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Maporal Hantavirus β-Integrin Utilization and Sensitivity to Favipiravir

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MAPORAL HANTAVIRUS: β-INTEGRIN UTILIZATION, GENETIC ANALYSIS, AND SENSITIVITY TO ANTIVIRALS

by

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ABSTRACT

Maporal Hantavirus β-integrin Utilization and Sensitivity to Favipiravir

by

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Hantaviruses are members of the *Bunyaviridae* family of viruses. Pathogenic hantaviruses are the etiologic agents of hemorrhagic fever with renal syndrome (HFRS), a disease principally endemic in the Old World, and hantavirus pulmonary syndrome (HPS), a disease primarily restricted to the Americas. Maporal virus (MPRLV), a recently isolated hantavirus, has been found to cause disease in hamsters that resembles HPS in humans. However, the virus has not been linked to human cases of HPS. Considerable evidence suggests that β-integrin usage mediating infection may serve to distinguish hantaviruses pathogenic to humans from nonpathogenic, but this receptor usage pattern information is not yet available for MPRLV. Although ribavirin has been shown to be effective in treating HFRS, it lacks specificity and has toxicity. Moreover, there are no effective antivirals for the treatment of HPS. Considering the above, we have investigated MPRLV 1) β-integrin-mediated mechanism of entry, 2) genetic determinants of pathogenicity,

and 3) susceptibility to the promising antiviral, favipiravir (T-705). Using antibodies targeting specific integrin chains, we found infection of Vero E6 cells with MPRLV to be dependent on β_3-integrins, similar to that reported for other pathogenic hantaviruses such as Dobrava virus (DOBV) included in our studies. β_1-integrin chain-specific antibodies and fibronectin did not block MPRLV or DOBV infectivity as observed with the nonpathogenic Prospect Hill Virus (PHV). Phylogenic analysis of characteristic degron sequences and ITAM motifs in the G1 cytoplasmic tails of MPRLV and other hantaviruses emphasizes the close genetic proximity of MPRLV to other HPS-causing hantaviruses. Favipiravir, a pyrazine derivative reported to be active against related bunyaviruses, was found to be active against MPRLV, DOBV, and PHV (EC_{50} = 65 - 93 μM) with therapeutic indexes of 74, 52, and 58, respectively. The data presented suggests that MPRLV may be pathogenic to humans and that it and other hantaviruses tested are sensitive to favipiravir in cell culture.

(59 pages)

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Kristin K. Buys

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CHAPTER 1

INTRODUCTION

Pathogenic hantaviruses cause two medically important diseases; hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS). HPS is a severe disease that primarily affects the lungs, but can cause residual damage in the cardiac and renal systems (Hallin et al., 1996; Jonsson et al., 2008; Peters et al., 1999). Certain hantaviral disease in humans, such as HPS caused by the Sin Nombre and Andes hantaviruses, have mortality rates as high as 50 percent (Doyle et al., 1998; Milazzo et al., 2002). Consequently, effective countermeasures for the prevention and treatment of hantavirus infections are much needed.

The hantaviruses that cause HPS are restricted geographically to the Americas, while HFRS-causing hantaviruses are mainly found in Europe and Asia and, thus, are separated into two categories; New World (NW) and Old World hantaviruses, respectively (Plyusnin and Morzunov, 2001). The focus of my research has been Maporal virus (MPRLV), a recently isolated NW hantavirus that has been found to cause disease in hamsters that is symptomatically and pathologically similar to HPS (Milazzo et al., 2002). Because there have been no documented HPS cases associated with MPRLV infection, it is unclear as to whether the virus is a human pathogen.

Critical to the success of my project was establishing a focus forming unit (FFU) assay to measure MPRLV, Dobrava (DOBV), and Prospect Hill virus (PHV)

replication in cell culture. Once able to quantify viral replication, I was able to test the susceptibility of these viruses to experimental (favipiravir) and licensed (ribavirin) drugs, and study their β-integrin receptor utilization for infection. PHV and DOBV were used as controls representing nonpathogenic and pathogenic HFRS-causing hantaviruses, respectively. I also developed a quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assay for MPRLV to verify the FFU assay findings. Phylogenetic analysis comparing MPRLV to other known hantaviruses at regions conserved across pathogenic hantaviruses was also performed to gain insights into the possible pathogenicity of MPRLV.

LITERATURE REVIEW

Hantaviruses

Hantaviruses are members of the *Bunyaviridae* virus family, divided into 5 genera: *Nairovirus, Orthobunyavirus*, *Phlebovirus*, *Tospovirus*, and *Hantavirus*. Hantaviruses are named after the first isolated virus in its genus, Hantaan, discovered near the Hantaan river, South Korea in 1976 (Lee et al., 1978). Hantaviruses are generally transmitted to people through aerosols from rodent feces, saliva, and urine, as well as through bites. Recent data has shown that person-to-person transmission may occasionally occur with the Andes hantavirus (ANDV) in Argentina (Padula et al., 1998); however, there is no evidence that this occurs with any other hantaviruses. Although asymptomatic in their rodent hosts, hantaviruses cause a spectrum of serious disease in humans. Ease of propagation and infectivity through aerosol exposure contribute to pathogenic hantaviruses

being on the NIAID Category A pathogens list as potential bioterror agents and HPS and HFRS are recognized as a global public health problems (Borio et al. 2002; Bronze et al., 2002; Jonsson et al., 2008).

Within the last few decades, a considerable number of hantaviruses have been identified throughout the world, with many being human pathogens (Table 1). Maporal virus (MPRLV) is a newly discovered hantavirus isolated from a fulvous pygmy rice rat in Venezuela (Fulhorst et al., 2004). It has not been determined whether MPRLV is a human pathogen, but previous studies have shown MPRLV to be phylogenetically related to and cause clinical and pathologic disease that resembles HPS in animal models (Milazzo et al., 2002; Peters et al., 1999). In a study done with hamsters, MPRLV-infected animals showed signs of cardiac depression and diffuse pulmonary edema with rapid onset comparable to findings in clinical cases of HPS. The prolonged incubation time typical of hantaviruses was also seen (Milazzo et al., 2002).

Virus genome and structure

Hantaviruses are enveloped, segmented negative-sense RNA viruses. Typically spherical, they have a diameter of 100 nm with 6 nm glycoprotein membrane projections (McCaughey and Hart, 2000). The genome consists of three RNA segments; the small (S) segment coding for the viral nucleocapsid protein, the medium (M) segment coding for the glycoprotein precursor, and the large (L) segment coding for an RNA-dependant RNA polymerase (Antic et al., 1992; Plyusnin and Morzunov, 2001). Electron micrographs of Puumula virus show the genome

Table 1. Hantavirus types, locations, and disease

Hantavirus	Location	Disease
Andes	Argentina	HPS
Bayou	North America	HPS
Bermejo	South America	ND
Black Creek Canal	North America	HPS
Bloodland Lake	North America	ND
Caño Delgadito	South America	ND
Calabazo	South America	HPS
Central Plata	South America	HPS
Choclo	North America	HPS
Dobrava	Balkans	HFRS
El Moro Canyon	North America	ND
Hantaan	Asia/Africa	HFRS
Isla Vista	North America	ND
Khabaroovsk	Asia	ND
Laguna Negra	South America	HPS
Lechiguanas	South America	HPS
Maporal	South America	ND
Muleshoe	North America	ND
New York	North America	HPS
Pergamino	South America	ND
Prospect Hill	North America	ND
Puumala	Europe	HFRS
Rio Mamore	South America	HPS
Saaremaa	Europe	HFRS
Seoul	World-wide	HFRS
Sin Nombre	North America	HPS
Topografov	Siberia	ND
Thailand	Thailand	ND
Thottapalayam	India	ND
Tula	Europe	ND

ND: none documented
HPS: Hantavirus pulmonary syndrome.
HFRS: Hemorrhagic fever with renal syndrome.
(Adapted from: McCaughey and Hart, 2000; Monroe et al., 1999; Schmaljohn and Hjelle, 1997; Zeier et al., 2005)

segments to have a coiled-bead appearance due to segments being complexed with the nucleocapsid protein to form ribonucleocapsid structures (Knipe and Peter, 2001).

