AN ABSTRACT OF THE DISSERTATION OF

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Title: Targeted Development of Antivirals against Influenza A and Respiratory Syncytial Virus.

Abstract approved:

Manoj K. Pastey

Influenza A and Respiratory Syncytial Virus (RSV) are both enveloped, negative strand RNA viruses which infect the respiratory mucosa of animals and humans. Despite decades of research and development of antivirals and vaccines, both of these viruses continue to be a major health concern throughout the world. The focus of my research was to examine alternative ways of developing antiviral which would be both faster and more effective than conventional methods of screening libraries of compounds. By examining the nucleotide sequences of influenza A virus my colleagues and I were able to determine conserved regions as candidates for antisense drug targets. Lead antisense compounds were effective at inhibiting influenza A virus replication in a Balb/C mouse model. Although resistance was observed in some strains, a combination of antisense targeting multiple conserved regions was effective. One limitation of the antisense used in these experiments was their inability to inhibit virus replication when administered

more than 2h after infection. For RSV, I examined the potential of targeting a cellular protein (ubiquitin) thought to interact with and be required for RSV replication. I utilized currently available drugs to interfere with ubiquitin recycling through the proteasome. Treatment with proteasome inhibitors reduced virus replication in cell culture and had an indirect effect on RSV virion formation. However, treatment of RSV infection with a proteasome inhibitor in a Balb/C mouse model resulted in an altered immune response, increased pathology in the lungs, and increased mortality. From these results I conclude that unless sufficient background information is available for the target of interest, the targeted development of antivirals for influenza A and RSV is no more effective than conventional methods.

Targeted Development of Antivirals against Influenza A and Respiratory Syncytial Virus

by

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

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Christopher Lupfer, Author

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CONTRIBUTION OF AUTHORS

David A. Stein assisted with the experimental design and writing of Chapter 2. Patrick L. Iversen assisted with the experimental design of Chapter 2. Dan V. Mourich assisted with flow cytometry and Samuel E. Tepper assisted with influenza PB1 sequencing for Chapter 2. Kristin M. Patton performed histopathological examination of mice lungs for Chapter 4.

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DEDICATION

This work is dedicated to my wife, mother, and father; as well as all the teachers and mentors who never gave up on me.

Targeted Development of Antivirals against Influenza A and Respiratory Syncytial Virus

Chapter 1:

General Introduction

Influenza A virus

Virus life cycle. Influenza A is an enveloped, segmented, single stranded, negative sense, RNA virus belonging to the family *Orthomyxoviridae*. The eight genomic RNA strands of influenza A encode a total of 11 proteins and are arranged as depicted in Table 1.1. These 11 proteins are capable of performing most functions necessary for virus genome replication and nascent virion formation, as well as interfering with the natural defense mechanisms of its host (Strauss and Strauss, 2002).

The influenza A virus life cycle begins when a virus enters the airways of an animal and binds to sialic acid residues which are present on the surface of most cells. The HA membrane glycoprotein is responsible for binding the cell surface receptor sialic acid (Bergelson et al., 1982; Nicholls et al., 2007; Shinya et al., 2006), and once HA is bound to the sialic acid it induces endocytosis. Next, the endocytic compartment begins to acidify as it progresses toward becoming a lysosome. As an ion channel, M2 protein allows for H+ ions to flow from the endocytic compartment into the core of the virus. This acidification has two effects. First, it induces a conformational change in the HA protein such that the HA protein inserts itself into the endosome membrane and mediates the fusion of the viral membrane with the endosome membrane (Takeuchi and Lamb, 1994; Wharton et al., 1994). Secondly, the acidification results in the disassociation and unpackaging of nucleocapsids from the M1 matrix protein (Bukrinskaya et al., 1982).

Table 1.1: Genome Organization and Proteins of Influenza A. Adapted from NCBI, 2004 and Strauss and Strauss, 2002.)

Genome segment	Length (nt)	Protein (Abrev.)	Protein Size (aa)	Function
1	2250-2350	Polymerase Basic 2 (PB2)	759	Cap snatching
2	2250-2350	Polymerase Basic 1 (PB1)	757-758	Major polymerase
		Frame 2 (PB1-F2)*	52-90	Induces apoptosis
3	2150-2250	Polymerase Acidic (PA)	716	Endonuclease for cap snatching
4	1600-1800	Hemagglutinin (HA)	552-570	Receptor binding, membrane fusion
5	1450-1600	Nucleoprotein (NP)	498	Forms helical structure w/ vRNA, genome replication
6	1350-1500	Neuraminidase (NA)	447-473	Sialidase, nascent virion escape
7	950-1050	Matrix 1 (M1)	252	Associates w/ cell membrane and NP
		Matrix 2 (M2)*	96-97	H^+ Ion Channel, unpackaging, Polymerase activ.
8	800-900	Non-structural (NS/NS1)	217-230	Inh. cell mRNA transport, cap snatching, and IFN
		Nuclear Export Protein (NEP/NS2)*	121	Nuclear export of nascent nucleocap.

* indicates spliced transcript or alternative reading frame

Once in the cytoplasm, the virus nucleocapsid, consisting of the virus genomic RNA (vRNA), the three polymerase proteins PB1, PB2, and PA, as well as the NP protein, is translocated to the nucleus (Bullido et al., 2000; Martin-Serrano et al., 2004). The first step in virus replication is mRNA production. The polymerase complex initiates mRNA production by snatching the first ~13nt of the 5'mG caps from cellular mRNA. This function is performed by the PB2 and PA proteins, with the actual nuclease activity residing in the PA protein (Dias et al., 2009; Guilligay et al., 2008). Once snatched, the cap is used to initiate transcription of the viral mRNA where the PB1 protein is the major

replicase. PB1 also adds a poly A tail to the 3' end of the mRNA by a stuttering process where it repeatedly pairs adenines with a series of uracils (Zheng et al., 1999). Virus mRNAs are then preferentially protected from cap snatching by the polymerase (Neumann et al., 2002) and exported from the nucleus to the cytoplasm by the NS protein (Satterly et al., 2007). After sufficient levels of viral proteins have accumulated, a switch in the virus polymerase occurs from mRNA synthesis and to genome replication. Specifically, NEP facilitates the switch and new polymerase performs the genome replication (Jorba et al., 2009; Robb et al., 2009). Genome replication is a two-step process that involves the production of a full length positive sense antigenome (cRNA). The cRNA is then used as a template to produce the full length negative sense genomic vRNA. In this process the cRNA is only an intermediate and it is thought to be produced at a much lower ration to the final vRNA (i.e. 1 cRNA can be used as a template multiple times to produce the desired vRNA). In addition, NP is needed to bind to newly synthesized genomes (Portela and Digard, 2002). NP associates with the vRNA and cRNA (in much the same way that single stranded binding protein does during DNA synthesis) to form the nucleocapsid. The association of NP with nascent RNA is necessary for polymerase activity and also serves to create a compact helical genome suitable for downstream packaging into nascent virions.

Following protein synthesis and genome replication, the virus begins to package itself for exit from the host cell. The first step in this process is the export from the nucleus of mature nucleocapsid, consisting of nucleoprotein encapsidated vRNA as well as the polymerase complex and the M1 protein. Nucleocapsid nuclear export is mediated by the virus NEP and NP in association with the M1 protein (Portela and Digard, 2002;

Robb et al., 2009). Once out in the cytoplasm, the main function for the matrix protein is to form a shell around the virus nucleocapsid and package the innards of the virus for exit from the cell. The M1 protein bridges the association of the nucleocapsid with the virus surface glycoproteins imbedded in the cell membrane. The M1 protein is thought to associate both with the cell membrane and directly with the HA, M2, and NA proteins which are localized to the apical surface of infected cells by exocytosis and preferentially accumulate in cholesterol rich lipid rafts. Once all these components are present at the cell membrane the virus buds out of the cell through a combination of viral and host factors (reviewed in Nayak, 2001). The NA protein performs its function at this final step in the virus life cycle. The NA protein is a sialidase enzyme and as the virus buds from the cell membrane it cleaves off any nearby sialic acid receptors thus preventing the nascent virus from binding to and reinfecting the cell it just left (Palese and Compans, 1976).

Genetic diversity and immune evasion. Influenza viruses are classified into subtypes based on the antigenic nature of their HA and NA surface glycoproteins. There are 16 antigenically distinct HA and 9 antigenically distinct NA proteins for a total of 144 possible subtypes of influenza A virus. In reality, however, not all HA and NA combinations are possible as the HA and NA proteins possess opposing functions. HA binds to sialic acid for cell entry and NA cleaves it off and prevent cell entry. Therefore, there are only certain combinations that allow for an appropriate combination of HA binding affinity and NA enzyme kinetics (Al Faress et al., 2008; Kaverin et al., 1998). Many combinations are possible and multiple subtypes exist which can infect humans from H1N1, H1N2, H2N2, H3N2, H3N8, H5N1, H7N2, H7N7, and H9N2. In addition to

different subtypes, there are different strains within a given subtype that may have slight changes in HA and NA, or other viral genes. Influenza viruses are classified as follows: influenza species (A,B,C)/Infected animal species (if not human)/geographical location/Isolate from that outbreak/year of isolate, and finally the HA and NA subtypes (e.g. Influenza A/Equine/Miami/1/63 H3N8: this virus is an influenza A virus from a horse in Miami, FL and was the first virus from a 1963 outbreak with an H3N8 subtype).

Antigenic variation occurs by two distinct methods in influenza A virus. The first is referred to as "antigenic shift". Antigenic shift occurs when two separate influenza A viruses infect the same cell simultaneously (Elliott et al., 2007). When virus nucleocapsid packaging occurs, the eight individual genomic segments can reassort in 64 different ways. This reassortment results in the formation of new HA and NA combinations which alters the antigenicity of the virus drastically. The production of novel HA and NA reassortants is what gives rise to global influenza pandemics, as the population of the world has little to no preexisting immunity to the new virus (Guan et al., 2004; Taubenberger and Morens, 2006). As mentioned earlier, however, most of the reassortant viruses fail to spread due to incompatibility between the HA and NA, or other viral proteins, as it is possible for all eight vRNA segments to reassort.

The second means of antigenic variation is much more subtle and is referred to as antigenic drift (Elliott et al., 2007). Antigenic drift occurs when the virus RNA polymerase makes mutations during the replication process. These point mutations occur with a frequency in RNA viruses of about one mutation per genome. Again, the vast majority of these mutations are silent or deleterious. On occasion though, a mutation which changes the antigenicity of the virus or improves its replication efficiency occurs

and provides that virus with a selective advantage. Antigenic drift is generally responsible for seasonal changes in virus antigenicity and results in the annual influenza epidemic patterns which are less severe due to some amount of preexisting immunity in the global population.

The main driving force for antigenic variation is the immune response of the host organism. HA and NA are the major surface antigens of the virus and the predominant targets of the humoral (antibody) immune response (Chen and Deng, 2009). The diversity of size and sequence of these proteins is likely due to constant pressure from the host to create antigenically distinct HA and NA proteins which will evade the adaptive immune response (Blackburne et al., 2008). To allow for the efficient replication of influenza A virus, it must also overcome the innate immune response of the cell. This involves the production of pro-inflammatory signals from infected cells which stimulate the localization of immune cells to the site of infection, as well as the development of an antiviral state in the infected cell (e.g. shutdown of protein translation due to phosphorylation of eIF2α by PKR and production of interferon regulated genes). The NS protein directly inhibits the interferon response (Haye et al., 2009). It also prevents the nuclear export of cellular mRNAs and assists in 5'mG cap snatching of cellular mRNAs (Satterly et al., 2007). These two processes have the result of limiting production of cellular proteins, including those that would be chemokines, cytokines and interferon regulated genes (reviewed in Hale et al., 2008).

Epidemiology. Influenza A is of importance to the human and veterinary medical communities. Millions of chickens, ducks and other birds have died or been euthanized during the H5N1 avian influenza outbreaks in Southeast Asia which has persisted for

over a decade (1997-2009) (Park and Glass, 2007; WHO, 2009b). In addition, hundreds of thousands of pigs were slaughtered during the swine flu scare in the spring of 2009 (Maamoun, 2009). There are also influenza A strains which infect equines and canines and produce regular outbreaks in the dog and horse racing industry which cost not only animal lives but millions of dollars in lost revenue (Crawford et al., 2005; Martella et al., 2007; Tremayne, 2006). On the human aspect, there have been 429 confirmed human infections with H5N1 avian influenza, and the mortality rate among these patients is 50-60% (WHO, 2009b). Finally, seasonal influenza annually results in the death of 300,000-500,000 people worldwide (WHO, 2009a). There has also been concern that the cyclic pattern of influenza pandemics, due to shifts in virus antigenisity, is long overdue and there are fears that the recent zoonotic transmission of H5N1 avian and H1N1 swine flu to humans could lead to increased worldwide mortality with socioeconomic ramifications (reviewed in Guan et al., 2004).

Respiratory Syncytial Virus

Virus life cycle. Respiratory Syncytial Virus (RSV) is an enveloped, single stranded, negative sense, RNA virus in the family *Paramyxoviridae*, subfamily *Pneumovirinae*. The genome structure of RSV consists of a single RNA of ~15,000bp with 10 genes (Tolley et al., 1996)which are made by the virus polymerase through a process known as transcriptional reinitiation (Reviewed in Cowton et al., 2006). This means that after termination of transcription of the first gene, the polymerase reinitiates at the second gene and this pattern repeats to produce 10 mRNA. One effect of transcription reinitiation is transcript attenuation; each subsequent mRNA transcribed is done so in diminishing

quantities compared to the first. The order of genes in the RSV genome is therefore synonymous with the abundance of each specific viral protein in the cell and the nascent virions. The 10 genes of RSV encode for 11 proteins. Nine genes encode for a single protein each, whereas one of the genes produces two proteins by means a process known as coupled translation. When a ribosome finishes translating the first open reading frame it reinitiates translation of a downstrame ORF by means of a secondary structure similar to an IRES, thus producing an additional protein (Gould and Easton, 2007). A synopsis of the 11 proteins of RSV and their known functions is shown in Table 1.2.

Table 1.2: Genome Organization and Proteins of RSV. Adapted from Tolley et al., 1996 and Ghildyal et al., 2006

Gene	Size (bp)	Protein Name	Size (aa, kD)	Proposed Functions
NS1	531	Nonstructural 1	124, 22	Interfere w/ innate immunity
NS2	501	Nonstructural 2	139, 18	Interfere w/ innate immunity
N	1204	Nucleoprotein	391, 44	Nucloecapsid formation, genome replication
P	889	Phosphoprotein	241, 34	RNA Polymerase cofactor
M	956	Matrix	256, 28	Transcription inhibition, virion structure
SH	409	Small Hydrophobic	64, 7.5	Ion channel ???, unknown
G	921	Glycoprotein	298, 90	Receptor binding, cytokine mimic
F	1902	Fusion	574, 70	Membrane fusion, virus entry
M2	960	Matrix 2-1	194, 22	Transcription anti-terminator Transcript. elongation factor
		Matrix 2-2	90, 11	Genome replication enhancer
L	6577	Large	2165, 200	Virus RNA polymerase

The first step in the RSV life cycle is also entry into the airways of the host and binding to its cell surface receptor. However, unlike influenza viruses, the cell surface receptor(s) for RSV are not well understood, though some possibilities are that it binds to the glucosaminoglycan heparin (Shields et al., 2003) or annexin II (Malhotra et al., 2003). Binding of the cell surface receptor then triggers the F protein to undergo a conformational change and insert into the cell membrane (Walsh and Hruska, 1983). This results in fusion of the virus and cell membrane and introduces the virus core into the cytoplasm.

The core is then unpackaged and mRNA and genome replication occurs in the cytoplasm instead of the nucleus by means of the RSV RNA polymerase L and its cofactors P and M2-1 and M2-2. Similar to influenza, the L protein first produces mRNA which requires the elongation factor and anti-termination functions of M2-1 (Bermingham and Collins, 1999). The L protein does not perform cap snatching, but instead possesses its own capping activity. Like influenza, though, it adds a poly-A-tail by stuttering on 3' uracil residues (Reviewed in Cowton et al., 2006). Once viral protein levels reach sufficient concentrations there is a transition from transcription to genome replication. This transition is mediated by the presence of *cis*-acting elements in the genome, the N protein which enhances polymerase activity by binding to genomic RNA (McGivern et al., 2005), and the M2-2 protein which regulates the switches to genome replication by an as yet undiscovered mechanism (Bermingham and Collins, 1999). Genome replication for RSV is also a two step process that first produces a cRNA which is subsequently used as a template for making more genomic vRNA.

Genome replication is terminated and virus packaging initiated by localization of the M protein to virus replication bodies (Ghildyal et al., 2002; Shields et al., 2003). The M protein possesses the ability to inhibit RNA synthesis (Shields et al., 2003) and also facilitates the interaction of virus nucleocapsids with the cell membrane and the F and G proteins (Ghildyal et al., 2005). Perhaps as a means of sequestering the M protein, it is localized to the nucleus until late in the infection cycle, when it is exported to the cytoplasm in preparation for virion assembly (Reviewed in Ghildyal et al., 2006).

Again, similar to influenza, RSV proteins prefer to associate with cholesterol rich lipid rafts and virus budding initiates from these sites (Malhotra et al., 2003). The F, G and SH proteins (like HA, NA and M2 proteins) are made in the endoplasmic reticulum and transported to the apical surface of the infected epithelial cells where the virus can bud back into the host airways for transmission to the next host. Prior to fusion of the transport vesicle to the cell membrane, the F protein is cleaved by the intracellular protease furin (Gonzalez-Reyes et al., 2001). This cleavage is necessary for the F protein to undergo the conformational changes necessary for membrane fusion during the next round of virus infection. A similar event occurs with the HA protein of influenza virus, however, the cleavage of HA occurs in the extra cellular space by trypsin like proteases (Zhirnov et al., 1982).

Genetic diversity and immune evasion. There are two main subtypes of RSV in humans, namely RSV A and RSV B. Despite the small genetic diversity of RSV, compared to influenza A, reinfection occurs frequently (Parveen et al., 2006); beginning early in life (6-24 months) and continuing throughout adulthood (Nokes et al., 2008). One cause for this is a lack of completely neutralizing antibodies. Though a humoral immune

response develops to RSV the development of antibodies which can prevent RSV from causing infection appears to be problematic (Kawasaki et al., 2004).

The NS1 and NS 2 proteins have known roles in inhibiting interferon signaling by inducing the polyubiquitination and proteasomal degradation of STAT-2, which is a transcription factor normally activated by interferon α or β signaling (Elliott et al., 2007). The NS proteins also interfere with IRF-3, RIG-I and IFN-γ signaling (Aoyagi et al., 2003; Bossert et al., 2003; Kotelkin et al., 2006; Ramaswamy et al., 2004; Ramaswamy et al., 2006; Schlender et al., 2000; Spann et al., 2004). The L protein inhibits apoptosis by activating Akt and signaling the destruction of p53 by polyubiquitination and proteasomal degradation (Groskreutz et al., 2007). The NS proteins also activate the NF-κB signaling pathway, presumably for the anti-apoptotic effects (Bitko et al., 2007; Choudhary et al., 2005; Fink et al., 2008; Indukuri et al., 2006; Thomas et al., 2002). An alternative method employed by RSV is inhibition of host mRNA synthesis by the matrix protein when it is localized to the nucleus (Shields et al., 2003). Cleavage of the F protein by furin results in a small peptide virokinin which mimics tachykinin and may result in smooth muscle contraction of airways (Zimmer et al., 2003). Finally, the G protein is also produced in a secreted form which lacks its membrane spanning domain. This form of G protein has immunomodulatory properties and results in a shift from the normal Th1 immune response to a viral infection, to a Th2 response (Arnold et al., 2004).

Epidemiology. Like influenza, RSV is also an important pathogen of the medical and veterinary communities. Bovine, ovine, caprine, and human variants exist. Bovine and ovine RSV are responsible for substantial losses each year in the cattle ranching industries (Grubbs et al., 2001; Pastey and Samal, 1997). In the case of human RSV, the

vast majority of humans are infected with this virus before the age of two, and it is one of the leading causes of infant mortality world wide with ~160,000 deaths annually (Girard et al., 2005). It is also a health concern in the elderly, most especially in nursing homes where seasonal outbreaks can take a heavy toll. In the United States, ~12,000 geriatric patients die each year from human RSV (Thompson et al., 2003).

Antivirals

Current treatment and prevention options. Influenza A and RSV are both pathogens of the respiratory tract resulting in sometimes severe illness. Influenza A is associated with high fever, cough, aches and chills and often predisposes the individual to secondary bacterial infections (McCullers, 2006; Peltola et al., 2005). RSV infection results in a moderate fever, cough and rhinitis and severe infection has been linked to child wheezing and an increased risk for developing allergies and asthma (Sigurs et al., 2005). With both viruses, the disease severity is a combination of damage as a direct result of the virus as well as collateral damage caused from a hyper-active or inappropriate immune response. Severe RSV infection is linked to a shift in the immune response from the desired Th1 response to a viral infection mediated by cytotoxic CD8+ T cells with an additional humoral antibody response, to a Th2 response characterized by a predominantly humoral response and the presence of IgE antibodies, eosinophil infiltration and mast cell activation (Aoyagi et al., 2003; Pinto et al., 2006), (reviewed in Becker, 2006). Complications during influenza infection can occur when a so called cytokine storm arises as a result of an overactive immune response. The classic example of this was the 1918 influenza pandemic where young, healthy adults were at an increased risk of death

due to the hyper-immune response to the virus (Ge et al., 2006), (reviewed in Taubenberger and Morens, 2006). Death frequently resulted from virus induced collateral damage such as edema and hemorrhaging in the lungs from the immune response (Yueh et al., 2006).

For the most part, severe cases of influenza A and RSV are in the minority. Influenza A and RSV infections are generally self limiting and the immune system resolves them with no more intervention than bed rest and over-the-counter medications. Treatment and prevention option are available but vary drastically for influenza A and RSV. There are seasonal influenza vaccines available which provide adequate protection in most cases. There are also four FDA approved antivirals: amantadine, rimantadine, oseltamivir, and zanamivir. RSV, on the other hand, has no approved vaccine and the only available antiviral drug is ribavirin, which has yet to demonstrate any significant clinical usefulness for RSV (Boeckh et al., 2007; Chen and Lamb, 2008; Kathleen and Adrienne, 2007). In the case of high risk patents (tissue transplant recipients, premature infants, and congenital heart defects, etc.), there is a prophylactic treatment consisting of passive immunization with anti-RSV fusion protein antibodies known as palivizumab (Synagis) (Cohen et al., 2008). For most, the only treatment option available in the event of severe RSV infection is supportive therapy consisting of administration of intravenous fluids and ventilation with oxygen (Cambonie et al., 2008; Martinon-Torres et al., 2008).

Despite treatment and prevention options, it is clear (especially for RSV) that additional resources are needed to combat these diseases. There are currently antiviral drugs and vaccines in clinical trials for both RSV (Chapman et al., 2007; DeVincenzo et al., 2008) and influenza A (reviewed inHayden, 2009). However, it is desirable to have a

continuous stream of products in the development pipeline as RNA viruses are notorious for their ability to mutate and develop resistance to antivirals and, in the case of influenza A, to evade seasonal vaccination by point mutations and reasortment. Influenza A vaccines did not match circulating strains three times in the last decade, which resulted in poor protection of vaccinated individuals (CDC, 2008; Herrera et al., 2007; Yang et al., 2001). During the last few years there has been an increase in the circulation of influenza A viruses which are resistant to the adamantine drugs amantadine and rimantadine, and these are no longer recommended for use in the United States (CDC, 2009b; Saito et al., 2007). There was also a jump in the level of resistance of H1N1 influenza A viruses to the neuraminidase inhibitor oseltamivir (CDC, 2009a; Dharan et al., 2009) despite some research indicating the development of resistance to this drug would result in decreased virulence (Simmons et al., 2007). In short, there appears to be only one antiviral which remains effective against all currently circulating influenza viruses (zanamivir), and the vaccine is only effective if predictions on circulating strains prove correct. Clearly there is a need for new treatment and prevention options for influenza and RSV.

