoviral entry, and give us insight on how to combat these deadly viruses.

The second focus of this study involved translational research to identify potential anti-viral therapies against filoviruses. The filoviruses Ebola and Marburg virus have caused many deadly outbreaks of viral hemorrhagic fever since their discoveries. Some of these outbreaks have reached mortality rates of up to 90%. The 2013-2015 outbreak in Western Africa is by far the most serious to date, with over 28,000 individuals infected and a case fatality rate of about 41% (CDC 2015b). Because there are no FDA approved treatments, it is important to continue researching potential treatments against these devastating viruses. To do so, our lab performed a HTS of a small molecule library, the Chembridge DIVERSet Chemical Library, to identify compounds with anti-filoviral properties. We are also evaluating natural plant extracts in attempts to identify plant fractions containing molecules that show anti-filoviral effects.
From our small molecule HTS, we identified 118 Marburg-specific hits from the primary screen, which was eventually narrowed down to 9 through further screens. These 9 compounds (compounds 5, 6, 8, 10, 21, 22, 23, 24, and 32) inhibited filovirus infection by at least 90%. To get a more thorough understanding of these nine anti-filoviral compounds, titrations were performed with both Marburg and Ebola pseudovirus to assess the IC$_{50}$s and CC$_{50}$s. Based on these IC$_{50}$ and CC$_{50}$ values, we were able to calculate the selectivity index (SI) for these ten compounds. The SI is calculated by dividing the CC$_{50}$/IC$_{50}$ and we look for SIs above 10. For Marburg pseudovirus, seven compounds had SIs above 10, with the highest SI being 131.7. For Ebola pseudovirus, four compounds had SIs above 10, with the highest SI being 108.5. We also tested these four of these compounds with infectious Marburg Ci67, and all of them against Ebola Kikwit virus to confirm that they are effective in inhibiting infection. When they were tested against infectious virus, they exhibited IC$_{50}$ values that were somewhat higher than seen with pseudovirus. This could be due to the difference in morphology between pseudovirus and infectious virus, or the genes not present in the pseudovirus could be having an effect on infection as well. When the SI was determined, 6 compounds had SIs above 10, proving their effectiveness against infectious virus as well.

To help determine the potential mechanisms these compounds are using for filoviral inhibition, we conducted a time-of-addition experiment. Compound 23 was the only compound which exhibited an inhibition profile which suggested it is inhibiting infection at the attachment stage. The remaining compounds inhibited infection at later time points suggesting they act at a post-attachment step, but prior to fusion during filoviral entry.
Finally, we performed a preliminary structure-activity relationship (SAR) analysis on the chemical structures of these compounds. From the original nine compounds, 76 derivatives were identified and ordered for testing against pseudotyped Marburg and Ebola virus. Of the 76 derivatives, 63 of them lost activity against the filoviruses. Eight of the derivatives caused a reduction in both Marburg and Ebola infection by at least 70%. Two of the compounds were able to reduce Ebola infection by more than 70% while having little effect on Marburg, and 3 of the derivatives had an effect on Marburg but not Ebola. None of the new derivatives were able to reduce Marburg infection greater than 90% (the original cutoff), and three were able to inhibit Ebola infection by more than 90%.

Together, these results suggest that these 9 compounds are promising leads for anti-filoviral therapeutics. This is significant because of the lack of approved therapies for those infected with filoviruses. These compounds could be used independently or in combination with other therapies that are currently being developed, similar to the highly active anti-retroviral therapy (HAART) drug cocktail used for patients with HIV.

While the results thus far are promising, there are still some questions that need to be answered in order to better understand these compounds. First, it will be very interesting to determine if any of the compounds are binding to the virus in order to exert their inhibitory effects. It is possible that compound 23, which exhibited it inhibitory effects early in infection, could be binding to either the Marburg virion itself or to a protein on the host cell to inhibit the attachment step. For the other compounds, they could possibly be binding to the GP or host proteins and preventing the fusion step from occurring, as discussed in chapter 5. We attempted to purify Marburg GP in order to test if binding was occurring, but ran into some difficulties. While we were able to purify soluble GP, when tested in a competition assay, the GP showed no activity. We are unsure why the protein was inactive, but perhaps
it had something to do with the HA tag attached to it or miss-folding of some sort. Once GP is successfully purified, it could be used for flow cytometry and a thermal shift assay to determine binding. It would also be interesting to perform microscopy to see at what point during infection the virus gets blocked. We would expect to see a similar profile between heparin and compound 23 with a block before the virus enters the cell. For the remainder of the compounds we would expect the block to occur while the virus is still in the endosome. Another issue to be addressed is the true CC_{50} values. Several of the compounds never reached 50% cytotoxicity even at 100 μM, which was the highest concentration tested. Determining the CC50 will allow us to more accurately calculate the SI for those compounds. Answering these questions will further our understanding of these compounds and potentially provide us with a successful and safe drug to use against filovirus infection.