Cell targets and mechanism of entry into cells

Integrins are cell receptors that mediate binding to the extracellular matrix (ECM) and other cells. Composed of a combination of two subunits, α and β, they specify ECM interaction, and cell-to-cell adherence (Hynes, 1987). Different combinations of the subunits have distinct binding and signaling properties. There are fifteen α, and eight β subunits that have been defined, and though some subunits have specific partners, many α and β subunits can associate with more than one subunit (Table 2)(Breuss et al., 1995; Hynes, 2002).

It has been found that hantaviruses enter host cells via interaction of the viral G1 glycoprotein and specific integrins on the host cell (Gavrilovskaya et al., 1998, 1999; Jonsson et al., 2008). Nonpathogenic viruses, such as PHV, utilize a β_1-integrin, whereas pathogenic viruses such as New York virus (NYV), Sin Nombre virus (SNV), or Dobrava virus (DOBV), use a β_3-integrin (Gavrilovskaya et al., 1998, 1999). In addition to the β-integrins, researchers have determined that hantaviruses also require a glycosylphosphatidylinositol (GPI)-anchored protein decay-accelerating factor (DAF) to be present on the host cell surface as a cofactor for entry (Buranda et al., 2010; Krautkramer and Zeier, 2008). At present, receptor utilization appears to be a good predictor of whether a hantavirus may be pathogenic to humans.

Table 2. Integrins and primary ECM ligands

Integrin	ECM ligand(s)
$\alpha_1\beta_1$	Collagens, Laminin
$\alpha_2\beta_1$	Collagens
$\alpha_3\beta_1$	Laminin
$\alpha_4\beta_1$	Fibronectin, Invasin,
$\alpha_4\beta_7$	MAdCAM-1, VCAM-1, Fibronectin
$\alpha_5\beta_1$	Fibronectin, Fibrinogen, Collagen,
$\alpha_6\beta_1$	Laminin, Invasin, Sperm fertilin
$\alpha_6\beta_4$	Laminin
$\alpha_7\beta_1$	Laminin
$\alpha_8\beta_1$	Cytotactin/tenascin-C, Fibronectin
$\alpha_9\beta_1$	Osteopontin, Cytotactin/tenascin-C,
$\alpha_{10}\beta_1$	Collagens
$\alpha_{11}\beta_1$	Collagens
$\alpha_v\beta_3$	Vitronectin, von Willebrand factor Osteopontin, Laminin, Prothrombin, Thrombospondin, Fibronectin, Fibrinogen,
$\alpha_v\beta_5$	Adenovirus penton base protein 5, Fibronectin, HIV Tat protein, Vitronectin
$\alpha_v\beta_6$	Cytotactin/tenascin-C, Fibronectin, vitronectin
$\alpha_v\beta_8$	Vitronectin, Fibronectin
$\alpha_{IIb}\beta_3$	Fibrinogen, Fibronectin, von Willebrand factor, Plasminogen, Prothrombin, Thrombospondi, Vitronectin,
$\alpha_L\beta_2$	ICAM-1 through 5
$\alpha_M\beta_2$	Fibrinogen, Factor X, iC3b, ICAM-1
$\alpha_x\beta_2$	Fibrinogen, iC3b
$\alpha_E\beta_7$	E cadherin
$\alpha_{Ib}\beta_3$	Collagens
$\alpha_v\beta_1$	Fibronectin, Vitronectin

(Adapted from Plow et al., 2000; Van der Flier and Sonnenberg, 2001)

Reservoirs and transmission

Each hantavirus is associated with a specific rodent species, and causes life-long, persistent infection in that species. The primary reservoir of MPRLV has been found to be the fulvous pygmy rice rat (*Oligoryzomys fulvescens)* in Venezuela, which is also a host for Choclo virus (CHOV) in Panama (Fulhorst et al., 2004; Vincent et al., 2000). Most primary reservoir hosts of pathogenic hantaviruses have been determined (Table 3)(Childs et al., 1994; Khan et al., 1996; Monroe et al., 1999; Nichol et al., 1993; Ravkov et al., 1995; Schmaljohn and Hjelle, 1997). Humans are considered incidental hosts.

Table 3. Selected hantaviruses and their primary rodent hosts

Virus	Host	Disease
Andes	*Oligoryzomys longicaudatus*	HPS
Bayou	*Oryzomys palustris*	HPS
Black Creek Canal	*Sigmodon hispidus*	HPS
Choclo	*Oligoryzomys fulvescens*	HPS
Dobrava	*Apodemus flavicollis*	HFRS
Hantaan	*Apodemus agrarius*	HFRS
Maporal	*Oligoryzomys fulvescens*	?
New York	*Peromyscus leucopus*	HPS
Prospect Hill	*Microtus pennsylvanicus*	Nonpathogenic
Puumala	*Clethrionomys glareolus*	HFRS
Sin Nombre	*Peromyscus maniculatus*	HPS

Knowledge of primary reservoirs is important in understanding the geographical constraints and potential for transmission of individual hantaviruses. In the example of the North American outbreak of HPS in 1993, increased rainfall and resulting forage availability that spring resulted in substantial growth of the rodent population, demonstrating that environmental and ecological changes can play a significant role in outbreaks of such diseases (Khan et al., 1996; Parmenter RR, 1993). To reduce risk of hantavirus infection, educational efforts for teaching at-risk populations about awareness of rodent infestation and precautions are needed.

Hantavirus determinants of pathogenicity

Hantaviruses infect human endothelial, and immune system cells such as dendritic cells and lymphocytes (Geimonen et al., 2003b; Raftery et al., 2002; Temonen et al., 1993). Lymphocytes are activated by recognition of specific antigens through binding of their receptors and subsequent intracellular signaling cascades. These B-cell and T-cell receptors contain conserved amino acid motifs consisting of paired tyrosine residues in the cytoplasmic domain known as immunoreceptor tyrosine-based activation motifs (ITAMs) (Geimonen et al., 2003b; Razzaq et al., 2004). ITAMs participate in intracellular signaling that directs cellular activation and proliferation. Some viruses can possess ITAM domains and regulate signaling responses of host cells. This is seen with a number of viruses including Epstein-Barr virus, bovine leukemia virus, Kaposi's sarcoma-associated herpes virus, human immunodeficiency virus and hantaviruses (Geimonen et al., 2003b; Lee et al., 1998; Miller et al., 1995; Willems et al., 1995; Xu et al., 1999).

Within the hantavirus genome, the M segment is translated into a polyprotein that is cleaved into N-Terminal G1 and C-terminal G2 glycoproteins (Antic et al., 1992; Sen et al., 2007). The G1 glycoprotein contains a long cytoplasmic tail that contains a conserved ITAM in all HPS-causing hantaviruses, but not in HFRS or nonpathogenic viruses (Geimonen et al., 2003b). Also associated with the conserved ITAM motifs, hantavirus G1 cytoplasmic tails can contain a C-terminal hydrophobic domain that directs proteasomal degradation. This 'degron' as it is called, directs ubiquitination and degradation of the cytoplasmic tail and is linked to the virulence of hantaviruses (Alff et al., 2006; Geimonen et al., 2003a; Sen et al., 2007). Reportedly, G1 tails of pathogenic hantaviruses facilitate proteosomal degradation, while the nonpathogenic viruses have G1 tails that are stable (Sen et al., 2007). The hantavirus degron has been shown to consist of tyrosines that reside within the ITAM (Y619 and Y632) as well as an additional eight residues (Geimonen et al., 2003a). Comparison of the G1 sequence of known pathogenic hantaviruses with that from a new virus such as MPRLV would be useful to provide insights into potential human pathogenicity.

Disease and clinical signs

Recognized as Korean hemorrhagic fever (KHF) since 1951, hemorrhagic fever with renal syndrome (HFRS) was first described in Europe and Asia in the mid 20th century (Lahdevirta, 1971; Lee et al., 1978; Myhrman, 1951). Many names were previously used to describe the diseases we now know are caused by Old World hantaviruses. These include hemorrhagic nephroso-nephritis in the Soviet Union, nephropathia epidemica in Scandinavia, Churilov's disease, and hemorrhagic

fever. HFRS was endemic in Korea from 1951 to 1976 wherein over 8000 hospitalized cases, including over 2000 cases among US troops, were documented (Lee et al., 1978). Today HFRS is prevalent in Europe and Asia with up to 200,000 hospitalized cases reported each year and a case fatality rate of 1-15% (Schmaljohn and Hjelle, 1997).