Targeted development of antivirals. The development of antivirals, and drugs in general, is usually performed by screening hundreds of thousands of compounds from libraries for potential activity against the desired protein or pathogen. Lead compounds are then taken and modified by the addition or substitution of functional groups to the parent and rescreened for increased activity. From this point the most active compounds are tested both in cell culture and animal models for toxicity and activity. For those compounds with promises, three phases of clinical trials are followed to demonstrate safety and efficacy in humans. If all barriers can be overcome the drug is then submitted

for FDA approval and subsequently marketed. Less than 1% of all drug candidates ever make it through this process, taking 12 years on average, with 3.5-9 years invested in discovery and preclinical development (California Biomedical Research Association, 2009).

An alternative approach is the target development of drugs. Rather than screen thousands of compounds, targeted development uses sequences of nucleic acids and proteins as well as structural information on active site and binding site conformations to design drugs that may be effective. Oseltamivir and zanamivir were specifically designed to fit into the sialic acid binding site of the influenza virus neuraminidase based on crystallographic and sequence data (Bossart-Whitaker et al., 1993). Another area where targeted drug development is useful is the emerging field of siRNA based treatments. In the case of RSV, there is currently an siRNA based drug undergoing clinical trials which targets the nucleoprotein gene (DeVincenzo et al., 2008). Development of siRNA drugs requires only the nucleotide sequence of the corresponding protein which is to be down regulated. A handful of potential sequences can then be tested for potency and safety (Gottwein et al., 2006). Regardless of the method for drug discovery, the testing of drugs for efficacy and toxicity in culture and in animal models, followed by clinical trials, will always be required. The use of targeted development for drug discovery may, however, reduce the time and resources needed for the initial step of lead compound discovery.

An additional tactic for drug development is the targeting of cellular factors involved in infectious disease rather than, or in addition to, standard practices of targeting the pathogen. The development of resistance to an antiviral that was years in the making is of great concern. The benefit of targeting cellular factors is the avoidance of pathogens

becoming resistant to a treatment. Again, oseltamivir, despite being developed through a targeted approach, has begun to lose its utility as a result of H1N1 influenza A viruses evolving resistance (CDC, 2009a; Dharan et al., 2009). It has therefore been proposed that drugs designed to target the interactions of a viral protein and a cellular protein required for its function would inhibit virus replication without the evolution of resistance (discussed in Müller and Kräusslich, 2009). This strategy also allows for the targeting of the immune response to a virus infection. As stated above, the immune response to influenza A and RSV are often involved in the pathology of the disease. It may therefore be possible to inhibit virus replication, in tandem with alteration of the immune response, to create a more favorable outcome to a viral infection. One such example is with hepatitis C virus (HCV) infection. Combination treatment with pegylated-interferon alpha and ribavirin is one of the most effective treatments to date (Manns et al., 2001). Ribavirin helps inhibit the virus replication directly by blocking genome replication as a nucleoside analog (Hui et al., 2006b). On the other hand, interferon alpha helps to stimulate the innate immune response which leads to the production of host proteins that inhibit virus replication or shut off host processes that are necessary for virus replication (Matsumoto et al., 2009). In this way, HCV is inhibited for long periods of time, or completely cleared from the body, with no apparent signs of resistance (Fried et al., 2002).

Targeted drug development and the targeting of host factors involved in virus replication have shown themselves to be effective ways of addressing the ever evolving RNA viruses. Based on the direct and logical nature for this strategy of therapeutic drug development, I made the following hypothesis: The targeted development of antivirals

against virus and host factors is the most rapid and effective means of drug development for influenza A and RSV.

Antisense PPMO. Peptide conjugated Phosphordiamidate Morpholine Oligomers are antisense compounds with a DNA-like structure which can base pair with target mRNA (Figure 1.1). PPMO do not act through the miRNA pathway to cleave target RNAs but instead sterically block sites of virus replication, translation initiation, or RNA splicing sites (Abes et al., 2006; Nelson et al., 2005). PPMO are resistant to nuclease cleavage due to the phospho-amine bond in place of the normal 5'-3' phosphate linkage.

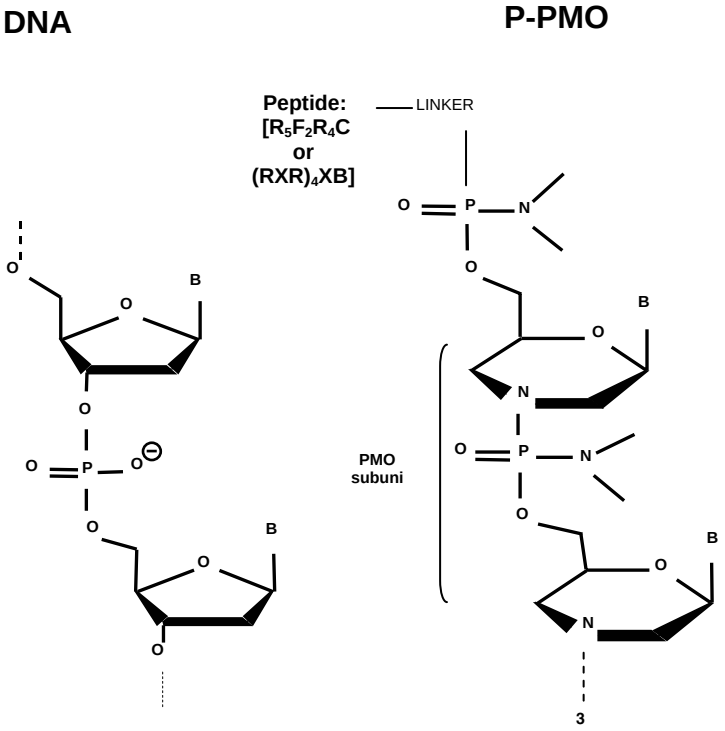

Figure 1.1 Structural comparisons of DNA and PPMO.
PPMO possess several differences in structure compared to DNA which makes them superior for antisense therapy. They are nuclease resistant, uncharged, and yet water soluble. They are also autonomously cell permeable due to the arginie rich peptide at the 5'end making transfection reagents unnecessary.

Furthermore, PPMO have a morpholine ring in place of the standard ribose ring of nucleic acids. The addition of an arginine rich peptide to the 5' end of the antisense sequence mediates with the entry of the PPMO into cells. The mechanism by which the peptide mediates cell entry is still under debate but possibilities include endocytosis or direct passage through the cell membrane (Abes et al., 2008).

To test my hypothesis that targeted development of antivirals is the most rapid and effective means of drug development for influenza A, I examined the ability of two PPMO compounds to inhibit influenza A virus replication. The PPMO utilized in my research were designed using a bioinformatics approach to target conserved regions of the translation initiation start site of influenza A PB1 polymerase or the 3' untranslated region of the NP vRNA. Initial testing of these compounds was a collaborative effort between Dr. Ge and Chen from MIT, AVI BioPharma, Corvallis, OR, Dr. Pastey and myself at OSU, Dr. Kobasa at the Public Health Agency of Canada, and Dr. Puthavathana at Mahidol University, Thailand. Cell culture screening revealed that a combination of these two antisense drugs was effective at inhibiting influenza A from various sources (human, equine and avian strains), at sub-toxic doses, and in a prophylactic or early therapeutic manner (Gottwein et al., 2006). My research continued with the in vivo development of these two antisense compounds in a Balb/C mouse model and tested the efficacy and toxicity against two equine influenza A viruses H3N8 and H7N7. Furthermore, I sequenced resistant strains for the presence of base misparings which conferred resistance to one but not both antisense compounds. My results demonstrate that targeted development of antisense compounds produces drugs that are capable of reducing influenza A virus replication in vivo at sub-toxic doses. However, treatment

must be initiated at an early stage during infection to observe any significant effect and some viruses naturally contain mutations that confer resistance to these antisense compounds.

Proteasome inhibitors. The matrix protein of RSV is an internal structural protein which is required for virus assembly and budding from the cell surface (Henderson et al., 2002; Li et al., 2008; Marty et al., 2004). In addition, evidence suggests the M protein inhibits host cell transcription and terminates virus genome replication in preparation of assembly (Li et al., 2008; Rodriguez et al., 2004; Shields et al., 2003). The cellular partners required for these actions are not well understood and Brendan Jeffrey (Dr. Rockey's lab, OSU) while rotating in Dr. Pastey's lab performed yeast-two-hybrid screens against a Hela cell cDNA library with the RSV matrix protein as the bait. His results indicated, among others, an interaction between the RSV matrix protein and the cellular protein ubiquitin (Table 1.3).

Table 1.3: Yeast-two-hybrid results for the RSV matrix protein.
AH109 yeast were transformed with the RSV matrix protein in the pGBKT7 vector and subsequently transformed with a HeLa cDNA library in the pGADT7 vector. Yeast were plated on TRP dropout medium for selection of cotransformed cells with a positive interaction and further screened by β-Gal filter lift assay. Plasmid was isolated from positive colonies and sequenced to determine interacting partner.

euk. translation elongation factor 1 alpha 1 (EEF1a1)
ubiquitin-conjugating enzyme E2I (UBE2I)
ubiquitin C (UBC)
phosphoglucomutase 1 (PGM1)
adaptor-related protein complex 1, µ1 subunit (AP1M1)
adaptor-related protein complex 3, µ1 subunit (AP3M1)

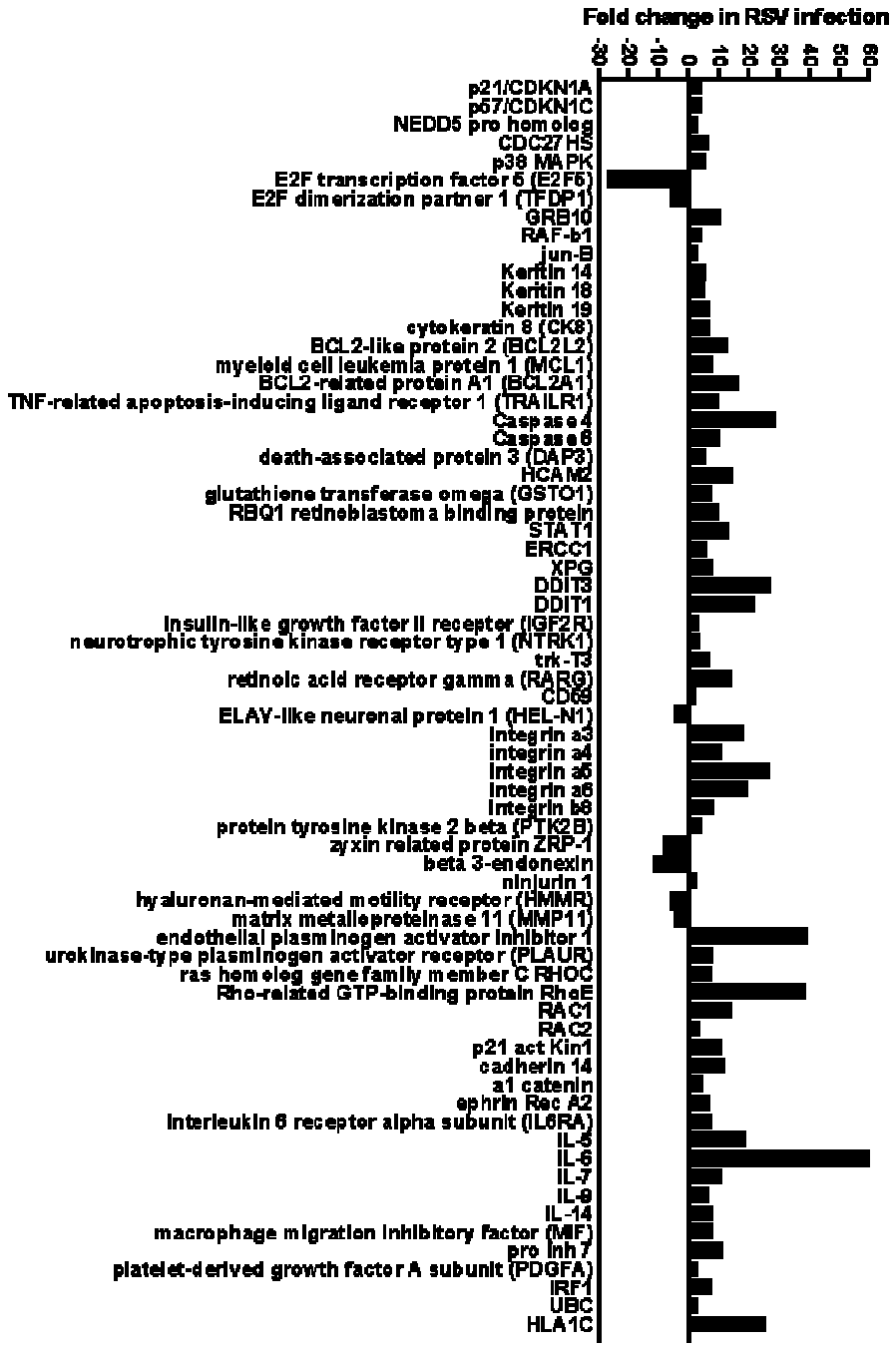

Figure 1.2: Effects of RSV infection on mRNA expression in HEp-2 cells.
HEp-2 cells were infected with RSV or left uninfected as a control and total RNA was isolated at 24h post infection and labeled with P^{32}. A membrane based Atlas Human Cancer cDNA Expression Array (Clontech) was used to analyze specific signaling pathways such as inflammation, cell cycle, and apoptosis. Results are depicted as fold change in mRNA of RSV infected cells compared to uninfected control cells.

Additional unpublished research by Dr. Pastey, while at the National Institute of Allergy and Infectious Disease, showed that specific ubiquitin mRNA levels (UbC) were upregulated during RSV infection in HEp-2 cells in culture (Figure 1.2) and were later confirmed by qRT-PCR (Figure 1.3) in our lab.

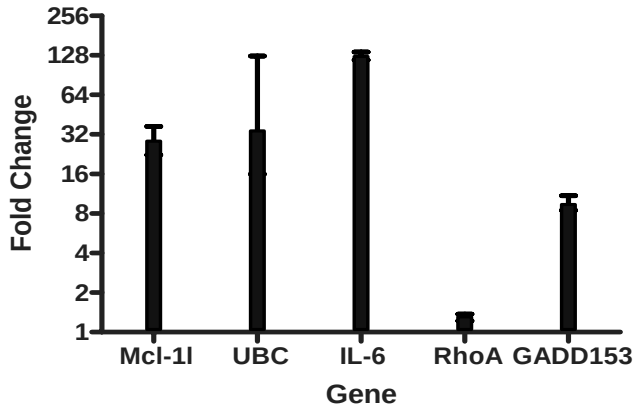

Figure 1.3: Verification of mRNA levels during RSV infection in HEp-2 cells. HEp-2 cells were infected with RSV or left uninfected as a control and total RNA was isolated at 24h post infection. Gene specific primers were designed for analysis of mRNA levels using Invitrogen's SuperScript III Platinum SYBR Green One-step qRT-PCR kit. Data is represented as GAPDH normalized fold change of RSV infected cells compared to uninfected controls (n=3 per group)

With this corroborating evidence, I tested my hypothesis: RSV replication can be targeted rapidly and effectively by inhibiting the cellular interacting partner ubiquitin. Inhibition of the ubiquitin/proteasome pathway in cell culture with proteasome inhibitors is the most commonly utilized method for interfering with the ubiquitination of proteins (Ott et al., 2000; Patnaik et al., 2002; Schmitt et al., 2005); aside from site directed mutagenesis of lysine residues which are the site of ubiquitination (Gottwein et al., 2006; Jager et al., 2007; Martin-Serrano et al., 2004; Ott et al., 2000). For my in vitro experiments I chose the often used proteasome inhibitor MG-132, which has been used

extensively in testing viruses for their requirement of ubiquitin. MG-132 is a proteasome substrate analog which works by competitive inhibition. For my in vivo experiments, I chose the FDA approved small molecule boronic acid proteasome inhibitor bortezomib due to reports of greater target specificity and increased potency compared to MG-132 (Neznanov et al., 2008). Its status as an FDA approved drug would greatly accelerate its development into a potential RSV therapy. Treatment of Vero cells in vitro with both drugs resulted in significant reductions in RSV replication and a potential defect in virus escape from infected cells. However, treatment of RSV infected Balb/C mice exacerbated the inflammatory response in their lungs and increased mortality rather than improving it.

Summary of results. The results of my research described in this compilation demonstrate the feasibility of both targeted drug design and/or the targeting of cellular factors necessary for virus replication. Both antisense compounds and the inhibition of ubiquitin were capable of inhibiting virus replication. However, a narrow time window in which to initiate PPMO treatment or the exacerbation of inflammation by inhibiting the ubiquitin/proteasome pathway resulted in little or no clinical usefulness for these drugs. These results demonstrate the need to better understand the workings of viruses, cells, and their interplay with each other before the targeted development of drugs or targeting of host proteins in infection can become a viable development strategy.

Chapter 2:

Inhibition of Influenza A H3N8 Virus Infections in Mice by Morpholino Oligomers

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Abstract

New methods to combat Influenza A Virus (FLUAV) in humans and animals are needed. The H3N8 subtype virus was the cause of the pandemic of 1890 and has recently undergone cross-species transmission from horses to dogs in the USA. In 2007 H3N8 spread to Australia, a continent previously devoid of equine influenza. Here we show that antisense peptide conjugated phosphorodiamidate morpholino oligomers (PPMO), delivered by intranasal administration, are able to inhibit the replication of FLUAV A/Eq/Miami/1/63 (H3N8) in mice by over 95% compared to controls. Monitoring of body weight and immune cell infiltrates in the lungs of noninfected mice indicated that PPMO treatment was not toxic at a concentration shown to be effectively antiviral in vivo. In addition, we detected a naturally occurring mutation within the PPMO target site of a viral gene that may be the cause of resistance to one of the two antisense PPMO sequences tested. These data indicate that PPMO targeting highly conserved regions of FLUAV are promising novel therapeutic candidates.

Introduction

Vaccine development has a critical role in inhibiting the spread of Influenza A Virus (FLUAV) between animals, humans, and from animals to humans. However, FLUAV vaccines are typically effective only on a seasonal basis and against anticipated strains (Brett and Johansson, 2005; Johansson and Brett, 2007). The circulation of unanticipated and highly pathogenic FLUAV strains has increased in recent years (Chutinimitkul et al., 2007). Furthermore, there has been a recent increase in the incidence of zoonotic transmission of FLUAV, notably highly pathogenic Avian influenza (HPAI), from birds to humans and other mammals (Adams et al., 1999; Kuiken et al., 2004). The discovery in 2005 of a cross-species transmission of the FLUAV H3N8 subtype from horses to dogs in the United States (Crawford et al., 2005; Yoon et al., 2005), in addition to the August 2007 spread of H3N8 equine influenza to the previously noninfected continent of Australia (Animal Health Australia, 2007), has increased concerns over this subtype as well.

FLUAV with the H3 hemagglutanin are also of significance to humans. Currently, the prominent subtype for seasonal influenza is H3N2. In addition, the influenza pandemic of 1890 is presumed to have been caused by an H3N8 subtype virus (Dowdle, 1999). Although it is unlikely that an H3N8 influenza virus will soon be the cause of a human epidemic or pandemic, this HA subtype has previously and will continue to be a cause of seasonal morbidity and mortality and is also useful as a model in the testing of potential drugs against influenza infections.

Small molecule inhibitors of influenza have been available for several years and have proven effective at reducing the severity and duration of illness (Hayden and Pavia,

2006). However, some of these drugs, notably the adamantanes, are now considered ineffective against many human FLUAV strains (Crawford et al., 2005). Recently, a small percentage of FLUAV strains have been shown to be resistant to neuraminidase inhibitors as well (Baz et al., 2006; Bermingham and Collins, 1999; de Jong et al., 2005b; Schmitt et al., 2005). The inadequacy of the current armament of FLUAV therapeutics is evident, and new therapeutics effective against a multiplicity of FLUAV subtypes are clearly desirable.

Phosphorodiamidate morpholino oligomers (PMO) (Summerton et al., 1997) are oligonucleotide analogs that can act as antisense agents by base pairing with complementary RNA target sequence and forming a steric block (Stein et al., 1997). PMO are water soluble and stable in cells (Hudziak et al., 1996; Nelson et al., 2005). Cellular uptake of PMO can be greatly enhanced through conjugation of a cell-penetrating peptide (CPP) (Deas et al., 2005; Moulton et al., 2004). CPP-PMO (PPMO) were recently shown to successfully inhibit several subtypes of FLUAV in cell cultures (Gottwein et al., 2006). Here we show that intranasal delivery of PPMO, at non-toxic doses, was able to reduce the replication of Influenza A/Eq/Miami/1/63 (H3N8) in the lungs of mice by over 95% compared to controls. The effective PPMO were designed against sequences in the polymerase basic 1 (PB1) and nucleoprotein (NP) genes that are highly conserved between FLUAV subtypes. These results indicate that PPMO may have utility as a novel therapeutic against a multiplicity of FLUAV subtypes.

Materials and Methods

Virus and cells. Influenza A/Eq/Miami/1/63 (H3N8) and A/Eq/Prague/56 (H7N7) were obtained from Rocky Baker (Veterinary Diagnostic Laboratory, Oregon State University) and grown in the allantoic cavity of ten day old embryonated chicken eggs for three days. Virus was harvested and stored at -80ºC for future use. Virus was titered by standard plaque assay on Madin Darby Canine Kidney (MDCK) cells (see below). MDCK cells were grown in minimum essential media (MEM) (Hyclone) supplemented with 100U/ml penicillin, 100µg/ml streptomycin (Gibco), and 10% fetal bovine serum (FBS) (Hyclone).

PMO and PPMO. PMO were synthesized at AVI BioPharma, Corvallis, OR, by previously described methods (Stein et al., 1997). PPMO were produced with the CPP $(RXR)_4XB$, as previously described (Abes et al., 2006). Two oligomer sequences specific to multiple strains of FLUAV RNA, along with a 'nonsense' sequence control having no significant homology to FLUAV or any human mRNAs, were prepared as both PMO and PPMO. The first of the two antisense sequences, PB1-AUG (5'-GACATCCATTCAAATGGTTTG-3'), is a 21mer complementary to the AUG translation start site region of the polymerase subunit PB1 mRNA. The second sequence, NP-v3' (5'-AGCAAAAGCAGGGTAGATAATC-3'), is complementary to the 22 terminal nucleotides at the 3' end of the NP virion RNA (vRNA). The nonsense sequence utilized as a negative control for both PMO and PPMO is a 20mer of 50% G/C content, named DScr (5'-AGTCTCGACTTGCTACCTCA-3'). In addition, the DScr sequence was prepared as a PPMO with fluorescein conjugated to its 3' end (DScr PPMO-Fl).

Mice. All animal experiments were performed with 6-8 week old female Balb/C mice (Simonsen Laboratories), which were allowed food and water ad libitum throughout the

studies. Infections and treatments of mice were performed under anesthesia with 67mg/Kg Ketamine and 4.5mg/Kg Xylazine and mice were euthanized by CO_2 asphyxiation for necropsy. All animal experiments were carried out under BSL-2 conditions with protocols approved by the Institutional Animal Care and Use Committee of Oregon State University.