The other aspect of our focus on anti-filoviral treatments is to find plants which have anti-filoviral properties. Plants have long been used for medicinal purposes, and a large number of medications used today came from plants. Plant derivatives have been used to treat malaria (quinine), pain (codeine and morphine), as well as a variety of other diseases (Fridlender et al. 2015; Atanasov et al. 2015; Cragg & Newman 2013; Taghizadeh et al. 2015). Plants are also being sought after to treat viral infections such as flaviviruses, herpes simplex virus type 2 (HSV-2), and influenza A and B viruses (Glatthaar-Saalmüller et al. 2015; Ojha et al. 2015; Sanna et al. 2015).

In this study, we identified 6 different plants, SHA5, SHA7, SHB5, SHB7, AEGZ, and HPARB, that exhibit anti-filoviral activity. We tested these plants using a bioassay directed fractionation approach to determine which extracts contained the active portions of the plants. Our collaborators began by collecting the plants in China, then fractionating them for us to test against either Marburg, Ebola, or influenza pseudovirus. The results of the fractions tested can be separated into 4 categories: those that
had no anti-filoviral activity, those that inhibited both Ebola and Marburg pseudovirus, those that reduced Ebola infection but not Marburg, and those that reduced Marburg infection but not Ebola. Most of the fractions inhibited both Ebola and Marburg infection, however there were a few fractions which had specific activity against either Ebola or Marburg. This highlights the fact that the viruses, while very similar, have certain differences between them which are still unknown.

Based on the results of the bioassays, we decided to determine the IC$_{50}$ and CC$_{50}$ values of several of the fractions through serial dilution with both pseudovirus and infectious Marburg and Ebola virus, and then calculated the SIs for these fractions. For Marburg pseudovirus, the SIs ranged from 12.9 (SHB5 F55) to >91 (HPARB AESP 9). For Ebola pseudovirus, the SIs ranged from 0.63 (SHB5 F55) to >47.6 (SHB5 F64). SHA7 F12 resulted in the best IC$_{50}$ values for both infectious Marburg and Ebola virus. It should be noted that these SIs are likely much higher due to the fact that almost all of the fractions did not reach their full CC$_{50}$, but maxed out at 20 µg/ml.

Overall, from this portion of work, we have found several plants that have anti-filoviral properties. Upon fractionation, we were able to determine which fractions were active against filoviruses. Some of these fractions have been further fractionated, while we are still working on sub-fractionating the others with our collaborators. While we were able to do a basic characterization of these fractions, there is still work that needs to be done. Further fractionations need to occur in order for us to be able to determine which fraction specifically contains the active components in the plant. Once we narrow the fractions down, it will be possible to run mass spectrometry on the fractions and hopefully determine the molecules present. Once we do this, it will be possible to synthesize the component and test it further. With this product we can then run additional experiments such as time-of-addition assays to help determine the mechanism by which these molecules are inhibiting virus
replication. We could also attempt binding assays as described above with purified GP, which would give us further insight into the mechanism. This work will help us get a better knowledge of these plant extracts and how they play a role in filoviral entry, and possibly give us an effective therapy for use against Marburg and Ebola infection.
REFERENCES


Nanbo, A. et al., 2010. Ebolavirus Is Internalized into Host Cells via Macropinocytosis in a Viral Glycoprotein-Dependent Manner. , **6**(9).


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“A Comparative High-Throughput Screening Protocol to Identify Entry Inhibitors of Enveloped Viruses”

“Discovery of 4-Aminopiperidines as novel anti-influenza entry inhibitors”

“Role of the Reticulon 4 Receptor in Influenza Entry”

“Role of Tropomyosin Receptor Kinase B in Filovirus Entry”

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