In the Americas, hantavirus pulmonary syndrome (HPS) was first recognized in 1993 when an outbreak of respiratory disease with high mortality was reported in the Four Corners region of the United States (Nichol et al., 1993). Before this time, hantaviruses had not been associated with severe respiratory illness, or with an outbreak of human disease in the Americas. Over 2000 cases of HPS have been confirmed in the Americas since 1993 with a mortality rate of over 40%. SNV and ANDV are the etiologic agents that cause the most severe and lethal HPS disease (Padula et al., 2004; Raboni et al., 2009; Schmaljohn and Hjelle, 1997).

HPS is characterized by the onset of fever, malaise and myalgia that progress with additional gastrointestinal complaints (vomiting and abdominal pain), other flulike symptoms, and dyspnea (Peters and Khan, 2002; Peters et al., 1999). Additional physical findings in HPS patients include tachypnea, tachycardia, and hypotension, with the clinical trademark being rapidly developing pulmonary edema caused by noncardiogenic vascular leakage (Duchin et al., 1994; Moolenaar et al., 1995).

Pathological studies conducted after autopsy of fatal HPS cases showed significant pulmonary edema and pleural effusions indicative of the lungs being the primary organ targeted by the disease (Duchin et al., 1994; Moolenaar et al., 1995;

Nolte et al., 1995; Zaki et al., 1995). This separates HPS from HFRS diseases that typically target the kidney, although minor amounts of plural edema and effusions have been found in some HFRS cases (Earle, 1954; Lukes, 1954). In HPS patients, the tissues of the kidney are histologically normal (Mori et al., 1999; Nolte et al., 1995; Zaki et al., 1995).

Due to the protein-rich nature of the fluid found in the lungs of HPS patients, capillary leakage has been considered responsible for the edema (Mori et al., 1999). Cytokine production by monocytes and T-lymphocytes may trigger the vascular leakage. Studies have shown high numbers of cytokine-producing cells in these edematous lungs of HPS patients producing cytokines such as IL-1α, IL-1β, IL-6, TNF-α, IFN-γ, IL-2, IL-4, and TNF-β (Mori et al., 1999). TNF (tumor necrosis factor) and interleukin (IL)-2 are particularly significant. TNF is produced by monocytes and macrophages, and studies have shown that high levels of TNF-α lead to increased pulmonary vascular permeability and edema (Stephens et al., 1988). Endothelial cells have a large amount of TNF receptors, so the large endothelial surface of lung may enhance effects of this particular cytokine (Mori et al., 1999; Stephens et al., 1988; Tracey and Cerami, 1993).

IL-2 has been shown to cause vascular leak syndrome, or microvascular permeability after infusion to cancer patients. It causes excessive fluid transport demonstrated by rapid clearance of radiolabeled albumin from circulation resulting in pulmonary edema (Thijs et al., 1990). This response has been observed in a number of experiments with mice, rats, and sheep (Klausner et al., 1989; Rabinovici et al., 1996; Welbourn et al., 1990), in addition to several human clinical trials (Lee

et al., 1989; Mier et al., 1988; Rabinovici et al., 1996; Thijs et al., 1990). IL-2 injection in cancer patients has also been observed to cause a release of TNF-α into circulation (Mier et al., 1988). There is evidence that TNF-α perpetuates its own production through a positive feedback mechanism, giving more explanation as to the sustained levels in pulmonary tissues, and a possible connection with IL-2 in causing lung disease (Rabinovici et al., 1996; Sherry and Cerami, 1988). It is not yet clear if TNF-α or IL-2 directly cause vascular permeability, but it is likely that they are central factors. Activated macrophages, lymphocytes, and complement components, have been recognized in IL-2 infused patients and implicated in vascular permeability, but as secondary players (Lee et al., 1989; Schoefl, 1972).

In fatal HPS cases, death consistently resulted from cardiogenic shock (Hallin et al., 1996; Saggioro et al., 2007). The main concern is that HPS is rarely diagnosed until in its advanced stages, and progression to respiratory failure and cardiogenic shock may occur within a couple days, or even hours. The incubation time and slow onset of initial symptoms may contribute to delayed diagnosis. With ANDV and SNV, time from exposure to onset of symptoms was 11-32 and 9-33 days, respectively (Mertz et al., 2006; Young et al., 2000). Due to this length of time, patients tend to seek medical care well after exposure as the viral load begins to decrease, rendering some antiviral treatments targeting viral replication largely ineffective (Jonsson et al., 2008). Mean time from first symptoms to cardiopulmonary failure is five days, illustrating the need for better physician awareness and early detection (Hallin et al., 1996; Peters et al., 1999).

Vaccines and Therapeutics

The high rate of mortality and lack of effective therapies for HPS underscores the need for effective therapies and vaccines. In Korea and China, vaccines for HFRS have been approved since the early 1990's. These vaccines are primarily inactivated HTNV and have been shown to be somewhat effective in clinical trials, but lack long-term persistence of antibody levels (Sohn et al., 2001). More recent studies have shown vaccinia virus (VACV) vaccines to enhance neutralizing antibodies to HTNV in VACV naive people, as well as produce seroconversion (McClain et al., 2000). Other modes of vaccination under investigation include vaccinia-vectored vaccines that express segments of the genome, DNA vaccines, and baculovirus-vectored vaccines (McClain et al., 2000; Sohn et al., 2001).

Using a lethal Syrian hamster model of HPS, adenovirus vectors expressing hantavirus nucleocapsid or envelope glycoprotein proteins were shown to confer immunity to ANDV. The study showed that all hamsters vaccinated with vectors expressing both glycoproteins had no detectable ANDV RNA in the blood or lungs after nine days of infection (Safronetz et al., 2009). Another promising study by Custer and co-workers describes the construction of a DNA vaccine containing the M genome segment of ANDV (Custer et al., 2003). Rhesus macaques injected with the vaccine developed neutralizing antibodies against ANDV that cross neutralized SNV. Serum taken from the monkeys conferred protection in Syrian hamsters when injected before, or even 5 days after, infection with ANDV (Custer et al., 2003). Clinical trials have yet to be initiated with either of these HPS vaccination strategies.

As with effective vaccines, robust therapeutics to treat hantaviral infections are needed. Ribavirin (1-β-D-ribofuranosyl-1, 2,4-triazole-3-caboxamide) is a broad-spectrum antiviral drug effective against both RNA and DNA viruses, and has been found to have antiviral activity against several members of the *Bunyaviridae* family (Huggins, 1989; Sidwell et al., 1972, 1988). It has been shown that against RNA viruses, ribavirin causes lethal mutagenesis by increasing the mutation rate of the virus and causing a "genetic meltdown" (Crotty et al., 2000, 2001; Day et al., 2005). It does this by forming ribavirin triphosphate (RTP), which acts as a nucleotide and is used by the viral RNA polymerase (Crotty et al., 2001; Crotty et al., 2000). When incorporated, RTP produces the antiviral effect through error catastrophe. Several other mechanisms may contribute to the antiviral activity of ribavirin including inhibition of viral capping by effecting or inhibiting the viral guanylyltransferase or viral methyltransferases, inhibition of viral helicase activity, and inhibition of inosine monophosphate dehydrogenase (IMPDH) that depletes nucleotide supply necessary for synthesis of viral progeny (Leyssen et al., 2008).

Ribavirin is effective against HFRS and HPS viruses *in vitro* (Huggins, 1989; Kirsi et al., 1983; Medina et al., 2007; Severson et al., 2003). It has been shown that against HTNV, and likely other HFRS viruses, ribavirin causes lethal mutagenesis of the virus (Chung et al., 2007; Severson et al., 2003; Sun et al., 2007). Nevertheless, ribavirin lacks specificity and reduces cellular RNA and DNA pools through its potent IMPDH inhibitory activity (Streeter et al., 1973). Some ribavirin derivatives are being studied against hantaviruses and show promise of less toxicity and similar efficacy (Chung et al., 2008; Kirsi et al., 1983).

In preparation for *in vivo* experiments, Medina and coworkers. demonstrated ribavirin's ability to inhibit SNV infection in Vero E6 cells (Medina et al., 2007). In a suckling mouse model, ribavirin effectively reduced viremia and mortality caused by HTNV challenge (Huggins et al., 1986; Kim and McKee, 1985). Ribavirin also reduced viral loads and inhibited seroconversion of deer mice (natural host) when infected with SNV (Medina et al., 2007). Although these studies demonstrate *in vivo* efficacy, these models are not considered to be reflective of human disease (Holbrook and Gowen, 2008). Moreover, toxicity is considered a problem at the concentrations required for effective therapy (>15mg/kg) (Booth et al., 2003; Chapman et al., 1999; Huggins et al., 1986; McKee et al., 1988).