Evaluation of intranasal administration as a method of PPMO delivery into lung tissue. To determine if intranasal instillation (i.n.) would effectively deliver PMO compounds into the lungs, 40µl of 0.9% saline or 40µl of 0.9% saline containing 3.75mg/Kg of DScr PPMO-Fl was administered i.n. to noninfected mice. At 16 or 24 h post treatment, PPMO-Fl and saline-treated animals were sacrificed and the lungs removed for imaging. One lung was imaged immediately by fluorescent whole mount microscopy with a Zeiss Stereo Discovery V8 microscope. The other lung was flash frozen in liquid nitrogen and then sectioned into upper, middle and lower lung samples for examination using a Leica DMLB fluorescent microscope and SPOT CCD camera (Diagnostic Instruments, Inc.).

Detection of pulmonary infiltrates. Noninfected mice were used to examine the effect that a prospective antiviral PPMO dosing regimen could have on inflammation of pulmonary tissue. Mice were administered two i.n. doses, 24 h apart, of a 50/50 mixture of PB1-AUG and NP-v3' PPMO at concentrations from 0-7.5mg/Kg. Mice were weighed at 24, 48 and 72 h after the initial dose, and sacrificed at 72 h for removal of the lungs and spleen. One lung was formalin fixed, sectioned, and hematoxylin and eosin (H&E) stained for histological examination. All histological examination was performed by pathologists at the Veterinary Diagnostic Lab, College of Veterinary Medicine, Oregon

State University. Leukocyte cellularity of the other lung, and of spleen tissue, was measured using flow cytometry with the following methodology. Fresh tissue samples were placed in PBS containing 1% FBS. Single cell suspensions were made by passing tissues through a 70 µm nylon mesh screen cell strainer (BD Falcon). The strainers were rinsed into 50 ml tubes and the suspension washed twice by room temperature centrifugation (1500 x g) and resuspension of the pellet in 10 ml of DMEM + 1% FBS. Red blood cells were lysed by suspending the pellet in 1 ml of cold 1 x RBC lysis buffer (eBioscience) followed by two washes in 10 ml DMEM + 1% FBS. Cell viability and enumeration was determined by trypan blue exclusion using a Vi-cell TM XP cell viability analyzer (Beckman Coulter). A final centrifugation and suspension of the cells (5×10^6/ml) in FACS Buffer (1X PBS+1% FBS+ 0.02 % NaN_3) was performed prior to subjecting the cells to antibody staining. Individual samples were stained using antibodies: CD4-PE (clone L3T4), CD8-PE (clone Ly-2), CD11b-PE (MAC-1), CD11c-PE (N418) (eBioscience), CD45R-FL (B220) (BD Pharmingen), GR-1 TriColor (Caltag) or manufacturer's corresponding isotype controls (Fl= fluorescein, PE= phycoerythrin). The stained samples were examined (minimum of 50,000 events) on a Cytomic FC 500 (Beckman Coulter) and data analyzed using FlowJo Software (TreeStar).

Viral inhibition experiments in mice. To compare the relative efficacy of non-conjugated PMO to that of PPMO, mice were treated i.n. with 100ul of saline, or 100ul of saline containing a 50/50 mixture of PB1-AUG/ NP-v3' PMO totaling 3.75mg/Kg or 3.75mg/Kg of DScr PMO. Additional groups were treated with saline containing either 3.75mg/Kg of 50/50 PB1-AUG/NP-v3' or 3.75mg/Kg of DScr PPMO. Treatments were performed at 4 h before and 20 h after infection with 2.5×10^5 PFU of Influenza

A/Eq/Miami/1/63 (H3N8). After infection, the mice were weighed every 24 h, and at 72 h euthanized and lungs harvested for determination of virus titer by plaque assay.

In a subsequent experiment, designed to determine which of the two antisense PPMO was more effective against H3N8 in vivo, mice were treated i.n. with 100µl saline, or 100µl saline containing 3.75mg/Kg of either PB1-AUG, NP-v3'or DScr PPMO. Treatment was again administered at 4 h before infection and 20 h post infection. Mice were weighed every 24 h, euthanized at 72 h pi, and lung tissue harvested for plaque assays.

To determine the effect of PPMO treatment timing in relation to the time of infection on antiviral efficacy, mice were treated with two doses of 3.75mg/Kg of NP-v3' PPMO under 5 different regimens: at either 4 h pre infection and 20 h post infection, 1, 2, or 4 h post infection and 20 h post infection, or 20 h post infection and 36 h post infection. Control mice were treated with DScr PPMO or saline on similar schedules. Body weights were recorded as above. All animals were euthanized at 72 h pi. and lungs then collected for plaque assays.

Plaque assays. Plaque assays were performed by grinding lungs in 1.5ml MEM supplemented with 100U/ml penicillin and 100µg/ml streptomycin and subjecting them to two freeze thaw cycles followed by centrifugation at 2300 x g for two minutes. Supernatant was serially diluted and standard plaque assays were performed on >90% confluent MDCK cells in 12-well plates. Briefly, MDCK cells in duplicate wells were infected with 100µl of serially diluted virus for one hour by incubation at 37ºC with periodic shaking. Virus-containing supernatant was removed by aspiration, and MDCK cell monolayer was overlaid with MEM containing 100U/ml penicillin, 100µg/ml

streptomycin, 3% BSA (Sigma), and 1µg/ml TPCK Trypsin (Sigma) with 1% Sea Plaque Agarose (Cambrex). Two days later agarose was removed and plaques were visualized by staining with 0.75% Crystal Violet (Alfa Aesar) diluted in 100% methanol, and quantified by determining the highest dilution in which duplicate wells contained at least 10 plaques.

Sequencing of PB1 gene. MDCK cells were infected with 0.01 MOI A/Eq/Miami/63 (H3N8) or A/Eq/Prague/56 (H7N7), and viral RNA isolated from cell culture supernatants at 48 h pi by centrifugation at 2300 x g for five minutes (to remove cellular debris), and Ambion's MagMAX™ -96 Viral RNA Isolation Kit, according to the manufacturer's protocol. Using primers PB1-forward (5'-GAATTCAGCTTAGCGAAAGCAG-3'), and PB1-reverse 248 (5'-TCCATCGATTGGATTAAGTTGTG-3'), or PB1-reverse 850 (5'-GGCAATCCTGATTGTTCAAG-3'), the gene of interest was converted to cDNA and amplified via one step RT-PCR (Superscript III One Step RT-PCR kit, Invitrogen). PCR products were then cloned into pCR8/GW/TOPO (Invitrogen) vector according to the manufacturer's protocol. Plasmid from five bacterial colonies containing each PCR product were isolated and purified using the PureLink Quick Plasmid Miniprep Kit (Invitrogen). Plasmids were sequenced at Oregon State University's Center for Genome Research and Biocomputing (CGRB) core facility. Sequence data is available in GenBank as accession numbers: EU236678 Influenza A/Eq/Miami/1/63 (H3N8) PB1 gene and EU236679 Influenza A/Eq/Prague/56 (H7N7) PB1 gene.

Statistics. All statistical analyses were performed using Prism 4 software (GraphPad Inc.) using One-Way ANOVA with Bonferroni or Dunnett's multiple comparison test.

Results

Toxicity of PPMO in mice. Our previous study showed that NP-v3' and/ or PB1-AUG PPMO were effective at inhibiting the replication of several strains of FLUAV replication in various cell lines, in a dose-dependant and sequence-specific manner, with minimal attendant cytotoxicity (Gottwein et al., 2006). Encouraged by these results we sought to evaluate the efficacy of these PPMO against A/Eq/Miami/1/63 (H3N8) replication in a mouse model. We first evaluated the potential toxicity of these two PPMO in noninfected mice under conditions similar to those being considered for virus inhibition experiments. No such preliminary assessment was carried out for PMO, as it has been shown to be safe in vivo at high doses (Amantana et al., 2007; Deas et al., 2005). None of the dosing regimens employed resulted in any significant weight loss over the 72 h period of monitoring (Figure 2.1A). Histological examination revealed no significant differences in the lung tissue of PPMO-treated compared to saline-treated mice (data not shown). To investigate whether PPMO induce pulmonary inflammation, we used flow cytometry to detect leukocytic infiltrates in a lung and the cellularity of spleen tissue isolated from each group shown in figure 2.1A. Our results indicate that there were no significant changes in spleen leukocyte populations for any PPMO treatment group compared to the saline-treated group (Figure 2.1B). The lungs of mice that received the highest PPMO dose (7.5mg/Kg) showed a statistically significant increase of 2.21 fold in macrophages (95%CI= 2.78 to 1.64 fold) and 3.38 fold in granulocytes (95%CI= 4.98 to 1.78 fold) (Figure 2.1C). It is noteworthy that the pulmonary infiltrates evident by flow cytometry were not associated with statistically significant weight loss or observable illness in mice over the three day duration of this experiment.

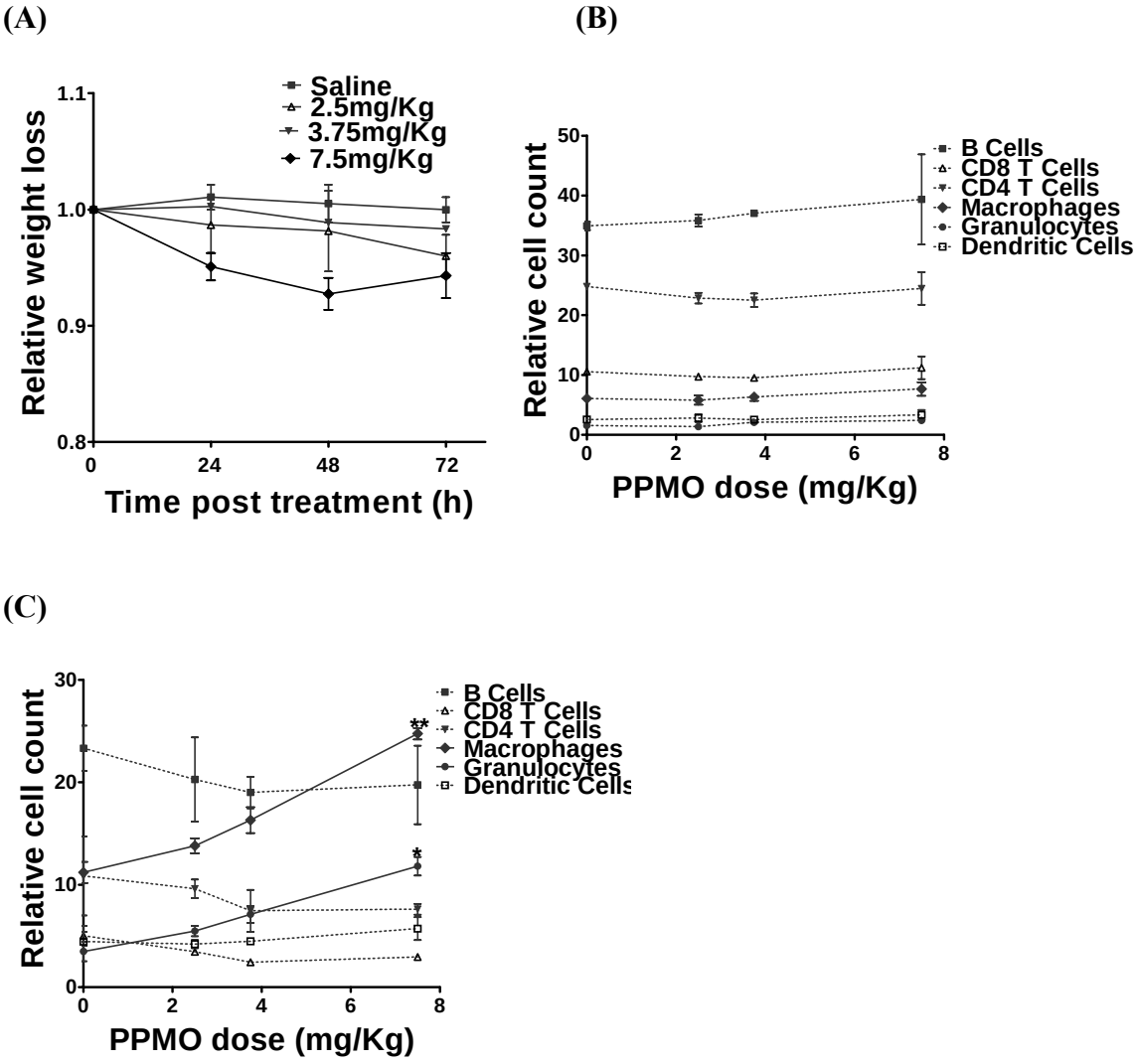

Figure 2.1: Evaluation of PPMO toxicity in mice. Duplicate mice were treated with various doses of 50/50 NP-v3'/PB1-AUG PPMO or 0.9% saline (control) through intranasal administration. (A) Mice were weighed every 24 h following treatment, for 72 h, to determine weight loss. At 72 h post treatment, all mice were sacrificed and spleen (B) and lung (C) were removed and flow cytometry was performed on single cell suspensions using antibodies targeting immune-cell specific markers. *P<0.5, **P<0.01 using One-Way ANOVA with Bonferroni's multiple comparison test.

Lung distribution of PPMO when delivered intranasally. FLUAV has been reported to replicate in both the upper and lower respiratory tract (Nicholls et al., 2007; Shinya et al., 2006; van Riel et al., 2006). Because the lungs are known to be the primary site of

viral replication during FLUAV infection, we explored i.n. administration as a way to maximize PPMO delivery to the lungs. We first investigated the distribution of PPMO in the lungs of noninfected mice after i.n. delivery to determine the feasibility of this route for treatment during an infection. Mice were given either saline or 3.75mg/Kg of PPMO-Fl, and necropsied at 16 or 24 h post administration for examination of lung tissue.

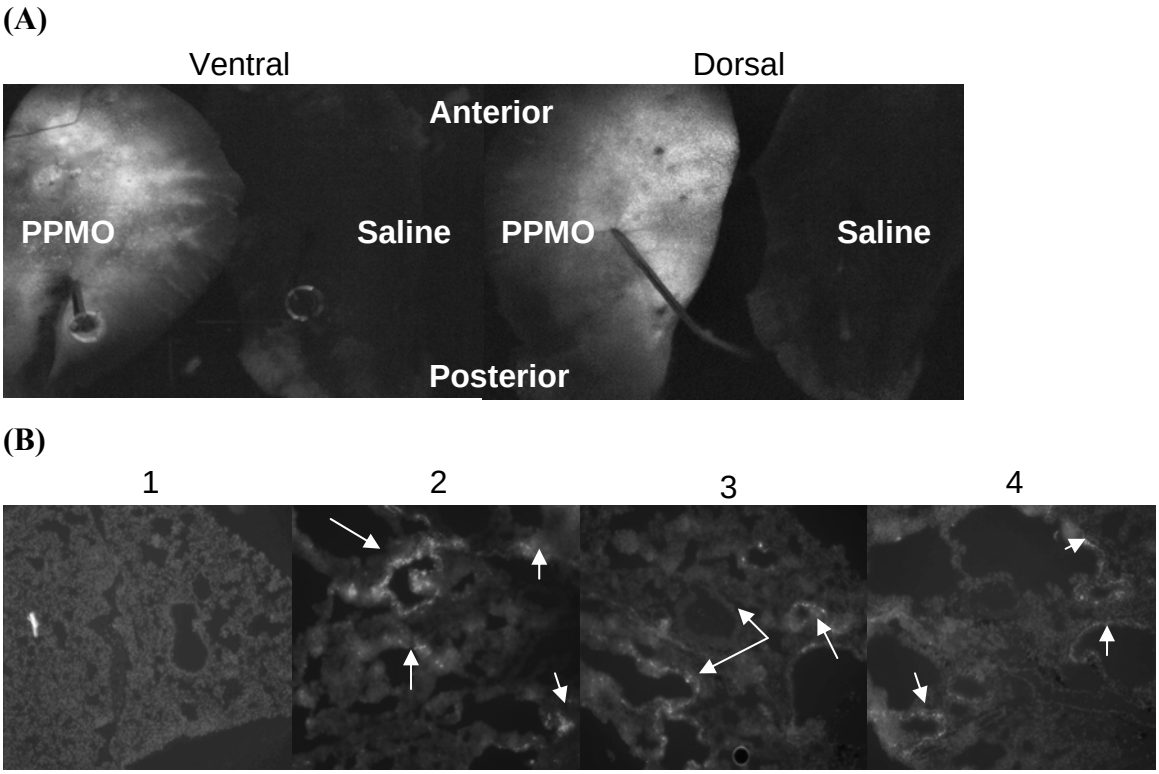

Figure 2.2: Visualization of PPMO-Fl in lungs after intranasal administration. Three mice were treated with either 3.75mg/Kg 3' fluorescein (Fl) conjugated PPMO, or 0.9% saline for determination of PPMO distribution in the lungs. (A) Fluorescent whole mount microscopy of left lung of PPMO-Fl and saline control treated mice necropsied at 16 h post treatment. (B) Fluorescent microscopy of cryo-sectioned right lung necropsied at 24 h post treatment. Section of saline control mouse lung (1), or PPMO-Fl treated mouse upper (2), middle (3), and lower (4) lung. Arrows indicate concentrations of fluorescent signal.

A view of the whole lung shows that PPMO-Fl is distributed in a gradated fashion from the upper to the lower lung. Signal from the PPMO-Fl is noticeably stronger in proximity to the major bronchioles (Figure 2.2A). Figure 2.2B includes higher magnification views of control lung tissue which received no PPMO-Fl (Figure 2.2B, panel 1) and tissue from the upper (Figure 2.2B, panel 2), middle (panel 3) and lower (panel 4) regions of the lung receiving PPMO-Fl. These images confirm that distribution of PPMO after i.n. administration is highest in the upper lung, but that a detectable amount of PPMO-Fl is present in the lower lung region (Figure 2.2B, panel 4). Together these data clearly show that PPMO compounds delivered by the i.n. route efficiently enter the lungs.

Determination of most active compound. Initially, we compared the efficacy of nonconjugated PMO to that of PPMO, to determine if conjugation to cell penetrating peptide had any effect on the ability of PMO to inhibit H3N8 replication in the lungs of mice. Mice were infected with the same strain of influenza, A/Eq/Miami/1/63 (H3N8), which had been used in previous cell culture experiments with PPMO (Gottwein et al., 2006). Based on the possible mild pulmonary inflammation evident from the 7.5mg/Kg PPMO dose (Figure 2.1C), we employed 3.75mg/Kg of PPMO per dose in this antiviral trial. Mice were dosed with a 50/50 mixture of PB1-AUG and NP-v3' in the PMO or PPMO form, or administered equivalent DScr or saline controls. Dosing was performed at 4 hours before and 20 hours after infection. There was no correlation between weight loss and treatment or weight loss and virus load during the 3 day duration of this experiment (data not shown). Plaque assays performed on lung tissue taken at 72 hours post infection showed a 1.5 $\log_{10}$ (95%CI=2.17 to 0.72) reduction in virus growth in mice treated with the PB1-AUG/NP-v3' PPMO combination in comparison to the DScr PPMO

treated group (Figure 2.3A). The PB1-AUG/NP-v3' PMO combination treatment appeared to produce a moderate decrease in titer compared to the DScr PMO treated group, however, the difference did not achieve statistical significance. These results indicate that there was a reduction in virus growth in vivo produced by the antisense PPMO combination treatment and that the conjugation of the $(RXR)_4XB$ peptide to PMO enhances the ability of PMO to inhibit H3N8 replication in vivo.

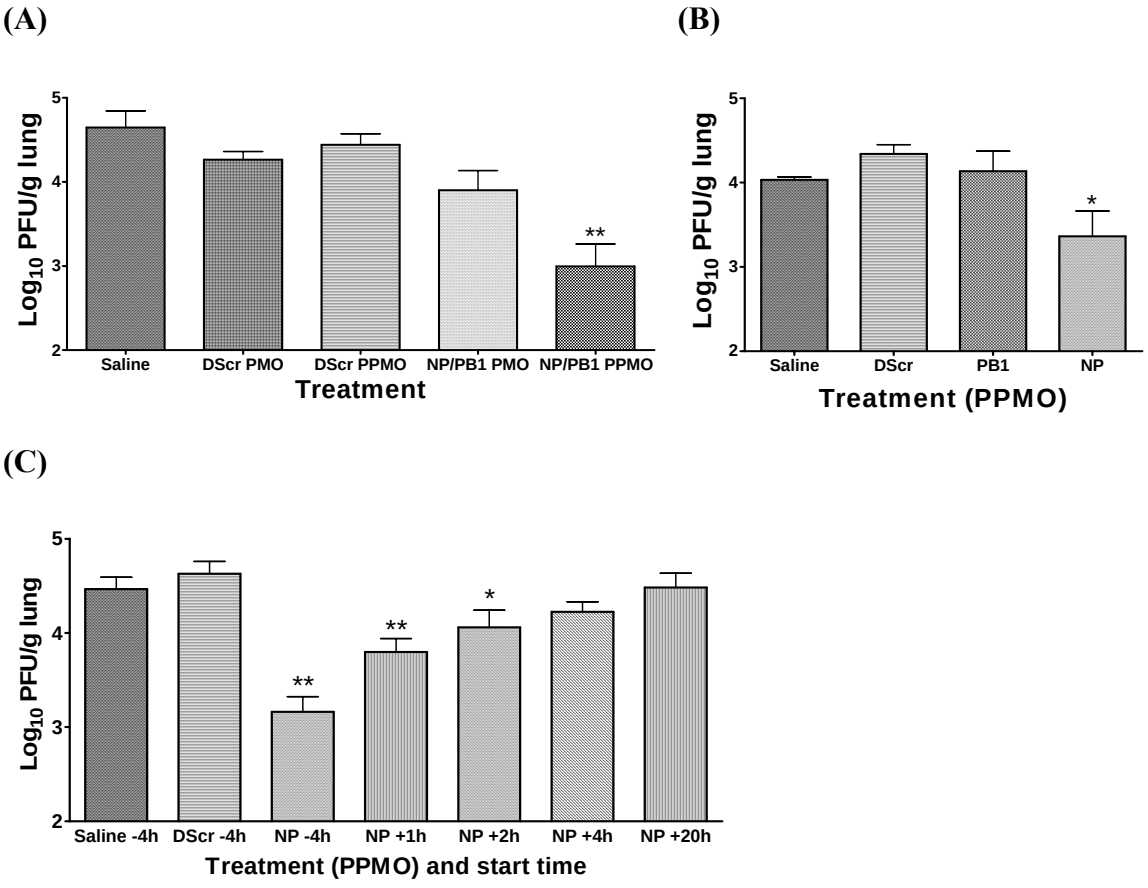

Figure 2.3: Reduction of influenza A H3N8 lung titer in mice treated with PPMO. Lung virus titers at 72h post infection were determined by plaque assays for all experiments. (A) Mice were treated with two doses of PMO or PPMO at 3.75mg/Kg as indicated, or with same volume of 0.9% saline, 24hrs apart, starting at 4 h pre infection. (B) Mice were treated with 3.75mg/Kg of indicated PPMO at 4 h before and 20 h after infection. (C) Mice were treated with two doses of PPMO NP-v3' 3.75mg/Kg at indicated start times. *P<0.5, **P<0.01 using One-Way ANOVA with Dunnett's multiple comparison test, n=4-5 mice per group.