Based on the limited success of *in vitro* and *in vivo* findings, the use of ribavirin was explored for treatment of HFRS. In a double-blind controlled study, ribavirin was shown to reduce the chance of mortality of a person with HFRS by seven-fold (Huggins et al., 1991). Clinical trials have been unsuccessful in evaluating ribavirin's efficacy in treating HPS. Out of the two trials attempted, one was not able to recruit enough subjects, and the other lacked a placebo control resulting in neither study having the statistical power to determine therapeutic effect (Chapman et al., 1999; Jonsson et al., 2008; Mertz et al., 2004).

Other than ribavirin, there are few antiviral treatment options for diseases caused by hantaviruses. Favipiravir (T-705; 6-flouro-3-hydroxy-2-pyrazinecarboxamide) is a novel pyrazine compound found to have inhibitory antiviral activity against a number of RNA viruses, including members of the *Bunyaviridae* family (Furuta et al., 2009; Gowen et al., 2007, 2010). The mechanism

of action of favipiravir varies from ribavirin in that it appears to specifically inhibit the viral RNA polymerase while having little to no effect on nucleic acid pools (Furuta et al., 2005). Studies evaluating the activity of favipiravir against hantaviruses have yet to be performed and are warranted.

In addition to favipiravir, other drug candidates including lactoferrin, FGI-106, and nitric oxide (NO) continue to be developed and are being evaluated against hantaviruses. Tested against the Seoul HFRS-causing hantavirus, bovine lactoferrin was found to inhibit virus to 15% of infected controls, and completely inhibit viral growth when used in combination with ribavirin (Murphy et al., 2000). Lactoferrin is also a protein commonly found in human saliva. One study found human saliva inhibited HTNV replication by 75%, and the salivary protein mucin was up to 90% effective (Hardestam et al., 2008). FGI-106, a small molecule compound that is postulated to interfere with viral egress has an antiviral effect on a number of bunyaviruses. The compound reduces HTNV and ANDV virus yield by at least 1 $\log_{10}$ PFU/mL and shows promise against HPS viruses based on preliminary findings that indicate FGI-106 enrichment within lung tissue at levels that may block hantavirus infections (Smith et al., 2010).

NO has shown strong antiviral activity against a number of viruses *in vitro* (Croen, 1993; Klingstrom et al., 2006; Lin et al., 1997), most likely through inhibition of viral proteases (Saura et al., 1999). HNTV was inhibited by NO in one *in vitro* study resulting in 85% less viable virus than in controls (Klingstrom et al., 2006). Perioxynitrate, an oxidizing reactive oxygen intermediate, also was shown to be

effective against HTNV *in vitro* inhibiting virus replication by 40% (Klingstrom et al., 2006).

An encouraging single patient case showed rapid improvement of oxygenation status in a 16 year old patient with HPS after treatment with NO (Rosenberg et al., 1998). As treatment for acute respiratory distress syndrome, persistent pulmonary hypertension of the newborn, and other conditions of pulmonary distress, NO is used to directly vasodilate vascular smooth muscle of the lungs and reduces pulmonary artery pressure without producing systemic hypotension (Frostell et al., 1991; Pepke-Zaba et al., 1991; Roberts et al., 1992; Rossaint et al., 1993). Reduction in vascular resistance may reduce pulmonary edema and with its additional antiviral effects, inhaled NO may be a promising treatment of HPS, especially in combination with a traditional antiviral that directly targets the viral replicative machinery such as favipiravir. With the lack of effective antiviral therapy for HPS, the growing incidence of the disease, the potential weaponization of viruses that cause HPS, and the significant case fatality rate of the disease, the development of effective countermeasures is paramount.

OBJECTIVES

It is not known if MPRLV is pathogenic to humans. In order to investigate this possibility, we compared known traits of pathogenic, and more specifically HPS-causing hantaviruses, which have been linked to pathogenicity. First, we tested the hypothesis that MPRLV infectivity would be mediated by the β_3-integrin receptor as with other pathogenic hantaviruses (Gavrilovskaya et al., 1998, 2002). We also

hypothesized that MPRLV would contain conserved ITAMs as well as the degron sequence within the G1 cytoplasmic tail, further supporting likelihood of pathogenicity. We examined these sequences at the amino acid level to measure phylogenetic relatedness to other known HPS-causing hantavirus. Lastly, we evaluated favipiravir against MPRLV, DOBV, and PHV in cell culture comparing its activity to ribavirin using FFU and qRT-PCR assays.

CHAPTER 2

METHODS AND RESULTS

MATERIALS AND METHODS

Cells and viruses

Vero E6 (African green monkey) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), and gentamicin (Sigma, St. Louis, MO). All media and serum were from HyClone Laboratories (Logan, UT) unless otherwise stated. PHV (MP40 TVP6042), DOBV (Sotkama), and MPRLV (HV9021050) were provided by Dr. Robert Tesh (World Reference Center for Emerging Viruses and Arboviruses, University of Texas Medical Branch, Galveston, TX). All three viruses were propagated in vero E6 cells and confirmed by quantitative RT-PCR (data not shown). Infections were performed in DMEM with 2% FBS and gentamicin. Biosafety level 3 (BSL-3) facilities were used for studies with DOBV and MPRLV, while BSL-2 containment was used for work with PHV.

Ligands, antibodies, and staining reagents

SNV hyperimmune mouse serum, kindly provided by Dr. Robert Tesh, was used as the primary anti-hantavirus antibody for FFU detection. The secondary antibody used was a goat anti-mouse horseradish peroxidase conjugated antibody from Kirkegaard & Perry Laboratories (KPL, Gaithersburg, MD). Both were used at a 1:500 dilution. Antibodies directed at β_1 and β_3-integrins for receptor usage

determination studies were purchased from Millipore (Chemicon, Temecula, CA) and used at 1 μg/ml concentrations. Fibronectin and vitronectin were purchased from Sigma and used at concentrations of 20 and 1 μg/ml, respectively. Acidin and biotinylated horseradish peroxidase complex (ABC) and 3,3'-diaminobenzidine (DAB) staining kits were purchased from Vector Laboratories (Burlingame, CA).

Integrin usage

To determine the β-integrin receptor used to mediate hantavirus infection, 96-well plates were seeded with Vero E6 cells. After culture for 24 hours, cells were pretreated with known β-integrin ligands or function-blocking antibodies for 1 hour (h) at 37^0C before viral infection. Ligands or antibodies were removed, monolayers were washed with warm phosphate buffered saline (PBS), and 1.1×10^4 FFUs of MPRLV or 4.25×10^4 FFUs of DOBV or PHV were adsorbed onto the cells for 90 minutes. After adsorption, virus inocula were removed, monolayers were washed with warm DMEM, and incubated an additional 24 h for MPRLV, 72 h for DOBV, and 5 days for PHV.

Immunoperoxidase staining of the nucleocapsid protein in infected cells has been previously described (Gavrilovskaya et al., 1998). In brief, cell monolayers were fixed with 100% methanol (100 μl/well) at 4^0C for 10 minutes, then washed gently with 0.2 ml warm DPBS. Cells were permeabilized with 0.25% Triton X-100 in PBS for 10 minutes, washed with DPBS, then blocked with 4% goat serum in 1% BSA in PBS for 60 minutes. Blocking sera were removed and monolayers were incubated with primary antibody, diluted in 1% BSA in PBS, for 90 minutes at 37^0C and subsequently with secondary antibody for 90 minutes. Following the antibody

incubation and washing steps, the ABC kit was used to prepare cells for staining. Monolayers were washed with 0.2 ml warm DPBS and stained with the working DAB solution for 5 to 30 minutes. After staining, monolayers were washed with water, FFUs quantified and integrin antibody or ligand treated experimental groups were compared to untreated controls.