Next we sought to determine which PPMO would produce the highest efficacy: PB1-AUG alone or NP-v3' alone. Previous data indicated that certain FLUAV strains (A/PR/8/34 (H1N1), A/Thailand/1(KAN-1)/04 (H5N1), and A/WSN/33 (H1N1)) were inhibited similarly by either PB1-AUG or NP-v3' PPMO, but that other strains (A/Eq/Miami/1/63 (H3N8), A/Eq/Prague/56 (H7N7), and A/Memphis/8/88 (H3N2)) were not (Gottwein et al., 2006). In addition, we sought to determine if our previous results with the PB1-AUG and NP-v3' PPMO against A/Eq/Miami/1/63 (H3N8) in cell culture would extend to in vivo conditions. Mice were treated with PB1-AUG, or NP-v3', as well as DScr or saline controls. Treatments were all performed at 4 hours before and 20 hours after infection with A/Eq/Miami/1/63 (H3N8). There was a statistically significant decrease of 1.0 $\log_{10}$ (95%CI= 1.886 to 0.06974) in virus titer produced by treatment with NP-v3' PPMO alone compared to DScr PPMO. In contrast, the PB1-AUG PPMO alone produced no significant reduction in virus titer compared to the DScr PPMO (Figure 1.3B). These data are consistent with those previously obtained with A/Eq/Miami/1/63 (H3N8) in cell cultures (Gottwein et al., 2006).

PB1-AUG target site sequence. Various experiments indicate that antiviral PPMO act through steric blockage of particular viral RNA regions, such as the AUG start site or critical regulatory motifs in noncoding regions (Deas et al., 2005; Holden et al., 2006; Neumann et al., 2002; Vagnozzi et al., 2007; van den Born et al., 2005; Yuan et al., 2006). Sequence analysis has indicated that FLUAV resistant to PPMO treatment can contain sequence mismatches between the PPMO and the RNA target. For example, the

NP-v3' PPMO-targeted region of A/Memphis/8/88 (H3N2) has two known mismatches and was resistant to this PPMO in cell culture (Gottwein et al., 2006) (see Table 1.1).

As shown here in vivo, and in previous work in cell-cultures, PB1-AUG PPMO is not effective against the A/Eq/Miami/1/63 (H3N8) strain. In an effort to gain insight into a possible explanation for the inactivity of PB1-AUG PPMO against this strain, we

Table 2.1: Comparison of PPMO target sequences in various strains of FLUAV. The nucleotide sequence of the PB1 gene of A/Eq/Miami/1/63 (H3N8) and A/Eq/Prague/56 (H7N7) targeted by the PB1-AUG PPMO was determined. The target sequence is shown to have a single base mutation (highlighted in bold italicized letter), compared to many other FLUAV strains. The NP-v3' PPMO target region of A/Eq/Miami/1/63 (H3N8) and A/Eq/Prague/56 (H7N7) viruses shows no mutations that could interfere with PPMO hybridization. All viruses shown have undergone cell culture testing with PPMO [17].

FLUAV strain	**PB1-AUG PPMO target (5' to 3')**
A/WSN/1933_(H1N1)	CAAACCAUUUGAAUGGAUGUC
A/Puerto Rico/8/34_(H1N1)	CAAACCAUUUGAAUGGAUGUC
A/Memphis/8/88_(H3N2)	CAAACCAUUUGAAUGGAUGUC
A/Thailand/1(KAN-1)/2004_(H5N1)	CAAACCAUUUGAAUGGAUGUC
A/Eq/Prague/56_(H7N7)	CAAAC***U***AUUUGAAUGGAUGUC
A/Eq/Miami/63_(H3N8)	CAAAC***U***AUUUGAAUGGAUGUC
	NP-v3' PPMO target (5' to 3')
A/WSN/1933_(H1N1)	*NNNNNNNNNNNNTAGATAATC
A/Puerto Rico/8/34_(H1N1)	AGCAAAAGCAGGGTAGATAATC
A/Memphis/8/88_(H3N2)	AGCAAAAGCAGGGT***TA***ATAATC
A/Thailand/1(KAN-1)/2004_(H5N1)	AGCAAAAGCAGGGTAGATAATC
A/Eq/Prague/56_(H7N7)	AGCAAAAGCAGGGTAGATAATC
A/Eq/Miami/63_(H3N8)	AGCAAAAGCAGGGTAGATAATC

* N denotes nucleotide of unknown sequence

sequenced the first 850nt of the PB1 gene, which was not previously available in the Influenza Sequence Database (NCBI, 2004). We compared the PB1-AUG PPMO target sequence of the A/Eq/Miami/1/63 (H3N8) strain used in this experiment to several other FLUAV strains used in previous experiments (Gottwein et al., 2006) (Table 1.1). The 6th

nucleotide in the PB1-AUG PPMO target region of A/Eq/Miami/1/63 (H3N8) has a C to U mutation compared to other strains of FLUAV. The identical mutation was also found to be present in A/Eq/Prague/56 (H7N7), a strain which PB1-AUG PPMO was also found to be ineffective against in cell culture testing (Gottwein et al., 2006). In contrast, no mutations in the PPMO target sequence were found in any of the viral strains for which PB1-AUG or NP-v3' PPMO have previously been shown to be effective (Table 2.1).

Time of treatment initiation on PPMO efficacy. Finally, we tested the effect of the timing of PPMO treatments, in relation to the time of viral infection, on antiviral efficacy in mice. One group of mice was treated initially at 4 h before infection, while the other four groups were treated initially at 1, 2, 4 or 20 h after infection, respectively, with NP-v3' PPMO. Infections and treatments were performed as before, with 3.75mg/Kg of PPMO per treatment. Figure 2.3C shows that treatment initiated at four hours pre-infection produced a 1.5 $\log_{10}$ (95%CI= 1.884 to 1.050) reduction in virus growth; similar to that observed in previous experiments (Figures 2.3A and 2.3B). With initial PPMO treatment occurring at one or two hours after infection we observed a significant decreases of 0.83 $\log_{10}$ (95%CI= 1.248 to 0.4136) and 0.57 $\log_{10}$ (95%CI= 0.9871 to 0.1528) respectively (Figure 2.3C), while treatment initiated at four or twenty hours post-infection produced no significant decrease in virus growth compared to mice treated with the DScr control PPMO on the same schedule. These results show that PPMO treatment before or early after infection with a non-lethal influenza strain is capable of significantly reducing virus growth in the lungs of mice.

Discussion

There is a pressing need for new antiviral drugs against FLUAV (McCullers, 2006). PPMO were previously shown to be effective at inhibiting the replication of multiple FLUAV subtypes in cell cultures, with high specificity and low cytotoxicity (Gottwein et al., 2006). Here we show that the PB1-AUG and NP-v3' PPMO in combination or NP-v3' alone, but not PB1-AUG alone, effectively reduced the growth of the A/Eq/Miami/1/63 strain of H3N8 by as much as 1.5 $\log_{10}$ when delivered into lung tissue of mice through an intranasal route of administration (Figures 2.3A-C).

The results show that the PPMO in this study were effective if administered before or shortly after viral infection, producing reductions in viral titer similar to those reported for other antisense- (Abe et al., 2001) or siRNA-based experiments (Ge et al., 2004; Tompkins et al., 2004). However, if treatment was delayed until 4 hours after viral infection, a statistically significant reduction in viral titer was not achieved (Figure 2.3C). It is possible that the PPMO is only effective if administered early during the course of an infection, before virus replication is fulminant in the lungs. However, we note that other antiviral studies with T-705 (Sidwell et al., 2007), RWJ-270201 (Sidwell et al., 2001), and Oseltamivir (Sidwell et al., 2007; Sidwell et al., 2001), have shown that although drug treatment did not reduce virus growth significantly early in infection, viral titers were indeed reduced later in infection, compared to controls, and protection from lethal infection was observed. In addition, the efficacy of anti-FLUAV drugs appears to be variable depending on the viral strain challenged (Govorkova et al., 2001; Rameix-Welti et al., 2006).

The A/Eq/Miami/1/63 (H3N8) strain used in these experiments does not produce a lethal infection in mice, and propagates only to ~10^5 PFU/g of lung tissue, as compared to a lethal infection with A/PR/8/34 (H1N1), from which we have observed a titer of ~10^7 PFU/g of lung (data not shown). Furthermore, mice infected with A/Eq/Miami/1/63 begin to recover by day 5 post infection without any treatment (data not shown). This study therefore reflects the conditions of, and treatment for, a seasonal influenza infection. Further testing in a mouse model using a lethal influenza infection is necessary to determine if treatment in a therapeutic manner is capable of increasing survivorship, and reducing virus load over a period longer than three days.

PPMO produced antiviral effects at concentrations that did not cause weight loss or induce a host immune response detectable in the lungs or spleen (Figures 2.1A-C). However, some abnormal infiltration of the lungs by immune-system cells was produced by a dose of PPMO higher than those employed in the anti-viral experiments. This potential toxicity was presumably due to the CPP, as previous studies have shown no toxicity for nonconjugated PMO (Amantana et al., 2007; Deas et al., 2007). It has been shown that administration of high dose poly-arginine via intratracheal injection results in a marked increase in eosinophil and neutrophil infiltration into the lungs of guinea pigs (Arseneault et al., 1999). Mechanistically this has been attributed to the ability of poly-l-arginine to mimic the effect of constituents stored in the secretory granules of eosinophils, especially major basic protein (MBP) (Arseneault et al., 1999). MBP is a 177 amino acid residue polypeptide rich in arginine. When MBP is released from granules, it mediates migration of inflammatory cells via the induction of ion flux and prostaglandin synthesis in airway epithelium as well as histamine release and airway

smooth muscle contraction in the bronchoalveolar compartment (Arseneault et al., 1999). It is possible that at high doses the arginine rich CPP mimics MBP and has an effect on lung epithelium similar to that observed with high doses of poly-l-arginine.

Sequencing of the A/Eq/Miami/63 strain of H3N8 used here showed that it has a single base mismatch with PB1-AUG PPMO at the target site. The PB1-AUG PPMO has effectively inhibited other FLUAV strains lacking the mismatch (Gottwein et al., 2006) (see Table 2.1). The NP-v3' PPMO sequence has perfect complementarity in A/Eq/Miami/63 (H3N8) (Table 2.1), and was highly effective. Taken together, these observations indicate that the C to U variation causing the mismatch may be the cause for failure of the PB1-AUG PPMO to inhibit A/Eq/Miami/63 (H3N8). However, that conjecture is not consistent with previously reported results of cell-free translation experiments utilizing in vitro transcribed RNAs to investigate the activity of a PPMO against RNAs with which it had a various number of mismatches (Gottwein et al., 2006; Zhang et al., 2006). In those experiments, one mismatch between a PPMO and RNA sequence had little impact on PPMO-mediated inhibition of translation. The discrepancy between the results of those in vitro translations compared to our cell culture or in vivo results with PB1-AUG PPMO and FLUAV suggest that PPMO and RNA can interact quite differently in a cell-free assay compared to intact biological systems. It seems unlikely, however, that the C to U mutation would be the sole reason why the PB1-AUG PPMO alone was ineffective, as the resulting U in the viral mRNA would still have two hydrogen bonds with the corresponding G in the PPMO, and therefore represents a minor penalty to hybridization potential.

The evolution of drug resistant strains of FLUAV has resulted in the number of anti-FLUAV drugs recommended for use in humans being cut in half (Saito et al., 2007). The use of antivirals administered in combination as a strategy to inhibit the development of drug resistance has been suggested previously (Ge et al., 2004; Gottwein et al., 2006; Ilyushina et al., 2006). As shown here, although resistant to the PB1-AUG PPMO alone, A/Eq/Miami/1/63 (H3N8) was susceptible to NP-v3' PPMO or a combination of NP-v3' and PB1-AUG PPMO (Figure 2.3). These results suggest that a combination of PPMO may provide superior protection against naturally occurring mutants, as well as reduce the likelihood of the development of viral escape-mutants. The choice of the NP and PB1 genes as targets for PPMO is supported by other reports showing that these same genes provided highly productive targets for antisense- and siRNA-based efforts at inhibiting FLUAV replication (Abe et al., 2001; Ge et al., 2004; Tompkins et al., 2004). PPMO targeting other RNA viruses have been shown to be effective when delivered intraperitoneally (Burrer et al., 2007; Deas et al., 2007), or intravenously (Yuan et al., 2006) as well, and it may become important to determine if routes of PPMO administration other than intranasal delivery are effective, as some FLUAV strains have been shown to produce systemic infection in humans (Belser et al., 2007; de Jong et al., 2005a).

The testing of NP-v3' and PB1-AUG PPMO here is intended to further their development as possible anti-FLUAV drugs. The H3N8 virus utilized in this study is of significance to the human and veterinary community. As mentioned previously, the H3N8 subtype was responsible for the influenza pandemic of 1890 (Dowdle, 1999). Despite vaccination, there are frequent outbreaks of H3N8 virus each year in horses

(Martella et al., 2007), costing the horse racing industry hundreds of millions of dollars (Animal Health Australia, 2007). In August 2007, an outbreak of H3N8 occurred in horses in Australia; a continent previously unaffected by Equine Influenza. The recent transmission of H3N8 from horses to domestic dogs (Crawford et al., 2005; Yoon et al., 2005) is of notable concern as well, as both species are in frequent and close contact with humans. In light of the inexorable evolution of FLUAV, efficacy of a potential anti-FLUAV drug against a range of FLUAV subtypes is clearly desirable. This study suggests that further testing of PPMO against a variety of strains in vivo is warranted.

Acknowledgments

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Chapter 3:

Decreased Replication and Maturation of Human Respiratory Syncytial Treated with the Proteasome Inhibitor MG-132

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Abstract

Many enveloped viruses have a requirement for the host protein ubiquitin for efficient budding from the cell membrane including members of the paramyxoviridae family of viruses (PIV5, SeV). Until recently, little has been known about the budding requirements of the subfamily pneumovirinae. We report here that treatment of Vero cells with the proteasome inhibitor MG-132 results in the reduction of Human Respiratory Syncytial virus (HRSV) titers by as much as $2.2Log_{10}$. These treatment effects were time dependant, with greatest inhibitory effects observed early in infection (4-14 hours post infection). Although western blots indicated a possible decrease of 52% in virus budding, we show by transmission electron microscopy, fluorescence microscopy, and treatment with cyclohexamide that any apparent inhibition in HRSV budding is the result of decreased viral protein levels and not an inhibition of late budding. Further, we demonstrate that inhibition of HRSV in Vero cells by MG-132 is due to eIF2α phosphorylation. However, phosphorylation of eIF2α by MG-132 treatment only occurred in HRSV infected cells, and not in GFP transfected controls. A combination of HRSV infection and MG-132 treatment therefore provides sufficient cellular stress to induce inhibition of protein synthesis.

Introduction

Human Respiratory Syncytial Virus (HRSV) is a member of the family *Paramyxoviridae* subfamily *Pneumovirinae*. In recent years, ongoing studies of the genetics and molecular mechanisms of the pathogenesis of RSV have opened the doors to new approaches for treatment and vaccine development. To date, however, there are no approved drugs for therapeutic treatment, and vaccine trials continue to struggle with the inability to elicit a protective immune response (reviewed in Girard et al., 2005) or the generation of a hyper-immune response to the natural infection following vaccination (Boelen et al., 2000; Castilow et al., 2008; Waris et al., 1996). These results indicate the necessity for increasing our understanding of HRSV and the host factors involved in infection to facilitate the development of effective treatments and vaccines.

Numerous papers have been published on the requirement paramyxoviruses have for the host protein ubiquitin. Ubiquitin has been implicated in processes such as degradation of specific host proteins that pose a threat to virus replication (reviewed in Gotoh et al., 2001; reviewed in Gotoh et al., 2002). The NS proteins of HRSV were found to down regulating type I interferon signaling. Notably, NS1 was shown to interact with Elongin/Cullin E3 ubiquitin ligase and direct the polyubiquitination of STAT-2, thus leading to its proteasomal degradation (Elliott et al., 2007; Lo et al., 2005; Spann et al., 2004). This action impedes the host innate immune response by blocking signaling from interferon alpha and beta. Similar functions have also been ascribed to the V protein of other paramyxoviruses (Andrejeva et al., 2002; Didcock et al., 1999; Precious et al., 2005a; Precious et al., 2005b; Ulane and Horvath, 2002). Further evidence for the importance of ubiquitin in paramyxoviruses has been shown by the ability of the

proteasome inhibitor MG-132, which depletes free ubiquitin in the cell by blocking the 26S proteasomal degradation of polyubiquitinated proteins, to inhibit the ability of human parainfluenza 5 (hPIV5) to bud from the plasma membrane (Schmitt et al., 2005). These results indicate that ubiquitin is important in the paramyxovirus life cycle.

A recent report by Utley et al. (2008) showed that HRSV utilizes alternative cellular machinery consisting of Rab11-Fip2 for budding from the cell membrane (Brock et al., 2003; Utley et al., 2008), which is independent from the ESCRT and VPS4 proteins required by many other viruses (Chen et al., 2007; Stuchell et al., 2004) and may not require ubiquitin. We show here that treatment of HRSV-infected Vero cells with MG-132 reduces virus titers by as much as 2.2 $\log_{10}$ when treatment was initiated at 4 hours post infection. In a similar fashion to the recent report on vesicular stomatitis virus (VSV) (Neznanov et al., 2008), we show that treatment with MG-132 reduces HRSV replication by interfering with viral protein synthesis. These data indicate that perturbation of the ubiquitin proteasome pathway is affects HRSV but does not induce any late budding defects.

Materials and Methods

Viruses and Cells. Vero cells were cultured in growth medium consisting of DMEM supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100U/mL penicillin, 100µg/mL streptomycin, and 500ng/mL amphotericin B (Invitrogen, Carlsbad, California). Cells were grown at 37ºC and 5% CO_2.

The laboratory strain VR-1540™ HRSV A2 (ATCC, Manassas, VA) or HRSV A16 2000 clinical isolate (Barney Graham, NIAID) were grown in HEp-2 cells by

infecting with a multiplicity of infection (MOI) of 0.1-0.01. At day 4 or 5 after infection cells were scraped and cells and medium vortexed for 1 minute. Cells were pelleted at 3000 x g for 10 minutes and clarified medium was overlaid on a 20% sucrose cushion in 1M $MgSO_4$, 50mM HEPES, and 150mM NaCl buffer (MHN buffer) and virus pelleted at 18,000rpm for 30 minutes in a SW28 rotor. Virus pellet was then resuspended in 20% sucrose MHN buffer and 100µL aliquots were snap frozen in a dry ice ethanol bath. Samples were stored at -80ºC until used. Stock virus titers were determined by plaque assay (see plaque assay below).

Effects of MG-132 on virus replication. MG-132 (Calbiochem, EMD biosciences, San Diego, CA**)** was resuspended in DMSO and stored at -20ºC. Vero cells were grown in 24-well plates until >90% confluent and then infected with RSV A16 2000 at 0.25 MOI for 1 hour at 37ºC and 5%CO_2 with periodic shaking. Following adsorption, infection medium was removed and 1mL of growth medium was added per well and plates were returned to the incubator. For dose response experiments, growth medium was removed at 4 hours post infection and 1mL fresh growth medium containing 1.0µM or 5.0µM MG-132 or 0.5% DMSO as control were added. Alternatively, growth medium was removed at 4, 14, or 24 hours post infection for time of addition experiments and 1mL fresh growth medium containing 5.0µM MG-132 or 0.5% DMSO as control were added. Cells were then incubated until 40 hours post infection when medium was analyzed immediately for virus titer by plaque assay (see below). For multiple time point growth curves, Vero cells were infected with RSV A2 at the indicated MOI and treated at 14 hours post infection with 5.0µM MG-132 or 0.5%DMSO as control. Virus titer was determined by sampling

medium every 12 hours as indicated and immediately performing plaque assays (see below).

For comparison of MG-132 effects on HRSV and influenza virus, Vero cells were seeded into 24-well plates and grown until confluent. Cells were infected with 0.25 MOI of RSV A16 2000 or influenza A/PR/8/34 H1N1 for 1h at 37ºC and 5% CO_2 with periodic shaking. Infection medium was then removed and 1mL of MEM supplemented with 100U/mL penicillin, 100µg/mL streptomycin, and 1.0µg/mL TPCK treated trypsin (USB, Cleveland, Ohio) was added to influenza infected wells and growth medium was added to RSV infected wells. Cells were treated with 5µM MG-132, or DMSO control, at 4h post infection. Medium was measured for virus titer at 40 hours post infection by plaque assay.

Plaque assays. HRSV plaque assays were performed in Vero cells seeded into 24-well plates and grown until confluent. Serial ten-fold dilutions of each sample from treated or control treated wells were made in growth medium. 50µl was plated in duplicate wells for each dilution and adsorbed for 1h at 37ºC and 5% CO_2 with periodic shaking. Wells were then overlaid with 0.5mL DMEM supplemented with 100U/mL penicillin, 100µg/mL streptomycin, and 0.75% methylcellulose. On day 3-4 post infection, cells were fixed with 0.5mL of 4% paraformaldehyde in PBS for 24 hours. Wells were then stained with hematoxylin and eosin and plaques counted. Plaque titer was determined by the highest dilution in which duplicate wells had at least 5 plaques.

Influenza plaque assays were performed in MDCK cells grown until confluent in 24-well plates. Serial ten-fold dilutions of each sample from treated or control treated wells were made in MEM containing 100U/mL penicillin and 100µg/mL streptomycin.

Duplicate wells were infected with 50µL of each dilution and virus adsorbed for 1h at 37ºC and 5% CO_2 with periodic shaking. Infection medium was removed and cells were overlaid with MEM containing 100U/ml penicillin, 100µg/ml streptomycin, 3% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO), and 1µg/ml TPCK trypsin with 1% SeaPlaque® agarose (Cambrex Bioscience Rockland, Inc., Rockland, ME). After 2 days, overlay was removed and cells were fixed and stained by adding 0.75% crystal violet (Alfa Aesar, Ward Hill, MA) diluted in 100% methanol**.** Virus titer was determined by the highest dilution in witch duplicate wells contained at least 5 plaques.

Virus maturation experiments. Vero cells in 6-well plates were infected with 2.5 MOI RSV A2 as previously indicated and incubated for 14 hours at 37ºC and 5% CO_2. Medium was then removed and 1.5mL fresh growth medium with 2.5 or 5.0µM MG-132, 1.0 or 0.5µg/mL cyclohexamide, or 0.5%DMSO as control were added. Cells were incubated until 40 hours post infection when cells and supernatant were collected by scraping cells from the plate and pipetting up and down several times. Cells and supernatant were transferred to 1.5mL microcentrifuge tubes, vortexed for 30 seconds, and cells were pelleted at 2500g for 5 minutes. Supernatant was then removed and virions partially purified by ultracentrifugation through a 30% sucrose cushion in 150mM NaCl, 50mM Tris-HCl, and 1mM EDTA (NTE buffer) for 30 minutes at 39,000rpm (~185,000g) in an SW50.1 rotor. After centrifugation, sucrose and medium were decanted. Virus pellets and corresponding cell pellets were lysed in 40µL Bugbuster (Novagen, EMD biosciences, San Diego, CA) with FOCUS protease arrest (G Biosciences, Maryland Heights, MO). Samples were frozen for future use at -80ºC.

Cell and virion lysates were thawed and centrifuged at 12,000g for 5 min to remove debris. 15µL of cell and virion lysate with the addition of 5X SDS loading dye were run on 4-12% Bis-Tris NuPAGE gels (Invitrogen) and transferred to nitrocellulose membranes (PALL corporation, East Hills, NY). Blots were blocked in 30mL near IR blocking buffer (IRBB)(Rockland, Gilbertsville, PA) overnight at 4ºC and then stained with polyclonal goat anti-RSV (Cambrex, East Rutherford, NJ) and rabbit anti-GAPDH (Rockland) antibodies diluted 1:1000 each in 50/50 IRBB/ tris-buffered saline with 0.05% tween 20 (TBST) for 2 hours at room temperature. Blots were washed 3X with TBST and then stained with AlexaFluor 680 donkey anti-goat (Invirtogen) and IRdye 800 donkey anti-rabbit (Rockland) antibodies for 2 hours at room temperature. Blots were washed 3X in TBST and imaged using a Li-Cor near IR imager and densitometry was performed using Odyssey 2.0 software (Li-Cor, Lincoln, Nebraska). Maturation efficiency was determined by comparing the amount of the HRSV matrix protein contained in virions to the total amount contained in both the virions and cell pellet (Schmitt et al., 2005).

Transmission Electron Microscopy. Vero cells were grown until >90% confluent in a 12-well plate. Cells were infected with 2.5 MOI of RSV A16 2000 for 2 hours at 37ºC and 5% CO_2 with periodic shaking. At 4 hours post infection, growth medium was removed and 1mL fresh growth medium containing 10µM MG-132 or 0.5% DMSO as control were added. Cells were then incubated until 40 hours post infection. Cells were collected by scraping from the plate and pipetting up and down several times. Cells and supernatant were then transferred to a 1.5mL microcentrifuge tube and cells were pelleted at 2000g for 5 minutes. Supernatant was then removed and cells were fixed in 1% EM

grade gluteraldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS followed by 1% osmium tetraoxide (Electron Microscopy Sciences). Cells were washed 3 times in PBS and then dehydrated by incubation in washes of increasing acetone concentration from 30-100%. Incubation in 70% acetone was accompanied with uranyl acetate staining. Following acetone dehydration, samples were embedded in 500uL Spurr's resin (Electron Microscopy Sciences). ~80nm cross sections were prepared on an ultramicrotome and placed on 200-count copper grids (Electron Microscopy Sciences). Samples were then post stained with urinal acetate and lead citrate and examined on a Philips CM-12 scanning transmission electron microscope (STEM).

Immunofluorescence. Vero cells were seeded onto glass cover slips in 6-well plates and grown until ~60% confluent. Cells were infected with 2.5 MOI RSV A16 2000 for 1 hour at 37ºC and 5% CO_2 with periodic shaking. Infection medium was removed and 2mL growth medium was added. Plates were incubated for four hours followed by removal of medium and addition of 2mL growth medium with 5μM MG-132 or 0.5% DMSO as control. Cells were incubated until 24 hours post infection and fixed with 4% formaldehyde in PBS for 15 min, washed 3X in PBS, and fixed in ice cold methanol for 10 min at -20ºC. Cells were blocked and permeablized in 30% fish blocking buffer (BioFX, SurModics In Vitro Diagnostic Products, Eden Prairie, MN) with 1% BSA in TBS with 0.3% Triton X-100 for one hour at room temperature. Cells were then stained for 2 hours with a 1:500 dilution in TBS with 0.3% Triton X-100 of Cy3 conjugated anti-RSV Fusion glycoprotein antibody (derived from Palivizumab/Synagis; MedImmune, Gaithersburg, MD). After an additional 3 washes, cover slips were mounted on slides using Vectashield with DAPI (Vector Laboratories Inc., Burlingame, CA) and examined

with a Leica DMLB fluorescent microscope and SPOT CCD camera and software(Diagnostic Instruments, Inc., Sterling Heights, MI). Images were analyzed in Adobe Photoshop CS (Adobe, San Jose, CA).

GFP production efficiency. Vero cells were grown in 24-well plates until ~80% confluent and transfected with 0.8µg pEGFP-N1 (Clonetech, Mountain View, CA) using Lipofectamine 2000 (Invitrogen) according to manufactures recommendations. Vero cells were treated with 2.5 or 5µM MG-132, 1.0 or 0.1µg/ml cyclohexamide, or 0.5% DMSO respectively, at 14h post transfection. At 30h post transfection cells were lysed with M-PER (Thermo Scientific, Rockford, IL) and 25µg total protein was run on NuPAGE 4-12% Bis-Tris gels. GFP expression was determined by western blot using rabbit anti-GAPDH (Rockland) and Living Colors anti-GFP primary antibodies (Clontech) and IRdye 700 donkey anti-mouse IgG and IRdye 800 donkey anti-rabbit secondary antibodies (Rockland), following the procedure outlined above for determining budding efficiency.

eIF2α Phosphorylation state. Cell lysate collected for virus maturation efficiency and GFP production efficiency experiments were subsequently analyzed by western blot using the primary antibodies mouse anti-eIF2α and rabbit anti-phopspo Ser-51 eIF2α (Cell Signaling Technologies) and IRdye 700 donkey anti-mouse IgG and IRdye 800 donkey anti-rabbit secondary antibodies (Rockland) and data analyzed as described above.

Statistics. All statistics were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA) by One-Way ANOVA and either Bonferroni or Dunnett post tests, or Two-Way ANOVA with Bonferroni post test.

Results

Proteasome inhibitor MG-132 inhibits HRSV replication in cell culture. To determine if ubiquitin is necessary for RSV replication, we infected Vero cells with RSV A16 2000 clinical isolate at a MOI of 0.25 and then treated cells with various concentrations or at various time points post infection with the proteasome inhibitor MG-132. Figure 3.1A and 3.1B show that there was a dose and time dependant decrease

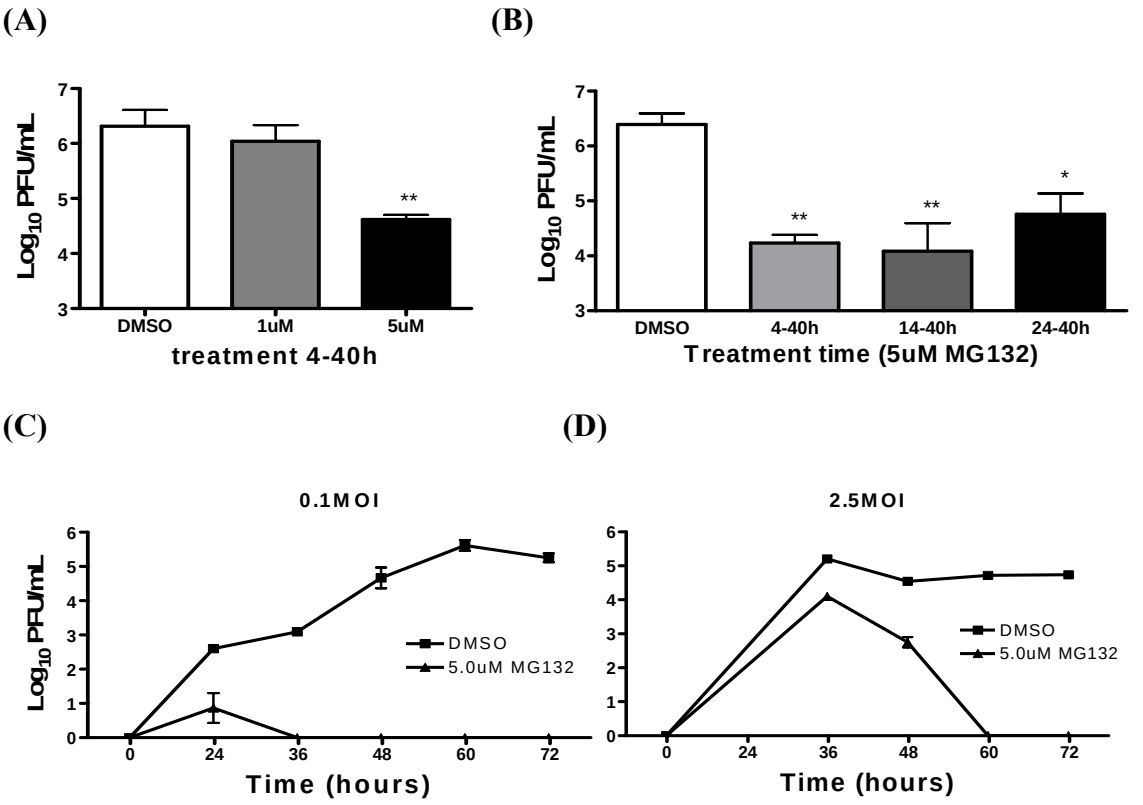

Figure 3.1: Reduction of RSV titer by MG-132. Vero cells were infected with RSV A16 2000 and treated with (A) various concentrations (B) or at different times after infection with MG-132. Controls were treated with 0.5% DMSO. Virus titer was determined at 40 hours post infection by plaque assay. (C-D) Vero Cells were infected at the indicated MOI with RSV-A2 and treated with 5μM MG-132 at 14 hours post infection. Virus titer was determined by plaque assay at 12 hour intervals. (n=3, ** $P<0.01$ based on One-way ANOVA with Dunnett's Multiple Comparison Test. All time points in C-D were significant by Two-way ANOVA with Bonferroni post test.)

observed in virus titer at 40 hours post infection of up to 2.2 $\log_{10}$ (95% Confidence Interval (CI)= 0.76 to 3.6).

Multiple time point growth curves were also generated by infecting Vero cells with 0.1 or 2.5 MOI of RSV A2 laboratory strain and examining virus titer at 12 hour intervals (Figures 3.1C and 3.1D respectively). These results show that by 24 hours post infection in the 0.1 MOI infection there was a significant reduction in virus titer of 1.73 $\log_{10}$ (95%CI= 0.7203 to 2.748). In the 2.5 MOI infection, virus titer was significantly reduced at 36 hours post infection by 1.10 $\log_{10}$ (95%CI= 0.1597 to 2.038). In both experiments, MG-132 treated groups reached maximal titers and then declined rapidly, whereas DMSO treated controls reached maximal titers and then plateaued. These data indicate that RSV is sensitive to proteasome inhibitor treatments in vitro.

MG-132 reduces virion maturation as a result of decreased protein synthesis. We determined if there was any effect of MG-132 on virion maturation by western blot. Mature virions were partially purified by ultracentrifugation through a 30% sucrose cushion. Virion pellets and cell pellets from MG-132 treated or control treated wells were lysed and examined by western blot to determine viral protein concentrations. The efficiency of virion maturation was then determined by the ratio of viral protein in the virions to the total amount of viral protein $\frac{VirionLysate}{virionLysate + CellLysate}$ (Schmitt et al., 2005). Figures 3.2A and 3.2B show a time dependant inhibition of virion maturation, as treatment initiated at 14 hours after infection had a significant effect on virion maturation (2.5μM -48%, 95%CI=-74% to -23% and 5.0μM -52%, 95%CI=-77% to -26%), but when treatment was delayed until 24 hours after infection there was no significant effect.

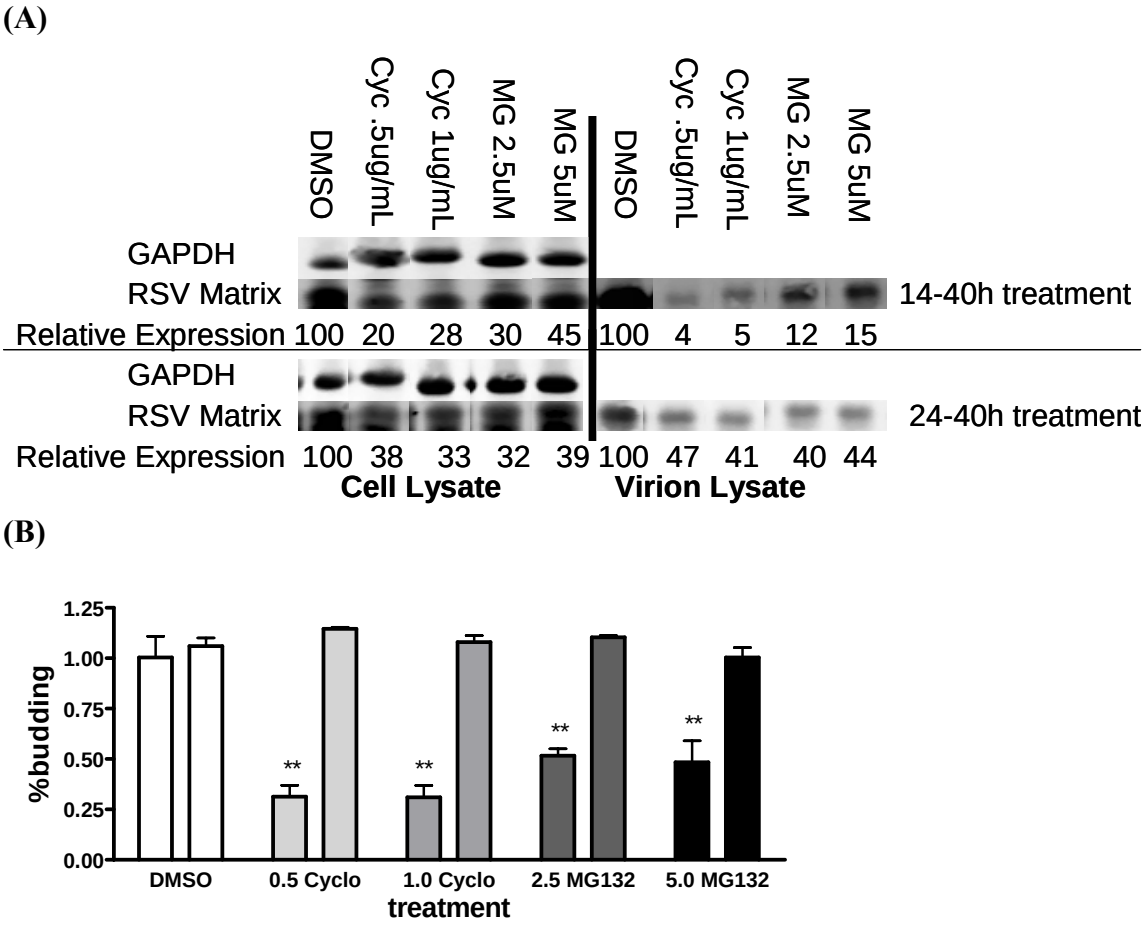

Figure 3.2: Inhibition of virion maturation and protein synthesis in Vero cells by MG-132. (A) Western blot of HRSV matrix protein from cell lysate and virion lysate fractions from Vero cells infected with 2.5 MOI RSV A2 and treated with the indicated concentrations of either MG-132, cyclohexamide, or DMSO control from 14-40 hours or 24-40 hours post infection. (B) The effect of time of addition of inhibitors on virion maturation was determined by comparing the amount of HRSV matrix protein in the virion lysate to the total amount of matrix protein present in the cell and virion lysate fractions. All samples were collected at 40 hours post infection. Data represents densitometry from triplicate samples. (** $P<0.001$ based on Two-way ANOVA with Bonferroni post test)

We also observed that treatment with MG-132 resulted in a decrease in viral protein synthesis (Figure 3.2A, cell associated). To test whether this was responsible for decreased virion maturation, we treated cells with the protein translation inhibitor cyclohexamide (Figures 3.2A and 3.2B). The results from cyclohexamide inhibition were

nearly identical to those obtained with MG-132 and indicate that the reduced efficiency of virion maturation seen here is due to an inhibition in viral protein synthesis. Treatment with either 1.0 or 0.5μg/mL cyclohexamide resulted in a 69% decrease in virion maturation (95%CIs=-95% to -44% and -94% to -43% respectively). These results are in contrast to reports on other paramyxoviruses (Schmitt et al., 2005; Taylor et al., 2007) which have shown a decrease in virus budding, but not a decrease in viral protein synthesis.

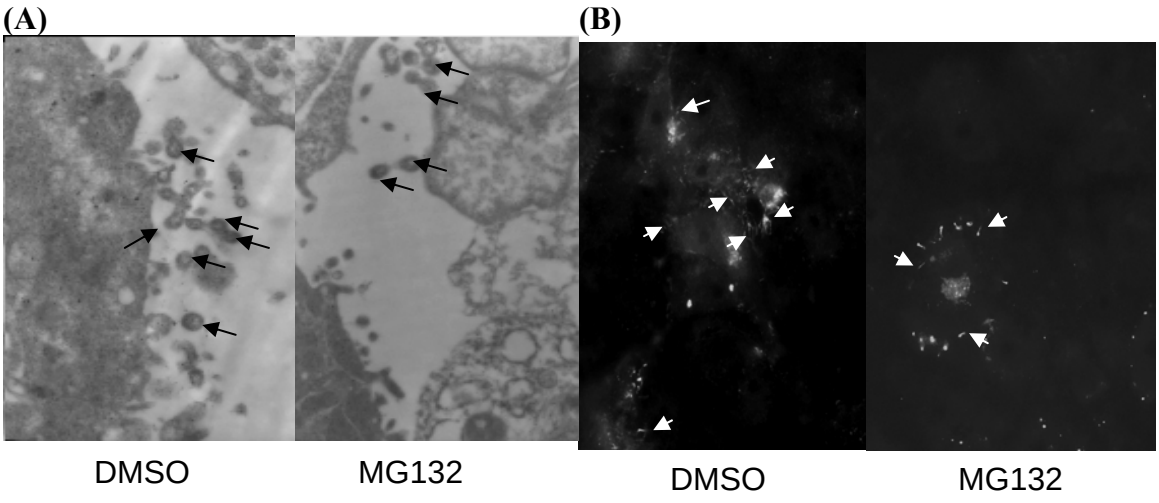

Figure 3.3: Electron and fluorescent microscopic examination of virion maturation in Vero cells treated with MG-132. (A) Electron micrographs (13k X) of Vero cells infected with 2.5 MOI RSV A16 2000 and treated with DMSO control or 10μM MG-132 from 4-40 hours post infection. (B) Fluorescence microscopy images of 2.5 MOI RSV A16 2000 infected Vero cells treated with 5.0μM MG-132 from 4-24 hours post infection and stained with Cy3 anti-RSV Fusion protein antibody. Arrows indicate virions.

MG-132 does not induce a late budding phenotype in HRSV. We examined the effects of MG-132 on HRSV maturation by transmission electron microscopy (TEM). Vero cells were infected with 0.25 MOI of HRSV A16 2000 and were then treated with 10μM MG-132 at 4 hours post infection to maximize any inhibition of virus budding. Multiple images were obtained from cells fixed at 40 hours post infection for each sample. As

shown in figure 3.3A, cells treated with MG-132 had a marked decrease in virus production compared to DMSO treated controls. In no case did we observe cells which demonstrated a late budding phenotype of viruses tethered to the plasma membrane. These results were also confirmed by immunofluorescence of HRSV A16 2000 infected Vero cells treated with 5μM MG-132 at 4 hours post infection. In this instance, cells were fixed and stained at 24 hours post infection with a Cy3 conjugated anti-HRSV Fusion protein antibody (Figure 3.3B). These results confirm that MG-132 treatment reduces the number of mature virions seen but does not induce a late budding phenotype.

Determination of nonspecific effect of MG-132 in Vero cells. Due to the long duration of treatments, we tested the toxicity of MG-132 on Vero cells for the maximum time period (36h) used in our experiments and found that there was no substantial toxicity at the concentrations employed in our experiments (Figure 3.4A). To further confirm that the reduction in viral replication seen in our work is specific to HRSV, we tested the effects of long term exposure of cells to MG-132 on another virus. Vero cells were infected with either RSV A16 2000 or influenza A/PR/8/34 H1N1 at 0.25 MOI and treated with 5.0μM MG-132 or 0.5% DMSO as a control. Examination of virus titers showed a decrease of 1.999 $\log_{10}$ (95%CI 1.797 to 2.200) in for HRSV (Figure 3.4B). Though there was a significant decrease in influenza titer of 0.39 $\log_{10}$ (95%CI= 0.21 to 0.57) it did not account for the large decrease seen in HRSV (Figure 3.4B). These data indicate that Vero cells treated for extended durations with MG-132 are capable of supporting replication of some viruses and that the inhibitory effect of MG-132 on HRSV is at least partially specific in Vero cells.

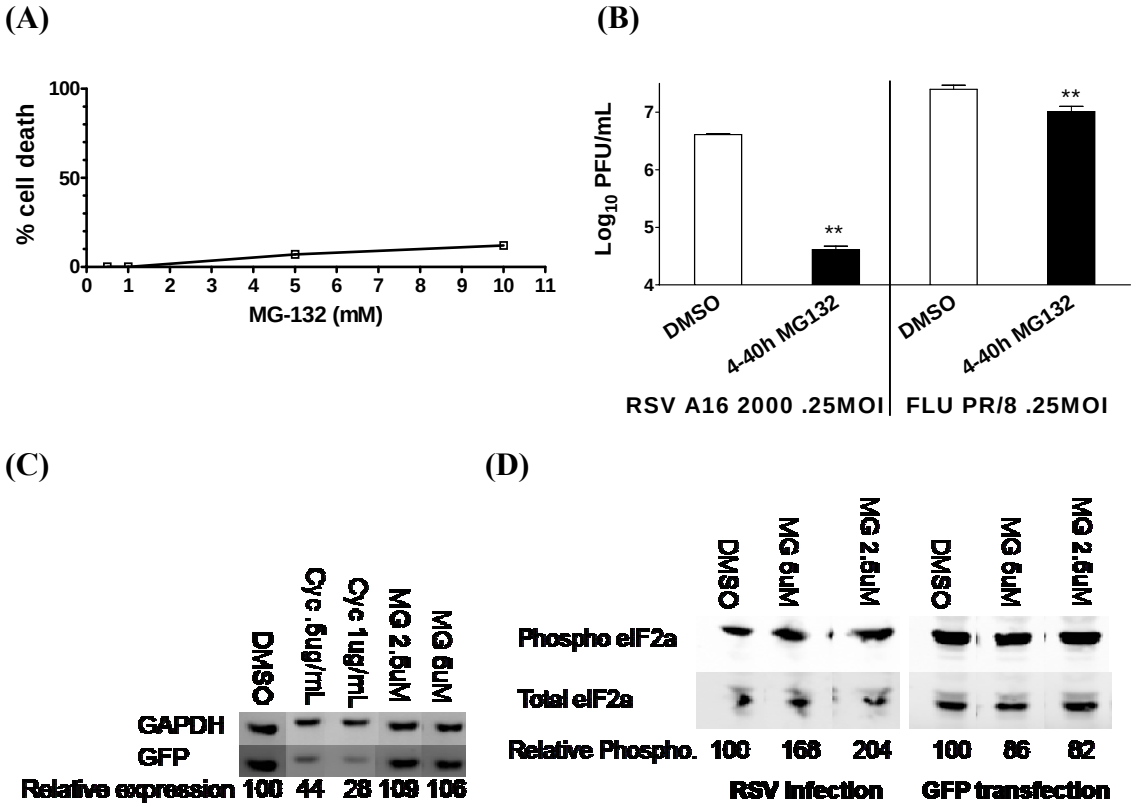

Figure 3.4: Specificity of MG-132. (A) Cell viability of Vero cells following 36 hour treatment with MG-132 as measured by CellTiter Blue (Promega). (B) RSV A16 2000 and Influenza A/PR/8/34 H1N1 virus titers at 40 hours post infection following treatment of Vero cells with DSMO control or 5μM MG-132 at 4 hours post infection. (C) Vero cells transfected with pEGFP N-1 and treated as indicated with Cyclohexamide (Cyc), MG-132 (MG) or DMSO control starting at 14 hours post transfection were examined for expression levels by western blot at 40 hours post transfection. (D) RSV A2 infected or GFP transfected Vero cells treated at 14h post infection with MG-132 were examined at 40h post infection for eIF2α phosphorylation. All data represent triplicate samples. (** $P<0.01$ based on One-way ANOVA with Bonferroni or Dunnett post tests)

It is possible that prolonged exposure to MG-132 may not be lethal to cells, but may affect cellular function during HRSV infection. We transfected Vero cell with a GFP expression vector and treated them at 14 hours post transfection with MG-132 or cyclohexamide. There was no significant decrease in GFP production in Vero cells treated with MG-132 (Figure 3.4C). However, treatment with 1.0 or 0.5μg/mL of

cyclohexamide did result in a 72% or 56% decrease in GFP expression respectively (95%CI= 25-119% decrease or 8.7-103% decrease).

It was reported that treatment with MG-132 leads to a global inhibition of translation due to phosphorylation of eIF2α by Heme-regulated Inhibitor (Yerlikaya et al., 2008). We examined GFP transfected or RSV A2 infected Vero cells for the level of eIF2α phosphorylation and found that there was a 69-104% increase in serine 51 phosphorylation only in RSV infected cell, whereas GFP transfected cells showed no increase (Figure 3.4D).These data indicate that the mode of action of MG-132 inhibition of HRSV protein synthesis is different from cyclohexamide. MG-132 inhibits translation specifically in HRSV infected cells by triggering the phosphorylation of eIF2α.