Phylogenetic analysis

To determine the genetic relationship of MPRLV to other hantaviruses, comparisons of conserved motifs present in pathogenic hantaviruses were made. Amino acid sequences from the Genbank database were compared using Molecular Evolutionary Genetics Analysis (MEGA) software (Center for Evolutionary Medicine and Informatics, Tempe, AZ). Analysis of phylogenetic relationships were made using the neighbor-joining and bootstrap consensus methods (Saitou and Nei, 1987) analyzing only the ITAM/degron coding regions (Geimonen et al., 2003a, 2003b) of the M segment of the genome at the protein level. The evolutionary distances were computed using the poisson correction method and are in the units of the number of amino acid substitutions per site (Zuckerkandl, 1965). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 30 positions in the final dataset.

Hantavirus M segment Genbank sequence accession numbers were as follows: Andes (AAO86638.1), Bayou (AAA61690.1), Bermejo (AAB87911), Choclo (ABB90558.1), Dobrava (AAY27875.1), Hantaan (AAK27683.2), Laguna (AAB87603), Maporal (AAR14889.1), New York-1 (AAC54560), Prospect Hill

(CAA38922.1), Puumula (AB297666.2), Seoul (NP_942557.1), Sin Nombre (AAG03036.1), and Tula (NP_942586).

Drug efficacy

Favipiravir was provided by the Toyama Chemical Co. (Toyama, Japan). Ribavirin was obtained from (ICN Pharmaceuticals, Costa Mesa, CA). The antiviral activity of favipiravir and ribavirin was determined as follows. Vero E6 cells were seeded in 96-well plates and incubated for 24 hours prior to the addition of drugs and viruses. Cells were then infected with hantavirus FFUs as specified above. MPRLV and DOBV inoculums were adsorbed for one hour, and PHV was absorbed for six hours. After infection, virus was removed and cell monolayers were treated with eight serial half-$\log_{10}$ dilutions of the drugs. Plates were incubated at 37°C with 5% CO_2 in DMEM with 2% FBS and gentamicin for 24 h - 5 days depending on the virus. Additional experiments were done with non-infected cells to determine drug toxicity under the same experimental conditions.

Drug efficacy was determined by quantification of infected cells and finding the 50% effective concentration (EC_{50}), defined as the concentration of the drug required to reduce viral FFUs by 50%. The 50% cell cytotoxic concentration (CC_{50}) was defined as the concentration of the drug that resulted in a 50% decrease in cell viability when compared to cell controls. The selectivity (or therapeutic) index (SI) is defined as the ratio of the CC_{50} to the EC_{50}.

Quantitative RT-PCR was also used to determine drug efficacy against MPRLV by measuring relative viral RNA concentrations after incubation of infected cells with the drugs. Triplicate wells of a 24-well plate were infected with MPRLV for six

hours after which virus innocula was removed and the cells were treated with $\log_{10}$ dilutions of ribavirin or favipiravir. After three days in culture, RNA was collected using an RNeasy kit following the manufacturer's recommendations (Qiagen, Germantown, MD).

The MGB Eclipse probe system from Epoch Biosciences (Bothell, WA) was used in combination with the SuperScript III Platinum One-Step Quantitative RT-PCR system from Invitrogen (Carlsbad, CA) following manufacturers' recommendations. The MPRLV primer and probe combination was designed using the MGB Eclipse Online Design software and targeted the nucleocapsid coding sequence. The forward primer used was 5'-GGA CAT TTC CAT AAC GCA GTG-3' and the reverse primer was 5'-TGG CAG CTC AGA AAC TGG CTT CAA A-3'. The probe target sequence was (MGB EDQ)-5'-GTC ATC AGG TTC AAG C-3'-(FAM). All reactions were performed in multiplex with β2-microglobulin primers and probe set for normalization of RNA loading. Reactions were run on a DNA Engine Opticon 2 Real-Time PCR detection system (Bio-Rad, Hercules, CA) using the following cycling conditions: 50°C for 20 min, 95°C for 2 min, 40 cycles of 95°C and 56°C, followed by a melting curve reading every 0.2° C from 50°C to 95°C.

Statistics

Statistical analysis was done using GraphPad Prism software (GraphPad Software, La Jolla, CA). Differences between untreated virus control infections and treated infections were evaluated by two-way analysis of variance (ANOVA) with Bonferroni post-test.

RESULTS

β_3-Integrin chain-specific antibodies and the extracellular matrix protein vitronectin, block MPRLV infectivity

β_3-integrin usage is a characteristic feature shared by pathogenic hantaviruses. We hypothesized that MPRLV would also require β_3-integrin receptors to mediate cell entry and infection. To assess MPRLV integrin usage, Vero E6 cell monolayers were treated with antibodies or integrin antagonists to block hantavirus infectivity. Following incubation, infected cells were quantified by FFU assay and experimental treatment groups were compared to untreated virus-infected controls. PHV infection was significantly inhibited by β_1-specific integrin antibodies ($P < 0.001$), but not β_3-integrin antibodies (Figure 1). Pretreatment of Vero E6 cells with polyclonal antibodies to β_3-integrins significantly decreased MPRLV and DOBV infectivity.

We next assayed the ability of specific integrin ligands to inhibit MPRLV infectivity. Vitronectin and fibronectin are ECM proteins with high affinity for $\alpha_V\beta_3$- and $\alpha_5\beta_1$-integrins, respectively. Consistent with the antibody studies, pretreatment of Vero E6 cells with vitronectin (1 μg/ml) significantly reduced MPRLV and DOBV infectivity ($P < 0.001$). In contrast, PHV infection was not significantly inhibited with vitronectin, only fibronectin (20 μg/ml) ($P < 0.001$) (Figure 2). Collectively, these data suggest that MPRLV host cell infection is facilitated principally by β_3-integrins, similar to the known pathogenic DOBV.

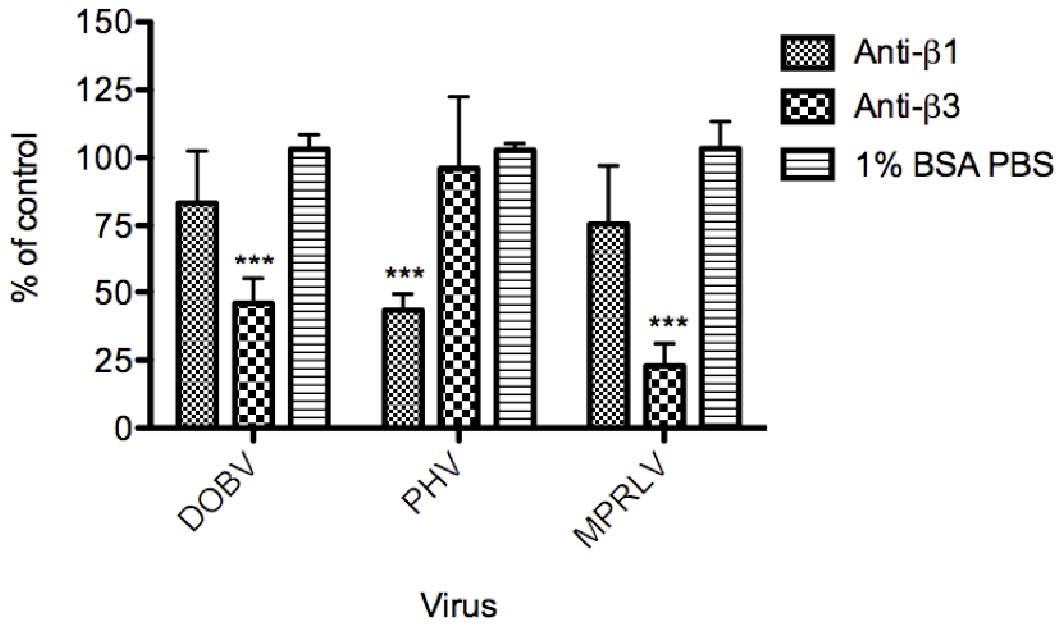

Figure 1. Hantavirus infection is dependent on specific integrins as receptors. Duplicate wells of Vero E6 cells were pretreated with integrin-specific antibodies (at 1 μg/ml) for 1 hour prior to viral infection. After infection and incubation for 1-5 days (depending on virus) cell monolayers were fixed and stained for FFUs. Viral FFUs were quantified and compared to infected and uninfected controls. Data are representative of three independent experiments. ***$P < 0.001$.