Discussion

The term budding is somewhat inappropriate in the context of our results, as we did not observe an increase in virions "tethered" to the plasma membrane by TEM or immunofluorescence (Figure 3.2A-B); as has been observed with retroviruses (Patnaik et al., 2000). We have therefore referred to any potential effects on HRSV by MG-132 as inhibitions of virus maturation, replication, or protein synthesis.

Recent studies into HRSV intracellular trafficking and virion budding show that this virus utilizes alternative cellular machinery consisting of Rab11-Fip2 and Myosin Vb for budding from the apical membrane in polarized lung epithelial cells (Brock et al., 2003; Utley et al., 2008). The report by Utley et al. also showed that there was no requirement in HRSV budding for known cellular factors; like the VPS4 proteins required by many other viruses. In addition they did not observe any effect on HRSV

maturation or replication in HEp-2 cells when treated with 1.0μM MG-132 starting at 24 hours post infection. We also observed that treatment at 24 hours after infection did not affect HRSV maturation (Figure 3.2B), and that maturation was only inhibited by treatment earlier during infection (14 hours post infection). In addition to our experiments being performed in a separate cell line, another reason for the difference in our reports is the concentration of MG-132 utilized. Figure 3.1A shows that 1.0μM MG-132 did not significantly reduce virus replication in Vero cells even when added at 4 hours post infection, whereas treatment with 2.5μM or higher had a significant effect (Figure 3.2B).

It is possible that the reductions in HRSV replication seen here are a result of this virus' utilization of the ubiquitin proteasome pathway to inhibit the innate immune response. As stated in the introduction, HRSV NS1 is known to mediate the proteasomal degradation of STAT-2 (Elliott et al., 2007). However, in relation to our results it is not evident how this would have an effect on virus replication as Vero cells are deficient in type-one interferon signaling. However, we tested the effect of shRNA against STAT-2 to inhibit HRSV replication in Vero cells and observed no effect (data not shown). In addition, HRSV inhibits premature apoptosis in infected cells by activating the Akt/MDM2 pathway leading to proteasomal degradation of p53. Enigmatically, though, inhibition of p53 degradation resulting in activation of apoptosis resulted in an increase in HRSV replication (Groskreutz et al., 2007).

Yerlikaya et al. (2008) showed that MG-132 treatment can result in general inhibition of protein synthesis by way of eIF2α phosphorylation by increased oxidative stress which signaled through Heme-regulated Inhibitor. This effect appears to be cell line specific, as treatment of MDCK cells for 18 hour did not reduce protein production

(Bush et al., 1997). A recent study on vesicular stomatitis virus (VSV) showed that treatment with MG-132 reduced virus protein synthesis as a result of phosphorylation of eIF2α by GCN2 (Neznanov et al., 2008). Alternatively, additional reports (Harty et al., 2001; Taylor et al., 2007) have shown that MG-132 inhibits VSV budding without a large decrease in viral protein levels.

It is evident that the ability to inhibit protein synthesis and virus replication with MG-132 is both virus specific and cell line specific. The ability of MG-132 to alter cellular function was somewhat specific to HRSV, as it had little to no effect on uninfected cells transfected with GFP or on the replication of influenza A virus; which has shown partial resistance to proteasome inhibitor treatment previously (Watanabe et al., 2005). We show here that treatment of HRSV infected Vero cells with MG-132 did result in increased eIF2α phosphorylation (Figure 3.4D) and we propose this as the mechanism for decreased HRSV protein levels and concurrent virus maturation. We hypothesize that the decreased efficiency with which mature HRSV virions were produced and escaped the cell (Figure 3.2 and 3.2) may indicate a minimum threshold level of viral proteins required for efficient trafficking or virion formation. Overall, our results demonstrate that any decrease in HRSV virion maturation is a byproduct of decreased viral protein levels and not due to a direct lack of ubiquitin or inhibition of late budding.

Acknowledgments

We would like to thank Hui Kim, Casey Ward, and Sam Tepper for technical assistance. We would also like to thank Mike Nesson and Al Soeldner at the Oregon State

University Electron Microscope Facility for training on the STEM and help with sample preparation.

Chapter 4:

Treatment of Respiratory Syncytial Virus infected Balb/C Mice with the Proteasome Inhibitor Bortezomib (Velcade®, PS-341) Results in Increased Inflammation and Mortality

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Manuscript in preparation

Abstract

Human Respiratory Syncytial Virus is an important pathogen and is associated with mortality in the young, old, and immuno-compromised patients. Due to the lack of effective therapeutic antivirals or a vaccine, there is a need for continued research in this field. Our previous work has shown that HRSV replication is sensitive to treatment with the proteasome inhibitor MG-132. Here we tested the ability of the FDA approved proteasome inhibitor bortezomib to inhibit HRSV in vitro and in vivo. We observed a significant inhibition of HRSV replication in Vero cells at concentrations from 20-40ng/mL. Bortezomib was well tolerated in mice when administered intranasally at concentrations of 0.3mg/kg or intraperitoneally at 1.0mg/kg. However, treatment of HRSV infected mice with doses as low as 0.01mg/kg resulted in increased mortality compared to mock treated infected control animals and an increase in lung pathology. Examination of cytokine expression levels from lung biopsies of HRSV infected mice revealed an increase in GCSF, MCP-1, and IL-6 levels and a decrease in IL-12 and TNF-α in bortezomib treated animals compared to mock treated infected controls, indicating an altered immune response in mice treated with bortezomib during HRSV infection.

Introduction

Human Respiratory Syncytial Virus (HRSV) is a member of the family *Paramyxoviridae* subfamily *Pneumovirinae*. HRSV is a major cause of infantile pneumonia and severe infection has been linked to childhood wheezing disease and asthma. Some 95,000 children are hospitalized and ~300 die each year in the United States (Thompson et al., 2003). With a world wide death rate of 160,000 deaths per year, HRSV is one of the leading causes of infant mortality (Girard et al., 2005). Reinfection in adulthood is common, as only partial immunity is established following initial infection, and the majority or the ~12,000 HRSV associated deaths in the United States are among the elderly (Thompson et al., 2003).

Current medical treatment focuses on the symptoms of severe HRSV bronchiolitis, including airway constriction and decreased blood oxygen levels, by standard ventilation or nasal continuous positive airway pressure (Cambonie et al., 2008; Martinon-Torres et al., 2008). There are currently HRSV vaccines in clinical trials (Falsey et al., 2008; Hui et al., 2006a). For decades, however, vaccine trials have struggled with the inability to elicit a protective immune response (reviwed in Girard et al., 2005) or the generation of a hyper-immune response to the natural infection following vaccination (Boelen et al., 2000; Castilow et al., 2008; Waris et al., 1996). Palivizumab (Synagis) is a monoclonal humanized antibody based therapy used for the prophylactic treatment of patients at high risk for complications from HRSV infection (Cohen et al., 2008). For the therapeutic treatment of HRSV, Ribovirin is the only approved drug available. However, multiple clinical trials have had mixed results and fail to conclusively demonstrate efficacy (Boeckh et al., 2007; Chen et al., 2008; Kathleen and

Adrienne, 2007). There are also several promising antivirals that are currently undergoing clinical trials including ALN-RSV01 and RSV604; both of which target the viral nucleocapsid protein (Chapman et al., 2007; DeVincenzo et al., 2008).

An ongoing problem with current antiviral therapies is the development of resistant strains. For instance, resistant strains of influenza have arisen against three of the four commonly used antivirals namely amantadine, rimantadine, and oseltamivir (Dharan et al., 2009; Saito et al., 2007). It has therefore been proposed that targeting host factors involved in virus infection may provide efficient inhibition without the appearance of resistance (discussed in Müller and Kräusslich, 2009). One cellular pathway which has been implicated in the life cycle of multiple viruses is the ubiquitin proteasome pathway (Harty et al., 2001; Martin-Serrano et al., 2004; Schmitt et al., 2005), (reviewed in Martin-Serrano et al., 2004). We have previously shown that treatment of HRSV infected Vero cells with the proteasome inhibitor MG-132 results in decreased replication efficiency and consequently a decrease in virus maturation (Lupfer and Pastey, 2009).

There is currently an FDA approved proteasome inhibitor, bortezomib (Velcade®, PS-341), which has been developed as a chemotherapeutic agent for relapsed or refractory multiple myeloma (Hideshima et al., 2001). In this report we evaluate the ability of bortezomib to inhibit the replication of HRSV in cell culture and Balb/C mice. We found that bortezomib is able to inhibit HRSV replication in Vero cell culture in a dose dependant manner. However, in vivo experiments showed that treatment of infected mice with bortezomib, which was not toxic in uninfected animals, resulted in an increase in inflammation and mortality with no discernable effect on virus replication. These

results indicate that bortezomib may elicit a hyper-immune response during infection with HRSV. To our knowledge this is the first paper to test the ability of a proteasome inhibitor to inhibit virus replication in vivo.

Materials and Methods

Virus and cells. HRSV A16 2000 clinical isolate (Barney Graham, NIAID) was grown in HEp-2 cells by infecting with a multiplicity of infection (MOI) of 0.1. On day 4 after infection, cells were scraped and cells and medium were vortexed for 1 minute. Cells were pelleted at 3000 x g for 10 minutes and clarified medium was overlaid on a 20% sucrose cushion in 1M $MgSO_4$, 50mM HEPES, and 150mM NaCl buffer (MHN buffer). Virus was pelleted at 18,000rpm for 30 minutes in an SW28 rotor. Each virus pellet was resuspended in 1.0mL of 20% sucrose in MHN buffer and 100µL aliquots were snap frozen in a dry ice ethanol bath. Samples were stored at -80ºC until used. Stock virus titers were determined by plaque assay (see plaque assay below).

Vero cells were grown in growth medium consisting of DMEM supplemented with 100U/mL penicillin, 100µg/mL streptomycin (Invitrogen, Carlsbad, California), and 10% fetal bovine serum (Hyclone, Logan, UT). Cells were grown at 37ºC with 5% CO_2.

In vitro effects of bortezomib. Bortezomib (Velcade®; Millennium Pharmaceuticals, Cambridge, MA) was resuspended to 5.0mg/mL in DMSO and used immediately. Vero cells were seeded into 24-well plates and grown until confluent**.** Cells were infected with 2.5 MOI RSV A16 2000 and triplicate wells treated with 10, 20, 30, or 40ng/mL bortezomib or 0.02% DMSO control at 4 hours post infection. Medium was collected at 40 hours post infection and analyzed by plaque assay (see plaque assay below).

Treatment of RSV infected mice with bortezomib. Mouse experiments were conducted using eight week old Balb/C mice from Simonsen Laboratories (Gilroy, California) and were approved by Oregon State University's institutional animal care and use committee. Group of 3-4 mice were anesthetized intraperitoneally with 67mg/kg Ketamine and 4.5mg/kg Xylazine. To determine toxicity of bortezomib, mice were treated with two intranasal (IN) doses 1.0, 0.5 or 0.1mg/kg of bortezomib diluted in 100µL PBS and administered 24 hours apart. An additional group was treated with one IN dose of 0.3mg/kg of bortezomib. Control groups were treated with PBS containing 3.2% DMSO. Mice were monitored daily for signs of illness including weight loss, malaise, and poor grooming.

The efficacy of bortezomib for in vivo inhibition of HRSV was performed by anesthetizing groups of seven mice each with 67mg/kg Ketamine and 4.5mg/kg Xylazine and infecting IN with 10^8 plaque forming units (PFU) of RSV A16 2000 in a 100µL volume. Four groups received 100µL administered IN of either 0.1, 0.03, or 0.01mg/kg of bortezomib respectively or PBS containing 3.2% DMSO as control at 4 hours before infection and again at 20 hours after infection. Three additional groups were treated IN with a single dose of 0.3mg/kg bortezomib in 100µL PBS at either 4, 24 or 48 hours after infection. Mice were weighed daily, and three mice from each group were euthanized and lung tissue removed on day 4 post infection for determination of virus titer by plaque assay, histopathological examination, and cytokine expression. The remaining four mice in each group were weighed and examined daily for 10 days for illness and mortality.

To test an alternative route of bortezomib administration and HRSV infectious dose, we tested the toxicity of 2 doses of 1.0mg/kg bortezomib or PBS containing 3.2%

DMSO as control by administering intraperitoneal (IP) injections, 24 hours apart with 2 mice per group and observed mice for 5 days for signs of illness including weight loss, malaise, and poor grooming. Five mice per group were then infected IN with 10^7 PFU of RSV A16 2000 in a 100µL volume. Each group was administered 2 doses at 4 hour before and 20 hours after infection by IP injection of either 1.0mg/kg bortezomib or PBS containing 3.2% DMSO as control and observed for 7 days for signs of illness including weight loss, malaise, and poor grooming.

Plaque assays. Vero cells were seeded into 24-well plates and grown until confluent. Lung biopsies were prepared by weighing lung tissue from each animal and then homogenizing lungs in 1.0ml DMEM supplemented with 100U/ml penicillin and 100µg/ml streptomycin. Lung homogenates were clarified by centrifugation at 2500 x g for 2 minutes. Serial ten-fold dilutions of cell culture medium, from in vitro experiments, or lung homogenates, from in vivo experiments, were made in growth medium. 50µl of each dilution was plated in duplicate wells of 24-well plates and adsorbed for 1 hour at 37ºC and 5% CO_2 with periodic shaking. Wells were then overlaid with 0.5mL DMEM supplemented with 100U/mL penicillin, 100µg/mL streptomycin, and 0.75% methylcellulose. On day 3-4 post infection, cells were fixed with 0.5mL of 4% paraformaldehyde in PBS for 24 hours. Wells were then stained with hematoxylin and eosin and plaques counted. Plaque titer was determined by the lowest dilution in which duplicate wells had at least 5 plaques.

Histopathology. Sections of lung tissue were fixed in 4% paraformaldehyde in PBS, embedded in paraffin and sectioned at 5 micron thickness via routine processing. Sections were stained with hemotoxylin and eosin. All sections were evaluated by a

veterinary pathologist (KMP). Tissue changes were subjectively ranked according to degree of inflammation, extent of inflammation, and degree of necrosis as 0= no inflammation, 1 = mild inflammation, 2= moderate inflammation and 3 =severe inflammation.

Cytokine antibody array. Cytokine levels in mouse lungs were determined using RayBio Mouse Cytokine Antibody Array I (RayBiotech, Norcross, GA). Membrane arrays were blocked for 30 minutes, then 400µL mouse lung homogenate (see plaque assays) diluted 1:2 in blocking buffer was added to each membrane for two hours at room temperature. Membranes were washed and 1mL biotin-conjugated anti-cytokine antibody cocktail was added and incubated at room temperature for two hours. Membranes were washed again and incubated at 4ºC overnight in HRP-conjugated streptavidin 1:1000 in blocking buffer. Membranes were washed a final time and chemiluminescent substrate added. Arrays were imaged and analyzed on a BioRad ChemiDoc XLS using Quantity One software (BioRad, Hercules, CA).

Statistics. All statistics were performed using Prism 4.0 (GraphPad Software, San Diego California) by One-Way ANOVA and Dunnett post tests for virus replication experiments, and student's T-test for cytokine expression.

Results

Bortezomib inhibits HRSV replication in Vero cells. Based on the inhibitory effects of MG-132 seen in our previous report (Lupfer and Pastey, 2009), we tested the ability of the FDA approved proteasome inhibitor bortezomib (Velcade®, PS-341) to inhibit HRSV replication in vivo. We chose bortezomib because it is a FDA approved drug and

data is available for toxicity and efficacy in mice (Adams et al., 1999; Mortenson et al., 2004). In addition, it is a far more specific and potent inhibitor of the proteasome than MG-132 (Neznanov et al., 2008). As a preliminary test, we infected Vero cells with 1.0 MOI HRSV A16 2000 clinical isolate and treated cells at 4 hours post infection with various concentrations of bortezomib or 0.02% DMSO as a control. Our data show that treatment of Vero cells with as little as 20ng/mL had a significant inhibitory effect on HRSV (Figure 4.1A). Treatment with 40ng/mL bortezomib resulted in a 2.8 $\log_{10}$ (95% Confidence Interval (CI) from 1.7 to 3.9 $\log_{10}$) decrease in virus titer measured at 40 hours post infection.

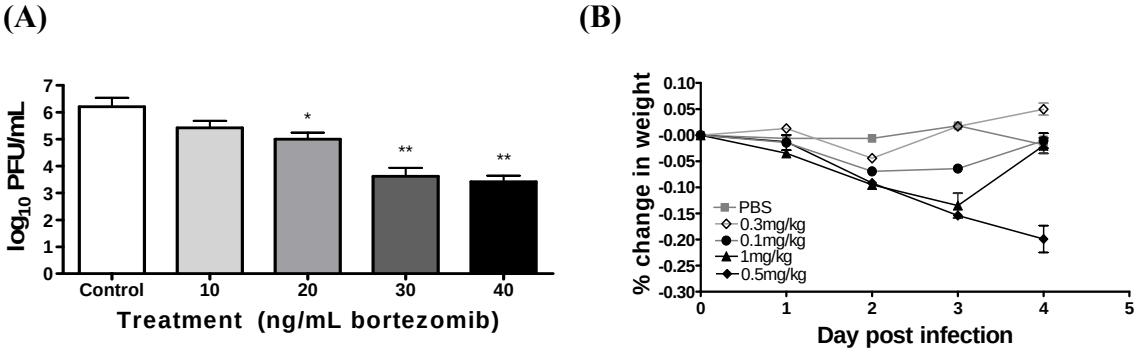


Figure 4.1: Efficacy of bortezomib in culture and preliminary in vivo toxicity. (A) Vero cells were infected with RSV A16 2000 and treated at 4 hours post infection with various concentrations of bortezomib. Samples were collected at 40 hours post infection for titration of virus by plaque assay. (B) Weight loss as a measure of toxicity from bortezomib administered IN to female Balb/C mice. PBS with 3.2% DMSO control, 1.0, 0.5 and 0.1mg/kg doses were administered twice, 24 hours apart. 0.3mg/kg dose was administered once at 0 hour. (*P<0.05, **P<0.01 by One-way ANOVA with Dunette post test)

Treatment of RSV-infected mice with Bortezomib. We next tested the toxicity of bortezomib when delivered by intranasal administration (IN) in Balb/C mice. Based on

weight loss (Figure 3.1B) and malaise with ruffled fur (data not shown), we determined there is some toxicity associated with a two dose regimen, 24 hours apart, using 1.0mg/kg or .5mg/kg. However, treatment with a single dose of 0.3mg/kg or a double dose of 0.1mg/kg , 24 hours apart, showed no significant weight loss or signs of illness (Figure 4.1B), nor was there any sign of inflammation when lungs were examined histopathologically (Figure 4.3A and 4.3B panels 1-3).

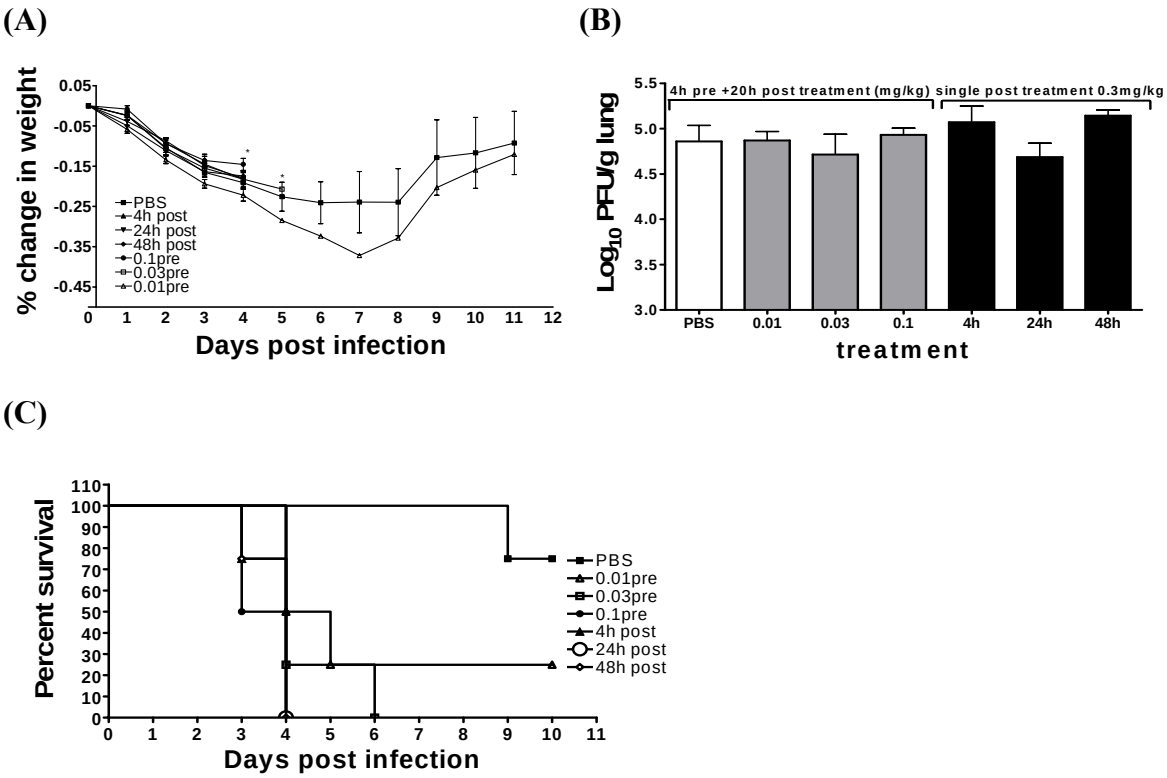


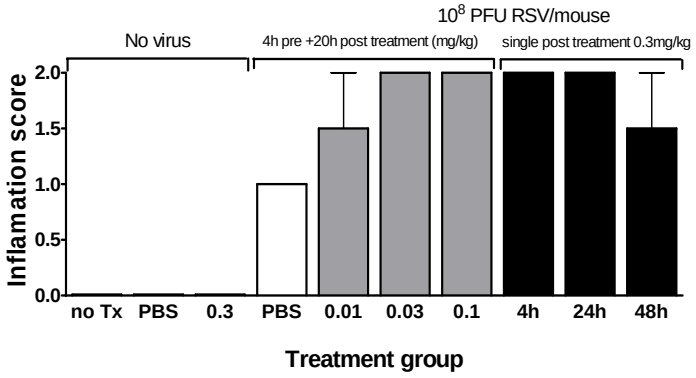
Figure 4.2: In-vivo effects of Bortezomib on RSV infection. Seven mice per group were infected with 10^8 PFU of RSV A16 2000 and treated with bortezomib or PBS with 3.2% DMSO control both 4 hours pre infection and 20 hours post infection (PBS, 0.1, 0.03 or 0.01mg/kg), or at a single time point post infection 4, 24 or 48 hours (0.3mg/kg). Mice were weighed daily and three mice were sacrificed on day four for lung virus titration by plaque assay. (A) Mouse weight loss (B) and virus titer show no improvement of treated animals over controls. (C) Survivorship shows that bortezomib treated infected mice had increased mortality compared with controls. (* all animals in these groups died before the end of the study)

For efficacy of bortezomib, 7 groups of 7 mice each were infected with 10^8 PFU RSV A16 2000 and treated with bortezomib. Four of the groups were treated 4 hours before infection and 20 hours post infection with 0.01, 0.03, or 0.1mg/kg of bortezomib or a PBS control containing 3.2% DMSO. The remaining 3 groups were treated with a single dose of 0.3mg/kg bortezomib at 4, 24, or 48 hours post infection, respectively. Three mice from each group were sacrificed on day 4 post infection for determination of lung virus titer and histopathological examination. We observed no significant changes in weight loss (Figure 4.2A) or virus titer (Figure 4.2B) in any of the bortezomib treated groups compared to the PBS control. In fact, we observed a significant increase in mortality associated with bortezomib treatment (Figure 4.2C). Based on histopathological examination, bortezomib treated mice had an increase in neutrophil and macrophage infiltration, as well as a general increase in inflammation (Figure 4.3A and 4.3B panels 4-6). HRSV infected PBS control treated mice were scored as having minimal, multifocal, suppurative, bronchopneumonia with intra-alveolar neutrophils present. Whereas bortezomib treated groups were almost exclusively scored as having moderate, diffuse, fibrinosuppurative, bronchopneumonia with intra-alveolar and interstitial neutrophils and macrophages.

We repeated our experiment using a lower infectious dose of 10^7 PFU RSV A16 2000 and delivering 1.0mg/kg bortezomib by intraperitoneal injection (a dose and delivery route used during development of bortezomib to inhibit tumor growth in mice (Teicher et al., 1999)). Though this dose and administration route had no negative effect

in uninfected control mice, it produced similar results to the intranasal treatment route and resulted in 100% mortality by day four post infection (data not shown).

(A)



(B)

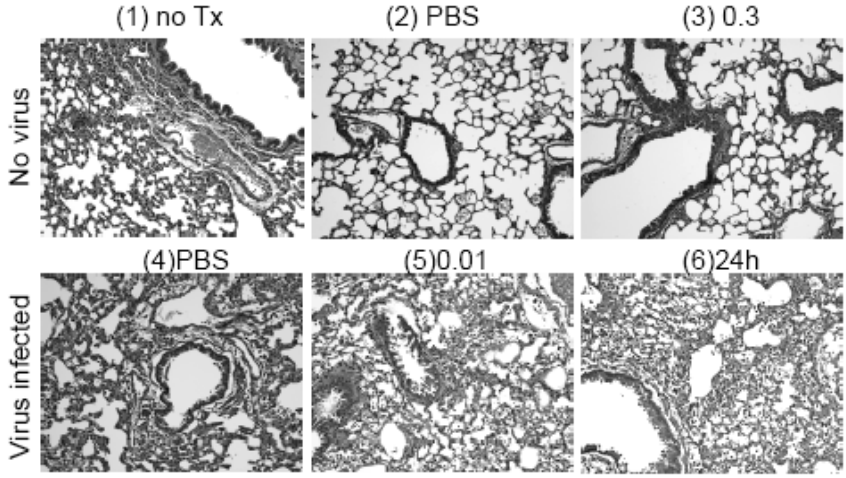


Figure 4.3: Histopahtological examination of lungs from mice. Lungs biopsies from bortezomib treated or PBS with 3.2% DMSO control treated mice were taken on day 4 post infection or post treatment initiation. (A)Average histopathological score shows that bortezomib treated mice had increased inflammation compared to controls only in HRSV infected animals but not in uninfected animals. (B) Photomicrographs of lungs from uninfected (panels 1-3) and infected (panels 4-6) animals treated with bortezomib or PBS with 3.2% DMSO control. n=2mice per group.

Bortezomib treatment during infection results in increased cytokine levels in lung tissue. Due to the increased inflammation seen in the lungs we examined the level of 22

cytokines in lung homogenates from infected and control or bortezomib treated mice by membrane based antibody array. There was a significant decrease in total IL-12 (1.8-2.3 fold reduction), SCF (1.1-3.6 fold reduction) and TNF-α (2.4-30.8 fold reduction) for at least two of the three treatment groups analyzed (Figure 4.4). We observed a significant increase in the level of several factors involved in granulocyte and macrophage proliferation and chemotaxis including GCSF (7.4-11.8 fold increase) in all bortezomib treatment groups and MCP-1 (1.4-2.3 fold increase) in both groups receiving 0.3mg/kg bortezomib. In addition, we also observed a significant increase in IL-6 (2.5-9.3 fold increase) in two of the three treatment groups (Figure 4.4). These results indicate that bortezomib may alter the inflammatory response during HRSV infection.

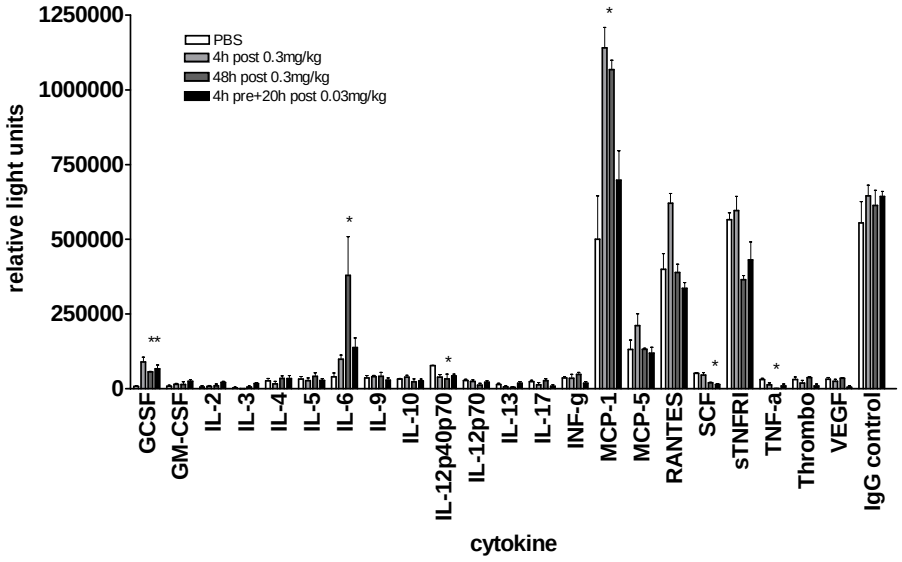


Figure 4.4: Lung cytokine levels
Mouse lung homogenates obtained from biopsies on day 4 post infection were used to determine the level of 22 cytokines simultaneously by a membrane based antibody array for groups infected with HRSV and treated with bortezomib or PBS with 3.2% DMSO control. Data is displayed as background subtracted densitometry (mean ±s.d.) (*P<0.05 for two of three treatment groups, **P<0.05 for all three treatment groups by students T-test; n=3 for all groups except 48h n=2)

Discussion

Based on our results obtained previously with MG-132 (Lupfer and Pastey, 2009), we examined the ability of bortezomib to inhibit HRSV replication in culture and in an animal model. An antiviral targeting a cellular protein required for virus replication is desirable as targeting host factors involved in virus infection may provide efficient inhibition without the appearance of resistance (Müller and Kräusslich, 2009). This method appears logical, as the host is not under selective pressure to mutate from the antiviral therapy, or mutates far more slowly, and resistance in the virus could therefore only develop by utilization of an alternative host factor. As intuitive as this approach may seem, it has been fraught with difficulties and setbacks. Recently for influenza, it was shown that treatment with alpha interferon afforded protection against a seasonal human H1N1 strain but was ineffective against a highly pathogenic H5N1 strain (Kugel et al., 2009). In HRSV, therapies have been tested using bronchodilators or anti-inflammatories to alleviate airway constriction and wheezing with little to no clinical benefit demonstrated (Almanzar et al., 2007; Ermers et al., 2009).

We observed here that treatment of uninfected mice with bortezomib at doses from 0.01-0.3mg/kg was not toxic (Figure 4.1B). However, treatment of HRSV infected mice, even at 0.01mg/kg, resulted in increased mortality and had no effect on virus growth (Figure 4.2B-C). In addition, treatment with bortezomib exacerbated the inflammation seen in infected mice (Figure 4.3A-B and 4.4).

Proteasome inhibitors have known immunomodulatory properties. MG-132 was shown to increase COX2 expression in an NF-κB independent NF-IL6 and CRE dependant fashion (Chen et al., 2005). Bortezomib is known to increase sensitivity to

apoptosis by TNF stimulation and the unfolded protein response pathway (Nowis et al., 2007). However, most of the known effects of bortezomib appear to be anti-inflammatory, such as decreased NF-κB activation (Kalogeris et al., 1999). Bortezomib was shown to modulate the immune response by altering the activity of CD4+ T cells and decreasing expression of TNF-α, IFN-γ, IL-4, and IL-5 (Berges et al., 2008). Although we observed a decrease in TNF-α, as well as total IL-12 and SCF in most treatment groups, we did not observe a decrease in IFN-γ, IL-4, or IL-5. In addition, the results of (Nencioni et al., 2006) showed that bortezomib treatment of dendritic cells reduced expression of MCP-1, whereas we observed a significant increase in the level of this chemokine in both groups receiving 0.3mg/kg bortezomib. Finally, all bortezomib treated mice had significant increases in GCSF.

Further research is needed to understand how bortezomib modulated the immune response and lung pathology during HRSV infection. A possible explanation may lie in how HRSV activates inflammation. It has been shown that treatment of HRSV infected cells with MG-132 had very little effect on NF-κB signaling due to the ability of HRSV to induce NF-κB signaling independent from the amount of IκBα present (Bitko et al., 2007; Fiedler and Wernke-Dollries, 1999). We propose that the increase in MCP-1 and GCSF resulted in increased macrophage and neutrophil infiltration into the lungs and resulted in airway constriction and respiratory failure. Increased levels of IL-6, which is involved in thermo regulation of the host, the acute phase protein response, and B cell maturation, may also have played a role in the disease severity. These results indicate the need for continued research and reporting for bortezomib, and caution during its administration if recipients show signs of a respiratory tract infection.

We demonstrated here that the ubiquitin-proteasome pathway is necessary for efficient replication of HRSV in Vero cell culture. However, inhibition of HRSV infection in vivo is not possible with currently available proteasome inhibitors due to toxic side effects. It is possible that a more targeted approach to inhibiting this pathway would prove beneficial. Discovery of other ubiquitin-proteasome pathway proteins such as E3 ubiquitin ligases or E2 ubiquitin conjugating enzymes involved in HRSV replication may provide more specific targets for drug development that would have fewer side effects.

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Chapter 5:

General Conclusion

The hypothesis for my dissertation research is that targeted development of drugs against influenza A virus and RSV, or their cellular interacting partners, would be more rapid and effective than conventional drug discovery methods of screening thousands of compounds. I hypothesized that the time to discovery of lead compounds would be reduced in the case of targeted drug development and that the viruses would be less likely to develop resistance when targeting cellular factor in place of viral.

Although we were able to rapidly design and test the PPMO anitsense compounds, there was limited clinical usefulness for these drugs as they had to be administered by 2 hours post infection to observe an effect on virus replication. The average person does not show signs of illness until ~48 hours after infection; PPMO could therefore only be used in a prophylactic manner. The existence of virus strains which were naturally resistant to the PPMO also calls into question their utility. However, no reports currently exist where a virus has shown resistance to both PB1-AUG and NP-v3' PPMO, and the treatment of patients with PPMO targeting multiple sites simultaneously could avoid the development of resistance or the inability to treat a naturally occurring variant. Clearly there is additional research which needs to be performed on influenza A to understand why subsequent rounds of replication are not inhibited by the PPMO and why treatment later in infection is not effective. One possibility is that influenza infection alters the uptake of the PPMO. It has been proposed that the arginine rich peptide which mediated cellular entry may do so by receptor mediated endocytosis (Abes et al., 2006). It is conceivable that the receptor or the

endocytic pathway is somehow perturbed during influenza infection and therefore limits drug availability once infection has occurred. However, there is also recent evidence indicating that the PPMO may enter cells by passing through the cell membrane (Fretz et al., 2007) in which case cell entry would not likely be the cause of diminished efficacy during post infection treatment regimes.

Targeting RSV replication by inhibiting the ubiquitin/proteasome pathway was rapid in development only due to the preexistence of the inhibitors; however, preexisting inhibitors to many cellular pathways are available and the key is knowing which pathway to target. Treatment in cell culture effectively inhibited virus replication and virion maturation when administered by 14 hours post infection. However, our lack of understanding of the complexities of the ubiquitin/proteasome pathway and its role in RSV infection lead to disastrous results in vivo. Despite a lack of toxicity on its own, bortezomib treatment during infection with RSV resulted in near 100% mortality in all treatment groups. As discussed in chapter 3, it appears that bortezomib altered the immune response to infection which resulted in increased lung damage and death. This result was surprising, as most data indicates that bortezomib is anti-inflammatory (Berges et al., 2008; Nencioni et al., 2006; Nowis et al., 2007). Due too the negative results obtained in vivo, we never tested the ability of RSV to develop resistance to this treatment, but continue to hypothesize that resistance would be unlikely as it would require evolution of an alternative interaction with cellular proteins performing a similar function as ubiquitin. This would not involve one or two point mutations; rather, it would likely require the acquisition of a new domain for the matrix protein.

My results also indicate that it is possible to observe what appears to be a decrease in budding efficiency based on western blot protein levels; however, the true culprit is not a decrease in budding but a decrease in virus and/or cellular protein levels which reduces mature virion formation. There are many points in virus egress which, when inhibited, would yield similar results by western blot examination of cell and virion associated virus protein levels. For example, inhibition of trafficking to the cell membrane of viral proteins or nucleocapsid packaging would also reduce the apparent budding efficiency if only examined by western blot. The best test of a proteasome inhibitor, or any other perturbation of the cell, on a virus' ability to bud is to examine cells and viruses by electron or fluorescence microscopy for the presence of increased numbers of virions tethered to the cell membrane (Gottwein et al., 2006; Martin-Serrano et al., 2004; Taylor et al., 2007; Zhadina et al., 2007). It is therefore possible that ubiquitin and the proteasome pathway do not affect the ability of some viruses to bud, but alternatively affect protein synthesis (Neznanov et al., 2008) or other earlier processes involved in virus egress like trafficking and packaging.

The results of my research indicate that using targeted drug development can be rapid (18-24 months from design to the completion of lead compound development in an animal model, compared to ~3.5 years for standard development). However, the results do not support my hypothesis that they will be more effective. I must therefore reject this hypothesis with one caveat. The amount of information available when performing targeted drug development is the key to utilizing this route to drug design. In the case of oseltamivir, highly specific structural information of the active site in complex with an antagonist provided sufficient information for rational design of this drug (Bossart-

Whitaker et al., 1993). In the case of the ubiquitin/proteasome pathway, it functions in cell signaling, protein turnover, cell cycle progression, apoptosis, the immune response, and potentially many others. Understanding all possible effects of inhibiting this pathway is therefore unlikely. As state previously, it may be possible, with further research, to discover the E2 ubiquitin conjugating enzyme(s) or E3 ubiquitin ligase(s) involved in the RSV lifecycle, and target these interactions with greater specificity and fewer unexpected and undesirable side effects. Based on my results I hypothesize that targeted drug discovery or targeting cellular factors can be effective when sufficient information is available to understand the pathways involved and accurately and selectively inhibit them.

Bibliography

Abe, T., Mizuta, T., Hatta, T., Miyano-Kurosaki, N., Fujiwarac, M., Takaia, K., Shigetad, S., Yokotac, T. and Takakua, H. (2001) Antisense therapy of influenza. Eur J Pharm Sci 13, 61-69.

Abes, R., Moulton, H.M., Clair, P., Yang, S.-T., Abes, S., Melikov, K., Prevot, P., Youngblood, D.S., Iversen, P.L., Chernomordik, L.V. and Lebleu, B. (2008) Delivery of steric block morpholino oligomers by (R-X-R)4 peptides: structure-activity studies. Nucl. Acids Res. 36(20), 6343-6354.

Abes, S., Moulton, H.M., Clair, P., Prevot, P., Youngblood, D.S., Wu, R.P., Iversen, P.L. and Lebleu, B. (2006) Vectorization of morpholino oligomers by the (R-Ahx-R)4 peptide allows efficient splicing correction in the absence of endosomolytic agents. Journal of Controlled Release 116(3), 304-313.

Adams, J., Palombella, V.J., Sausville, E.A., Johnson, J., Destree, A., Lazarus, D.D., Maas, J., Pien, C.S., Prakash, S. and Elliott, P.J. (1999) Proteasome Inhibitors: A Novel Class of Potent and Effective Antitumor Agents. Cancer Res 59(11), 2615-2622.

Al Faress, S., Ferraris, O., Moules, V., Valette, M., Hay, A. and Lina, B. (2008) Identification and characterization of a late AH1N2 human reassortant in France during the 2002-2003 influenza season. Virus Research 132(1-2), 33-41.

Almanzar, G., Herndler-Brandstetter, D., Chaparro, S.V., Jenewein, B., Keller, M. and Grubeck-Loebenstein, B. (2007) Immunodominant peptides from conserved influenza proteins--a tool for more efficient vaccination in the elderly? Wien Med Wochenschr. 157(5-6), 116-21.

Amantana, A., Moulton, H., Cate, M., Reddy, M., Whitehead, T., Hassinger, J., Youngblood, D. and Iversen, P. (2007) Pharmacokinetics, biodistribution, stability and toxicity of a cell-penetrating peptide-morpholino oligomer conjugate. Bioconjug Chem 18(4), 1325-31.

Andrejeva, J., Young, D.F., Goodbourn, S. and Randall, R.E. (2002) Degradation of STAT1 and STAT2 by the V Proteins of Simian Virus 5 and Human Parainfluenza Virus Type 2, Respectively: Consequences for Virus Replication in the Presence of Alpha/Beta and Gamma Interferons. J. Virol. 76(5), 2159-2167.

Animal Health Australia. (2007) Disease strategy: Equine influenza (Version 3.0). Australian Veterinary Emergency Plan (AUSVETPLAN), Edition 3, Primary Industries Ministerial Council, Canberra, ACT.

Aoyagi, M., Shimojo, N., Sekine, K., Nishimuta, T. and Kohno, Y. (2003) Respiratory syncytial virus infection suppresses IFN-gama production of gama-delta T cells. Clinical & Experimental Immunology 131(2), 312-317.

Arnold, R., König, B., Werchau, H. and König, W. (2004) Respiratory syncytial virus deficient in soluble G protein induced an increased proinflammatory response in human lung epithelial cells. Virology 330(2), 384-397.

Arseneault, D., Maghni, K. and Sirois, P. (1999) Selective inflammatory response induced by intratracheal and intravenous administration of poly-L-arginine in guinea pig lungs. Inflammation 23(3), 287-304.

Baz, M., Abed, Y., McDonald, J. and Boivin, G. (2006) Characterization of Multidrug-Resistant Influenza A/H3N2 Viruses Shed during 1 Year by an Immunocompromised Child. Clin. Infect. Dis. 43, 1555–61.

Becker, Y. (2006) Respiratory syncytial virus (RSV) evades the human adaptive immune system by skewing the Th1/Th2 cytokine balance toward increased levels of Th2 cytokines and IgE, markers of allergy—a review. Virus Genes 33(2), 235-252.

Belser, J.A., Lu, X., Maines, T.R., Smith, C., Li, Y., Donis, R.O., Katz, J.M. and Tumpey, T.M. (2007) Pathogenesis of avian influenza (H7) virus infection in mice and ferrets: enhanced virulence of eurasian H7N7 viruses isolated from humans. J Virol 81, 11139-47.

Bergelson, L.D., Bukrinskaya, A.G., Prokazova, N.V., Shaposhnikova, G.I., Kocharov, S.L., Shevchenko, V.P., Kornilaeva, G.V. and Fomina-Ageeva, E.V. (1982) Role of Gangliosides in Reception of Influenza Virus. Eur. J. Biochem. 128(2-3), 467-474.

Berges, C., Haberstock, H., Fuchs, D., Miltz, M., Sadeghi, M., Opelz, G., Daniel, V. and Naujokat, C. (2008) Proteasome inhibition suppresses essential immune functions of human CD4+ T cells. Immunology 124(2), 234-246.

Bermingham, A. and Collins, P.L. (1999) The M2-2 protein of human respiratory syncytial virus is a regulatory factor involved in the balance between RNA replication and transcription. PNAS 96(20), 11259-11264.

Bitko, V., Shulyayeva, O., Mazumder, B., Musiyenko, A., Ramaswamy, M., Look, D.C. and Barik, S. (2007) Nonstructural Proteins of Respiratory Syncytial Virus Suppress Premature Apoptosis by an NF-{kappa}B-Dependent, Interferon-Independent Mechanism and Facilitate Virus Growth. J. Virol. 81(4), 1786-1795.

Blackburne, B.P., Hay, A.J. and Goldstein, R.A. (2008) Changing Selective Pressure during Antigenic Changes in Human Influenza H3. PLoS Pathog 4(5), e1000058.

Boeckh, M., Englund, J., Li, Y., Miller, C., Cross, A., Fernandez, H., Kuypers, J., Kim, H., Gnann, J. and Whitley, R. (2007) Randomized Controlled Multicenter Trial of Aerosolized Ribavirin for Respiratory Syncytial Virus Upper Respiratory Tract Infection in Hematopoietic Cell Transplant Recipients. Clinical Infectious Diseases 44(2), 245-249.

Boelen, A., Andeweg, A., Kwakkel, J., Lokhorst, W., Bestebroer, T., Dormans, J. and Kimman, T. (2000) Both immunisation with a formalin-inactivated respiratory syncytial virus (RSV) vaccine and a mock antigen vaccine induce severe lung pathology and a Th2 cytokine profile in RSV-challenged mice. Vaccine 19(7-8), 982-991.

Bossart-Whitaker, P., Carson, M., Babu, Y.S., Smith, C.D., Laver, W.G. and Air, G.M. (1993) Three-dimensional Structure of Influenza A N9 Neuraminidase and Its Complex with the Inhibitor 2-Deoxy 2,3-Dehydro-N-Acetyl Neuraminic Acid. Journal of Molecular Biology 232(4), 1069-1083.

Bossert, B., Marozin, S. and Conzelmann, K.-K. (2003) Nonstructural Proteins NS1 and NS2 of Bovine Respiratory Syncytial Virus Block Activation of Interferon Regulatory Factor 3. J. Virol. 77(16), 8661-8668.

Brett, I.C. and Johansson, B.E. (2005) Immunization against influenza A virus: Comparison of conventional inactivated, live-attenuated and recombinant

baculovirus produced purified hemagglutinin and neuraminidase vaccines in a murine model system. Virology 339, 273 – 280.

Brock, S.C., Goldenring, J.R. and Crowe, J.E. (2003) Apical recycling systems regulate directional budding of respiratory syncytial virus from polarized epithelial cells. Proceedings of the National Academy of Sciences of the United States of America 100(25), 15143-15148.

Bukrinskaya, A.G., Vorkunova, N.K. and Pushkarskaya, N.L. (1982) Uncoating of a Rimantadine-resistant Variant of Influenza Virus in the Presence of Rimantadine. J. Gen. Virol. 60(1), 61-66.

Bullido, R., Gomez-Puertas, P., Albo, C. and Portela, A. (2000) Several protein regions contribute to determine the nuclear and cytoplasmic localization of the influenza A virus nucleoprotein. J Gen Virol 81(1), 135-142.

Burrer, R., Neuman, B.W., Ting, J.P., Stein, D.A., Moulton, H.M., Iversen, P.L., Kuhn, P. and Buchmeier, M.J. (2007) Antiviral effects of antisense morpholino oligomers in murine coronavirus infection models. J Virol 81, 5637-48.

Bush, K.T., Goldberg, A.L. and Nigam, S.K. (1997) Proteasome Inhibition Leads to a Heat-shock Response, Induction of Endoplasmic Reticulum Chaperones, and Thermotolerance. J. Biol. Chem. 272(14), 9086-9092.

California Biomedical Research Association. (2009) Fact Sheet: New Drug Development Process. www.ca-biomed.org/pdf/mediakit/CBRADrugDevelop.pdf(accessed on May 28, 2009).