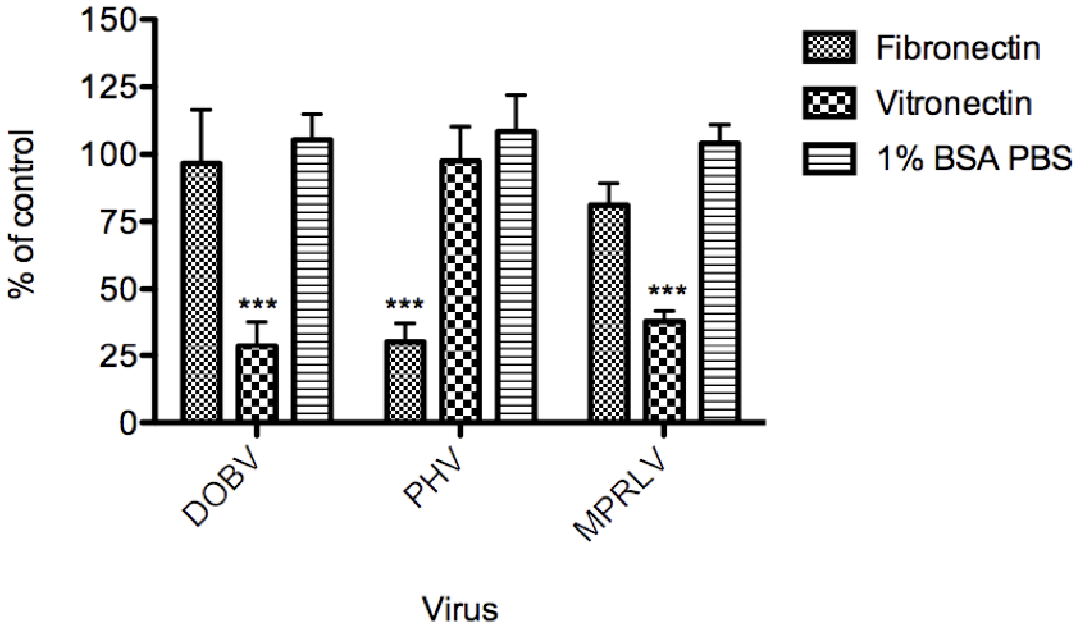

Figure 2. The effect of endogenously added extracellular matrix proteins on hantavirus infection. Duplicate wells of Vero E6 cells were pretreated with extracellular matrix proteins vitronectin and fibronectin (at 1 and 20 μg/ml, respectively) for 1 hour prior to viral infection. Virus was quantified by FFU assay. Data are representative of three independent experiments. ***$P < 0.001$.

Phylogenetic analysis

MPRLV shares ITAM and Degron sequence homology with pathogenic hantaviruses

We performed phylogenetic analysis to compare MPRLV gene sequences at the protein level with thirteen other hantaviruses, as a means to obtain insights into potential pathogenicity. Pathogenic hantaviruses have been found to contain conserved ITAMs in the G1 cytoplasmic tail, while nonpathogenic viruses lack this feature (Geimonen et al., 2003b). Pathogenic hantaviruses also contain a degron sequence that directs proteosomal degradations, whereas nonpathogenic hantaviruses have stable cytoplasmic tails (Sen et al., 2007). Alignment of hantavirus sequences obtained from Genbank that contain the overlapping ITAM and degron regions is shown (Figure 3).

The alignment, conducted using ClustalW, indicated that the G1 cytoplasmic tail of MPRLV contained the characteristic ITAM sequence found in all HPS-causing hantaviruses (Figure 3). The comparison also indicated that MPRLV does not contain the conserved residues that stabilize the G1 tail in nonpathogenic hantaviruses, but does contain residues consistent with pathogenic hantaviruses (Figure 3). In looking at the entire sequence alignment of 30 amino acids, MPRLV was found to be more closely related to the HPS-causing hantaviruses (Figure 4). These findings suggest that MPRLV shares motifs characteristic of pathogenic HPS-causing hantaviruses.

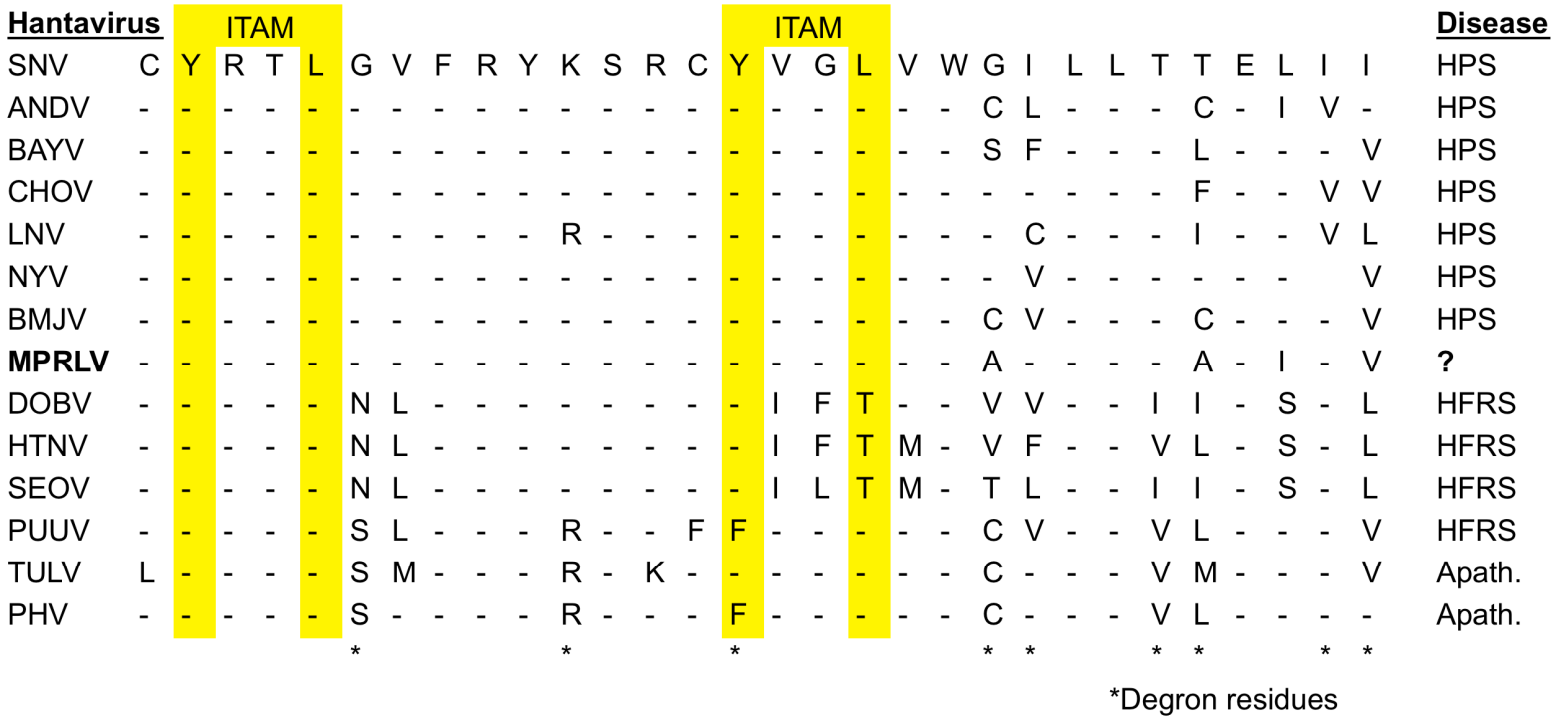

Figure 3. Amino acid comparison of ITAM/degron motifs. Alignment of G1 protein cytoplasmic tail of hantaviruses.

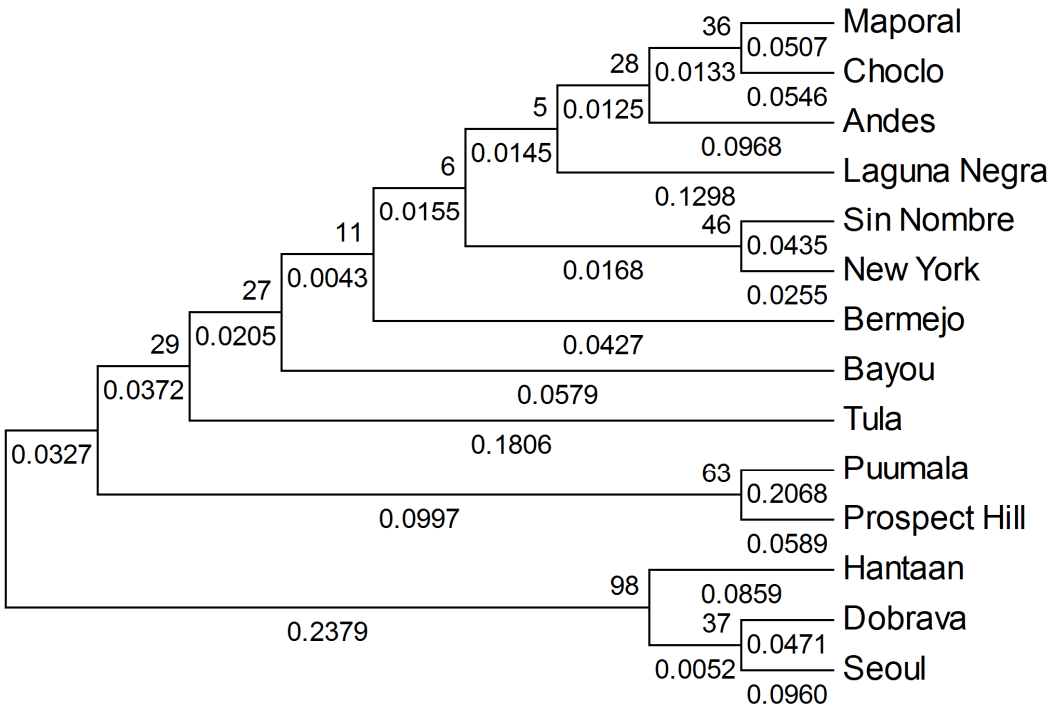

Figure 4. The phylogenetic relationships among the ITAM/degron sequences of thirteen different hantaviruses. The analysis was done with MEGA4 software using the neighbor-joining and bootstrap consensus methods. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary relationships of the taxa analyzed (Felsenstein, 1985). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein, 1985). The tree is not drawn to scale, but indicates branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Drug efficacy studies

Efficacy of ribavirin and favipiravir against MPRLV

The inhibitory activity of ribavirin and favipiravir against hantaviruses was determined by infectious FFU assay (Table 4) and qRT-PCR (Figure 5). Ribavirin, known to be active against HFRS- and HPS-causing hantaviruses *in vitro* (Murphy et al., 2000; Severson et al., 2003; Sun et al., 2007), inhibited MPRLV infectivity in a dose-dependant manner, with an EC_{50} of 47 μM and therapeutic index of 22 (Table 4). As expected, ribavirin was also active against DOBV and PHV. Favipiravir, a pyrazine derivative reported to be effective inhibiting related bunyaviruses (Gowen et al., 2007) was also found to be active against MPRLV with an EC_{50} of 65 μM and therapeutic index of 74 (Table 4). Notably, favipiravir was also effective against DOBV and PHV.

QRT-PCR was also employed to evaluate the anti-MPRLV activities of ribavirin and favipiravir. As shown in Figure 5A, ribavirin reduced MPRLV RNA levels in a dose-dependent manner and with an EC_{50} of 16 μM, lower than with the FFU assay data. The activity of favipiravir was also verified by the qRT-PCR assay with an EC_{50} less than the lowest tested dose of 8 μM (Figure 5B).

TABLE 4. *In vitro* inhibitory effects of favipiravir and ribavirin against hantaviruses[a] (FFU)

Virus	Favipiravir			Ribavirin		
	CC_{50}± SD[b]	EC_{50} ± SD[b]	SI[c]	CC_{50}± SD[b]	EC_{50} ± SD[b]	SI[c]
PHV	3819 ± 64	66 ± 26	58	1018 ± 866	23 ± 1.9	44
DOBV	4816 ± 662	93 ± 18	52	1215 ± 628	72 ± 2.4	17
MPRLV	4795 ± 1186	65 ± 17	74	1051 ± 135	47 ± 2.9	22

[a] Data are the mean and standard deviations from three separate data sets in Vero E6 cells

[b] CC_{50} and EC_{50} values are in μM.

[c] SI (selectivity index) = CC_{50}/EC_{50}

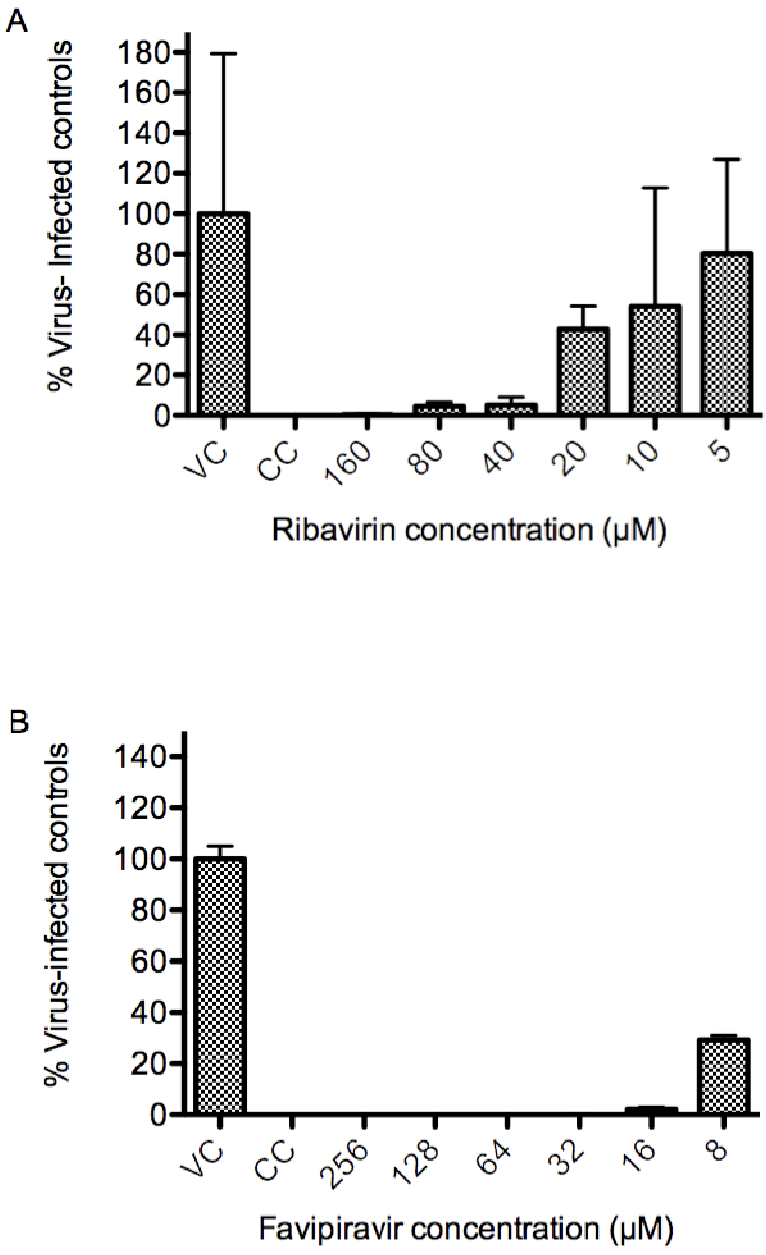

Figure 5. MPRLV sensitivity to favipiravir. MPRLV RNA was measured by qRT-PCR from total RNA extracted from infected Vero E6 cells and treated with varying concentrations of ribavirin (A) and favipiravir (B).

CHAPTER 3

DISCUSSION AND CONCLUSIONS

Worldwide, hantaviruses cause over 200,000 hospitalized cases of illness annually with mortality rates exceeding 40% in HPS cases (Schmaljohn and Hjelle, 1997). The need for further research into therapeutic interventions increases as exposure to rodent hosts is ever present and person-to-person transmission has been documented in the case of ANDV (Padula et al., 1998). Although there are several high-profile hantaviruses that receive the most attention, new serotypes are continually being discovered as awareness of the genus grows. MPRLV is a recently discovered member identified in Venezuela. It has not yet been determined if MPRLV is a pathogenic hantavirus capable of infecting and causing severe disease in humans. However, MPRLV has been reported to cause HPS-like disease in a hamster model (Milazzo et al., 2002) similar to that caused by ANDV (Hooper et al., 2001). In the present study, we have presented data that supports the hypothesis that MPRLV may be a human pathogen. We also describe data on the susceptibility of MPRLV and other representative hantaviruses to a licensed (ribavirin) and an investigational new drug (favipiravir), with known activity against related *Bunyaviridae* family members (Gowen et al., 2007, 2010)

Previous studies have shown that hantavirus infection of host cells is mediated through interaction of the viral G1 glycoprotein with specific integrins (Gavrilovskaya et al., 1998, 1999; Jonsson et al., 2008). Importantly, pathogenic hantaviruses utilize β_3-integrins, while their nonpathogenic counterparts use β_1-

integrins. In our studies characterizing MPRLV, pretreatment of cells with function-blocking β_3-integrin antibodies and vitronectin significantly reduced infectivity. Nevertheless, complete inhibition could not be achieved, suggesting other mechanisms of virus entry are likely.

Antibodies against integrins have been used to inhibit viral entry into cells (Vero, Vero E6, and rhabdomyosarcoma) (Chu and Ng, 2004; Gavrilovskaya et al., 1998, 1999; Heikkila et al., 2009; Medina et al., 2007). Effective concentrations of integrin-blocking antibodies against β_3-integrins with hantaviral infection were previously found to be between 1-20 μg/ml (Gavrilovskaya et al., 1998). Therefore, the amounts used for blocking in the present experiments were within the effective range. In previous work (Gavrilovskaya et al., 1998), and our optimization experiments, there was a dose dependent response with inhibition decreasing at lower concentrations of blocking antibody. We did not see greater inhibition using concentrations higher than 1 μg/ml in our studies. Similarly, previous ECM protein blocking studies used a concentration range of 1-40 μg/ml (Gavrilovskaya et al., 1998). We found that 20 μg/ml of fibronectin and 1 μg/ml of vitronectin were optimal for blocking β_1- and β_3-integrin interaction with hantaviruses, respectively.

Recent studies have shown that in addition to the β-integrins, hantaviruses also exploit DAF to successfully infect cells (Buranda et al., 2010; Krautkramer and Zeier, 2008). This may explain why, after incubation with ECM proteins or antibodies to the β-integrins, viruses still were able to infect cells with 'blocked' receptors resulting in less than complete inhibition. Studies blocking both β-integrin and DAF proteins would presumably result in more complete blockage of hantaviral

infection. Partial inhibition of pathogenic hantavirus following treatment of cells with β_1-integrin-specific blocking antibodies may also be attributed to steric hindrance of viral binding due to conformational change of the integrin as seen in experiments with West Nile virus and other hantaviruses (Chu and Ng, 2004; Gavrilovskaya et al., 1998). Notably, experimental methods by Gavrilovskaya et al. differed slightly from ours, including the use of fewer FFUs of virus for infections, staining reagents, and timing of infections.

In addition to integrin utilization pattern as a predictor of pathogenicity, several motifs present in the cytoplasmic tail of the G1 glycoprotein of hantaviruses can also be distinguishing features. Our comparative analysis of the stretch of amino acids encompassing both the ITAM and degron sequences clearly demonstrate extensive similarity between MPRLV and other HPS-hantaviruses. ITAM motifs participate in intracellular signaling that directs cellular mechanisms. Viruses can mimic, contain, or even regulate ITAM signaling responses of host cells (Geimonen et al., 2003a). The presence of such motifs almost exclusively in pathogenic HPS-causing hantaviruses may suggest an additional mechanism by which virulence and host cell function can be regulated by the virus. Notably, the apathogenic hantavirus, Tula, contains the ITAM sequence. However, its G1 tail is missing an adjacent cysteine residue conserved in all other hantaviruses that likely alters the conformation of the ITAM in the Tula G1 tail (Geimonen et al., 2003b). The degron sequence is a c-terminal hydrophobic domain of the G1 cytoplasmic tail that directs proteasomal degradation and is also linked to the virulence of hantaviruses (Geimonen et al., 2003a, 2003b). Experiments to confirm tail-directed proteasomal

degradation of the MPRLV sequence would further support the idea that MPRLV may emerge as a human pathogen.

In our analysis restricted to the ITAM and degron sequences, Puumala, a hantavirus that causes a mild form of HFRS (Elliott, 1997), segregates with the apathogenic PHV. Phylogenetic analysis not only serves to reveal relationships between hantaviruses that cause the same disease, but also a correlation with the phylogeny of their rodent hosts (Antic et al., 1992; Monroe et al., 1999; Plyusnin et al., 1996). To this end, the sharing of rodents hosts such as that of PHV and Puumala virus hantaviruses (*Arvicolinae*) likely drives these associations. MPRLV had significant similarities with pathogenic, and more specifically HPS-causing, hantaviruses such as SNV and ANDV. With population growth and spreading of urbanization in regions of Venezuela where the virus is harbored in rodents may ultimately result in human cases of HPS. At present, the lack of medical infrastructure, and awareness by physicians of HPS disease likely contribute to the lack of cases reported. As occurred in the four corners region of the southwestern United States with the initial SNV outbreak, the potential for a disease outbreak certainly exists based on the evidence collected thus far by our characterization of MPRLV.

There are currently no effective antivirals or other therapies for clinical treatment of HPS, and ribavirin is the only antiviral somewhat effective against HFRS. Ribavirin is licensed for the treatment of hepatitis C and respiratory syncytial virus infections in infants, but considered to be relatively nonspecific and is associated with toxicity primarily in the form of hemolytic anemia (Leyssen et al.,

2008). Clinical trials have been unsuccessful in evaluating the efficacy of ribavirin treatment of HPS (Chapman et al., 1999; Jonsson et al., 2008; Mertz et al., 2004). The results from our studies with the investigational new drug, favipiravir, are very encouraging as they indicate susceptibility of MPRLV and DOBV in cell culture. First described as an anti-influenza agent (Furuta et al., 2002), the spectrum of favipiravir's antiviral activity has since grown to include arenaviruses, and several bunyaviruses (Furuta et al., 2002; Gowen et al., 2007, 2010). Here we have expanded the spectrum to include *in vitro* antiviral activity against hantaviruses.

We found favipiravir to be active against representative hantaviruses, DOBV, PHV, and MPRLV, in a dose-dependant manner with EC_{50} values in the range of those reported for other bunyaviruses including La Crosse virus, Punta Toro virus, Rift Valley fever virus, and sandfly fever virus (EC_{50} range of 32 to 191 μM) (Gowen et al., 2007). The variation in EC_{50} values between different viruses may be attributed to differences in favipiravir phosphorylation to the active triphosphate form of the drug; a process that can vary between cell lines used in the assays (Furuta et al., 2005), as well as differences in methodologies. In comparison to ribavirin, the data indicate that favipiravir is comparably active, but far less cytotoxic in our hantavirus experimental systems. The present study supports future pre-clinical development of favipiravir in the hamster ANDV or MPRLV infection models or the mouse HNTV infection model as proof-of-concept for *in vivo* efficacy.

ANDV and MPRLV are the only pathogenic hantaviruses for which there is an animal model that closely resembles human disease (Hooper et al., 2008; Milazzo et

al., 2002). The Syrian golden hamster produces symptoms very similar to human HPS when infected with these viruses. Both MPRLV and ANDV have been shown to cause disease when infected intramuscularly, with subcutaneous, intranasal, and intragastric injections also effective with ANDV (Hooper et al., 2008; Milazzo et al., 2002). Using these animal models, challenge efficacy studies with ANDV or MPRLV, or alternatively, the mouse HNTV infection model will provide important data towards advancing a much needed antiviral candidate towards the clinic to combat HPS. Ultimately, favipiravir or other antiviral drug therapy would need to be combined with a biological response modifier that curtails the vascular leak that contributes to disease severity and lethality in the HPS hantavirus hamster models.

In summary, MPRLV shares distinctive qualities with other pathogenic, and especially HPS-causing hantaviruses, suggesting that human infection with the virus could result in severe disease. Though the clinical significance of effective *in vitro* compounds such as favipiravir is uncertain at present, studies in animal models and clinical trials may prove them to be effective in the future. Considering together the continued discovery of new pathogenic hantaviruses, the lack of effective therapies, and potential weaponization of these viruses (Borio et al., 2002), a better understanding of hantaviral disease and the development of prophylactic and therapeutic treatments is urgently needed.

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