Cambonie, G., Milési, C., Jaber, S., Amsallem, F., Barbotte, E., Picaud, J.-C. and Matecki, S. (2008) Nasal continuous positive airway pressure decreases respiratory muscles overload in young infants with severe acute viral bronchiolitis. Intensive Care Medicine 34(10), 1865-1872.

Castilow, E.M., Meyerholz, D.K. and Varga, S.M. (2008) IL-13 Is Required for Eosinophil Entry into the Lung during Respiratory Syncytial Virus Vaccine-Enhanced Disease. J Immunol 180(4), 2376-2384.

CDC. (2008) Interim within-season estimate of the effectiveness of trivalent inactivated influenza vaccine--Marshfield, Wisconsin, 2007-08 influenza season. MMWR 57(15), 393-8.

CDC. (2009a) 2008-2009 Influenza Season, Week 17, ending May 2, 2009

Flu View http://www.cdc.gov/h1n1flu/pubs/pdf/FluView_Week17.pdf(accessed May 27, 2009).

CDC. (2009b) Interim Guidance on Antiviral Recommendations for Patients with Novel Influenza A (H1N1) Virus Infection and Their Close Contacts. May 6, 2009 http://www.cdc.gov/h1n1flu/recommendations.htm(accessed May 27, 2009).

Chapman, J., Abbott, E., Alber, D.G., Baxter, R.C., Bithell, S.K., Henderson, E.A., Carter, M.C., Chambers, P., Chubb, A., Cockerill, G.S., Collins, P.L., Dowdell, V.C.L., Keegan, S.J., Kelsey, R.D., Lockyer, M.J., Luongo, C., Najarro, P., Pickles, R.J., Simmonds, M., Taylor, D., Tyms, S., Wilson, L.J. and Powell, K.L. (2007) RSV604, a Novel Inhibitor of Respiratory Syncytial Virus Replication. Antimicrob. Agents Chemother. 51(9), 3346-3353.

Chen, B.J. and Lamb, R.A. (2008) Mechanisms for enveloped virus budding: Can some viruses do without an ESCRT? Virology 372(2), 221-232.

Chen, B.J., Leser, G.P., Morita, E. and Lamb, R.A. (2007) Influenza Virus Hemagglutinin and Neuraminidase, but Not the Matrix Protein, Are Required for Assembly and Budding of Plasmid-Derived Virus-Like Particles. J. Virol. 81(13), 7111-7123.

Chen, C.-H., Lin, Y.-T., Yang, Y.-H., Wang, L.-C., Lee, J.-H., Kao, C.-L. and Chiang, B.-L. (2008) Ribavirin for respiratory syncytial virus bronchiolitis reduced the risk of asthma and allergen sensitization. Pediatric Allergy and Immunology 19(2), 166-172.

Chen, J.-J., Huang, W.-C. and Chen, C.-C. (2005) Transcriptional Regulation of Cyclooxygenase-2 in Response to Proteasome Inhibitors Involves Reactive Oxygen Species-mediated Signaling Pathway and Recruitment of CCAAT/Enhancer-binding Protein and REB-binding Protein. Molecular Biology of the Cell 16, 5579–5591.

Chen, J. and Deng, Y.-M. (2009) Influenza virus antigenic variation, host antibody production and new approach to control epidemics. Virology J. 6(1), 30.

Choudhary, S., Boldogh, S., Garofalo, R., Jamaluddin, M. and Brasier, A.R. (2005) Respiratory Syncytial Virus Influences NF-{kappa}B-Dependent Gene Expression through a Novel Pathway Involving MAP3K14/NIK Expression and Nuclear Complex Formation with NF-{kappa}B2. J. Virol. 79(14), 8948-8959.

Chutinimitkul, S., Songserm, T., Amonsin, A., Payungporn, S., Suwannakarn, K., Damrongwatanapokin, S., Chaisingh, A., Nuansrichay, B., Chieochansin, T., Theamboonlers, A. and Poovorawan, Y. (2007) New Strain of Influenza A Virus (H5N1), Thailand. Emerg. Infect. Dis. 13(3), 506-507.

Cohen, S., Zanni, R., Cohen, A., Harrington, M., VanVeldhuisen, P., Boron, M. and for the Palivizumab Outcomes Registry, G. (2008) Palivizumab Use in Subjects with Congenital Heart Disease. Pediatric Cardiology 29(2), 382-387.

Cowton, V.M., McGivern, D.R. and Fearns, R. (2006) Unravelling the complexities of respiratory syncytial virus RNA synthesis. J. Gen. Virol. 87(7), 1805-1821.

Crawford, P.C., Dubovi, E.J., Castleman, W.L., Stephenson, I., Gibbs, E.P.J., Chen, L., Smith, C., Hill, R.C., Ferro, P., Pompey, J., Bright, R.A., Medina, M.-J., Group, I.G., Johnson, C.M., Olsen, C.W., Cox, N.J., Klimov, A.I., Katz, J.M. and Donis, R.O. (2005) Transmission of Equine Influenza Virus to Dogs. Science 310, 482-485.

de Jong, M.D., Cam, B.V., Qui, P.T., Hien, V.M., Thanh, T.T., Hue, N.B., Beld, M., Phuong, L.T., Khanh, T.H., Chau, N.V.V., Hien, T.T., Ha, D.Q. and Farrar, J. (2005a) Fatal Avian Influenza A (H5N1) in a Child Presenting with Diarrhea Followed by Coma. New Eng. J. Med. 352, 686-691.

de Jong, M.D., Thanh, T.T., Khanh, T.H., Hien, V.M., Smith, G.J.D., Chau, N.V., Cam, B.V., Qui, P.T., Ha, D.Q., Guan, Y., Peiris, J.S.M., Hien, T.T. and Farrar, J. (2005b) Oseltamivir Resistance during Treatment of Influenza A (H5N1) Infection. New Eng. J. Med. 353(25), 2667-72.

Deas, T.S., Bennett, C.J., Jones, S.A., Tilgner, M., Ren, P., Behr, M.J., Stein, D.A., Iversen, P.L., Kramer, L.D., Bernard, K.A. and Shi, P.Y. (2007) In vitro resistance selection and in vivo efficacy of morpholino oligomers against West Nile virus. Antimicrob Agents Chemother 51, 2470-82.

Deas, T.S., Binduga-Gajewska, I., Tilgner, M., Ren, P., Stein, D.A., Moulton, H.M., Iversen, P.L., Kauffman, E.B., Kramer, L.D. and Shi, P.Y. (2005) Inhibition of flavivirus infections by antisense oligomers specifically suppressing viral translation and RNA replication. J Virol 79, 4599-609.

DeVincenzo, J., Cehelsky, J.E., Alvarez, R., Elbashir, S., Harborth, J., Toudjarska, I., Nechev, L., Murugaiah, V., Vliet, A.V., Vaishnaw, A.K. and Meyers, R. (2008) Evaluation of the safety, tolerability and pharmacokinetics of ALN-RSV01, a novel RNAi antiviral therapeutic directed against respiratory syncytial virus (RSV). Antiviral Research 77(3), 225-231.

Dharan, N.J., Gubareva, L.V., Meyer, J.J., Okomo-Adhiambo, M., McClinton, R.C., Marshall, S.A., St. George, K., Epperson, S., Brammer, L., Klimov, A.I., Bresee, J.S., Fry, A.M. and O.R.W.G. (2009) Infections With Oseltamivir-Resistant Influenza A(H1N1) Virus in the United States. JAMA 301(10), 1034-1041.

Dias, A., Bouvier, D., Crepin, T., McCarthy, A.A., Hart, D.J., Baudin, F., Cusack, S. and Ruigrok, R.W.H. (2009) The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. Nature 458(7240), 914-918.

Didcock, L., Young, D.F., Goodbourn, S. and Randall, R.E. (1999) The V Protein of Simian Virus 5 Inhibits Interferon Signalling by Targeting STAT1 for Proteasome-Mediated Degradation. J. Virol. 73(12), 9928-9933.

Dowdle, W.R. (1999) Influenza A virus recycling revisited. Bulletin of the World Health Organization 77(10), 820-8.

Elliott, J., Lynch, O.T., Suessmuth, Y., Qian, P., Boyd, C.R., Burrows, J.F., Buick, R., Stevenson, N.J., Touzelet, O., Gadina, M., Power, U.F. and Johnston, J.A. (2007) Respiratory Syncytial Virus NS1 Protein Degrades STAT2 by Using the Elongin-Cullin E3 Ligase. JOURNAL OF VIROLOGY 81(7), 3428–3436.

Ermers, M.J.J., Rovers, M.M., van Woensel, J.B., Kimpen, J.L.L., Bont, L.J. and R.S.V.C.S.G. (2009) The effect of high dose inhaled corticosteroids on wheeze in infants after respiratory syncytial virus infection: randomised double blind placebo controlled trial. BMJ 338(mar31_2), b897-.

Falsey, Ann R., Walsh, Edward E., Capellan, J., Gravenstein, S., Zambon, M., Yau, E., Gorse, Geoffrey J., Edelman, R., Hayden, Frederick G., McElhaney, Janet E., Neuzil, Kathleen M., Nichol, Kristen L., Simoes, Eric A.F., Wright, Peter F. and Sales, Valerie M.-P. (2008) Comparison of the Safety and Immunogenicity of 2 Respiratory Syncytial Virus (RSV) Vaccines- Nonadjuvanted Vaccine or Vaccine Adjuvanted with Alum- Given Concomitantly with Influenza Vaccine to High-Risk Elderly Individuals. The Journal of Infectious Diseases 198(9), 1317-1326.

Fiedler, M.A. and Wernke-Dollries, K. (1999) Incomplete Regulation of NF-kappa B by Ikappa Balpha during Respiratory Syncytial Virus Infection in A549 Cells. J. Virol. 73(5), 4502-4507.

Fink, K., Duval, A., Martel, A., Soucy-Faulkner, A. and Grandvaux, N. (2008) Dual Role of NOX2 in Respiratory Syncytial Virus- and Sendai Virus-Induced Activation of NF-{kappa}B in Airway Epithelial Cells. J Immunol 180(10), 6911-6922.

Fretz, M.M., Penning, N.A., Al-taei, S., Futaki, S., Takeuchi, T., Nakase, I., Storm, G. and Jones, A.T. (2007) Temperature-, concentration- and cholesterol-dependent

translocation of L- and D-octa-arginine across the plasma and nuclear membrane of CD34+ leukaemia cells. Biochem. J. 403(2), 335-342.

Fried, M.W., Shiffman, M.L., Reddy, K.R., Smith, C., Marinos, G., Goncales, F.L., Jr., Haussinger, D., Diago, M., Carosi, G., Dhumeaux, D., Craxi, A., Lin, A., Hoffman, J. and Yu, J. (2002) Peginterferon Alfa-2a plus Ribavirin for Chronic Hepatitis C Virus Infection. N Engl J Med 347(13), 975-982.

Ge, Q., Filip, L., Bai, A., Nguyen, T., Eisen, H.N. and Chen, J. (2004) Inhibition of influenza virus production in virus-infected mice by RNA interference. PNAS 101(32), 8676–8681.

Ge, Q., Pastey, M., Kobasa, D., Puthavathana, P., Lupfer, C., Bestwick, R.K., Iversen, P.L., Chen, J. and Stein, D.A. (2006) Inhibition of Multiple Subtypes of Influenza A Virus in Cell Cultures with Morpholino Oligomers. Antimcrob. Agents Chemother. 50(11), 3724–3733.

Ghildyal, R., Ho, A. and Jans, D.A. (2006) Central role of the respiratory syncytial virus matrix protein in infection. FEMS Microbiol. Rev. 30(5), 692-705.

Ghildyal, R., Li, D., Peroulis, I., Shields, B., Bardin, P.G., Teng, M.N., Collins, P.L., Meanger, J. and Mills, J. (2005) Interaction between the respiratory syncytial virus G glycoprotein cytoplasmic domain and the matrix protein. J Gen Virol. 86(Pt 7), 1879-84.

Ghildyal, R., Mills, J., Murray, M., Vardaxis, N. and Meanger, J. (2002) Respiratory syncytial virus matrix protein associates with nucleocapsids in infected cells. J Gen Virol. 83(Pt 4), 753-7.

Girard, M.P., Cherian, T., Pervikov, Y. and Kieny, M.P. (2005) A review of vaccine research and development: Human acute respiratory infections. Vaccine 23(50), 5708-5724.

Gonzalez-Reyes, L., Ruiz-Arguello, M.B., Garcia-Barreno, B., Calder, L., Lopez, J.A., Albar, J.P., Skehel, J.J., Wiley, D.C. and Melero, J.A. (2001) Cleavage of the human respiratory syncytial virus fusion protein at two distinct sites is required for activation of membrane fusion. PNAS 98(17), 9859-9864.

Gotoh, B., Komatsu, T., Takeuchi, K. and Yokoo, J. (2001) Paramyxovirus Accessory Proteins as Interferon Antagonists. MICROBIOLOGY and IMMUNOLOGY 45(12), 787-800.

Gotoh, B., Komatsu, T., Takeuchi, K. and Yokoo, J. (2002) Paramyxovirus strategies for evading the interferon response. Reviews in Medical Virology 12(6), 337-357.

Gottwein, E., Jager, S., Habermann, A. and Krausslich, H.-G. (2006) Cumulative Mutations of Ubiquitin Acceptor Sites in Human Immunodeficiency Virus Type 1 Gag Cause a Late Budding Defect. J. Virol. 80(13), 6267-6275.

Gould, P.S. and Easton, A.J. (2007) Coupled Translation of the Second Open Reading Frame of M2 mRNA Is Sequence Dependent and Differs Significantly within the Subfamily Pneumovirinae. J. Virol. 81(16), 8488-8496.

Govorkova, E.A., Leneva, I.A., Goloubeva, O.G., Bush, K. and Webster, R.G. (2001) Comparison of Efficacies of RWJ-270201, Zanamivir, and Oseltamivir against H5N1, H9N2, and Other Avian Influenza Viruses. Antimcrob. Agents Chemother. 45(10), 2723–2732.

Groskreutz, D.J., Monick, M.M., Yarovinsky, T.O., Powers, L.S., Quelle, D.E., Varga, S.M., Look, D.C. and Hunninghake, G.W. (2007) Respiratory syncytial virus decreases p53 protein to prolong survival of airway epithelial cells. J Immunol. 179(5), 2741-7.

Grubbs, S.T., Kania, S.A. and Potgieter, L.N.D. (2001) Validation of synthetic peptide enzyme immunoassays in differentiating two subgroups of ruminant respiratory syncytial virus. J Vet Diagn Invest 13, 123-127.

Guan, Y., Poon, L.L.M., Cheung, C.Y., Ellis, T.M., Lim, W., Lipatov, A.S., Chan, K.H., Sturm-Ramirez, K.M., Cheung, C.L., Leung, Y.H.C., Yuen, K.Y., Webster, R.G. and Peiris, J.S.M. (2004) H5N1 influenza: A protean pandemic threat. Proceedings of the National Academy of Sciences of the United States of America 101(21), 8156-8161.

Guilligay, D., Tarendeau, F., Resa-Infante, P., Coloma, R., Crepin, T., Sehr, P., Lewis, J., Ruigrok, R.W.H., Ortin, J., Hart, D.J. and Cusack, S. (2008) The structural basis for cap binding by influenza virus polymerase subunit PB2. Nat. Struct. Mol. Biol. 15(5), 500-506.

Hale, B.G., Randall, R.E., Ortin, J. and Jackson, D. (2008) The multifunctional NS1 protein of influenza A viruses. J. Gen. Virol. 89(10), 2359-2376.

Harty, R.N., Brown, M.E., McGettigan, J.P., Wang, G., Jayakar, H.R., Huibregtse, J.M., Whitt, M.A. and Schnell, M.J. (2001) Rhabdoviruses and the Cellular Ubiquitin-Proteasome System: a Budding Interaction. J. Virol. 75(22), 10623-10629.

Hayden, F. (2009) Developing New Antiviral Agents for Influenza Treatment: What Does the Future Hold? Clinical Infectious Diseases 48(s1), S3-S13.

Hayden, F.G. and Pavia, A.T. (2006) Antiviral Management of Seasonal and Pandemic Influenza. J. Infect. Dis. 194, S119–26.

Haye, K., Burmakina, S., Moran, T., Garcia-Sastre, A. and Fernandez-Sesma, A. (2009) The NS1 Protein of a Human Influenza Virus Inhibits Type I Interferon Production and the Induction of Antiviral Responses in Primary Human Dendritic and Respiratory Epithelial Cells. J. Virol. 83(13), 6849-6862.

Henderson, G., Murray, J. and Yeo, R.P. (2002) Sorting of the respiratory syncytial virus matrix protein into detergent-resistant structures is dependent on cell-surface expression of the glycoproteins. Virology. 300(2), 244-54.

Herrera, G.A., Iwane, M.K., Cortese, M., Brown, C., Gershman, K., Shupe, A., Averhoff, F., Chaves, S.S., Gargiullo, P. and Bridges, C.B. (2007) Influenza vaccine effectiveness among 50-64-year-old persons during a season of poor antigenic match between vaccine and circulating influenza virus strains: Colorado, United States, 2003-2004. Vaccine 25(1), 154-160.

Hideshima, T., Richardson, P., Chauhan, D., Palombella, V.J., Elliott, P.J., Adams, J. and Anderson, K.C. (2001) The Proteasome Inhibitor PS-341 Inhibits Growth, Induces Apoptosis, and Overcomes Drug Resistance in Human Multiple Myeloma Cells. Cancer Res 61(7), 3071-3076.

Holden, K.L., Stein, D.A., Pierson, T.C., Ahmed, A.A., Clyde, K., Iversen, P.L. and Harris, E. (2006) Inhibition of dengue virus translation and RNA synthesis by a morpholino oligomer targeted to the top of the terminal 3' stem-loop structure. Virology 344(2), 439-52.

Hudziak, R.M., Barofsky, E., Barofsky, D.F., Weller, D.L., Huang, S.B. and Weller, D.D. (1996) Resistance of morpholino phosphorodiamidate oligomers to enzymatic degradation. Antisense Nucleic Acid Drug Dev 6, 267-72.

Hui, E.K., Barman, S., Tang, D.H., France, B. and Nayak, D.P. (2006a) YRKL sequence of influenza virus M1 functions as the L domain motif and interacts with VPS28 and Cdc42. J Virol. 80(5), 2291-308.

Hui, E.K., Smee, D.F., Wong, M.H. and Nayak, D.P. (2006b) Mutations in influenza virus M1 CCHH, the putative zinc finger motif, cause attenuation in mice and protect mice against lethal influenza virus infection. J Virol. 80(12), 5697-707.

Ilyushina, N.A., Bovin, N.V., Webster, R.G. and Govorkova, E.A. (2006) Combination chemotherapy, a potential strategy for reducing the emergence of drug-resistant influenza A variants. Antiviral Res 70(3), 121-31.

Indukuri, H., Castro, S.M., Liao, S.-M., Feeney, L.A., Dorsch, M., Coyle, A.J., Garofalo, R.P., Brasier, A.R. and Casola, A. (2006) Ikkepsilon regulates viral-induced interferon regulatory factor-3 activation via a redox-sensitive pathway. Virology 353(1), 155-165.

Jager, S., Gottwein, E. and Krausslich, H.-G. (2007) Ubiquitination of Human Immunodeficiency Virus Type 1 Gag Is Highly Dependent on Gag Membrane Association. J. Virol. 81(17), 9193-9201.

Johansson, B.E. and Brett, I.C. (2007) Changing perspective on immunization against influenza. Vaccine 25, 3062–3065.

Jorba, N., Coloma, R. and Ortin, J. (2009) Genetic trans-Complementation Establishes a New Model for Influenza Virus RNA Transcription and Replication. PLoS Pathogen 5(5), e1000462.

Kalogeris, T.J., Laroux, F.S., Cockrell, A., Ichikawa, H., Okayama, N., Phifer, T.J., Alexander, J.S. and Grisham, M.B. (1999) Effect of selective proteasome inhibitors on TNF-induced activation of primary and transformed endothelial cells. Am J Physiol Cell Physiol 276(4), C856-864.

Kathleen, V. and Adrienne, R. (2007) Ribavirin for respiratory syncytial virus infection of the lower respiratory tract in infants and young children. Cochrane Database Syst Rev 4, CD000181.

Kaverin, N.V., Gambaryan, A.S., Bovin, N.V., Rudneva, I.A., Shilov, A.A., Khodova, O.M., Varich, N.L., Sinitsin, B.V., Makarova, N.V. and Kropotkina, E.A. (1998) Postreassortment Changes in Influenza A Virus Hemagglutinin Restoring HA-NA Functional Match. Virology 244(2), 315-321.

Kawasaki, Y., Hosoya, M., Katayose, M. and Suzuki, H. (2004) Role of serum neutralizing antibody in reinfection of respiratory syncytial virus. Pediat. Intern. 46(2), 126-129.

Kotelkin, A., Belyakov, I.M., Yang, L., Berzofsky, J.A., Collins, P.L. and Bukreyev, A. (2006) The NS2 Protein of Human Respiratory Syncytial Virus Suppresses the Cytotoxic T-Cell Response as a Consequence of Suppressing the Type I Interferon Response. J. Virol. 80(12), 5958-5967.

Kugel, D., Kochs, G., Obojes, K., Roth, J., Kobinger, G.P., Kobasa, D., Haller, O., Staeheli, P. and von Messling, V. (2009) Intranasal Administration of Alpha

Interferon Reduces Seasonal Influenza A Virus Morbidity in Ferrets. J. Virol. 83(8), 3843-3851.

Kuiken, T., Rimmelzwaan, G., Riel, D.v., Amerongen, G.v., Baars, M., Fouchier, R. and Osterhaus, A. (2004) Avian H5N1 Influenza in Cats. SCIENCE VOL 306 306, 241.

Li, D., Jans, D.A., Bardin, P.G., Meanger, J., Mills, J. and Ghildyal, R. (2008) Association of Respiratory Syncytial Virus M Protein with Viral Nucleocapsids Is Mediated by the M2-1 Protein. J. Virol. 82(17), 8863-8870.

Lo, M.S., Brazas, R.M. and Holtzman, M.J. (2005) Respiratory Syncytial Virus Nonstructural Proteins NS1 and NS2 Mediate Inhibition of Stat2 Expression and Alpha/Beta Interferon Responsiveness. JOURNAL OF VIROLOGY 79(14), 9315–9319.

Maamoun, Y. (2009) Egypt orders slaughter of all pigs over swine flu. The Associated Press. The Independent. http://www.independent.co.uk/news/world/middle-east/egypt-orders-slaughter-of-all-pigs-over-swine-flu-1676090.html.(accessed May 26, 2009).

Malhotra, R., Ward, M., Bright, H., Priest, R., Foster, Martyn R., Hurle, M., Blair, E. and Bird, M. (2003) Isolation and characterisation of potential respiratory syncytial virus receptor(s) on epithelial cells. Microb. Infect. 5(2), 123-133.

Manns, M.P., McHutchison, J.G., Gordon, S.C., Rustgi, V.K., Shiffman, M., Reindollar, R., Goodman, Z.D., Koury, K., Ling, M.-H. and Albrecht, J.K. (2001) Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. The Lancet 358(9286), 958-965.

Martella, V., Elia, G., Decaro, N., Trani, L.D., Lorusso, E., Campolo, M., Desario, C., Parisi, A., Cavaliere, N. and Buonavoglia, C. (2007) An outbreak of equine influenza virus in vaccinated horses in Italy is due to an H3N8 strain closely related to recent North American representatives of the Florida sub-lineage. Vet. Microbiol. 121, 56–63.

Martin-Serrano, J., Perez-Caballero, D. and Bieniasz, P.D. (2004) Context-Dependent Effects of L Domains and Ubiquitination on Viral Budding. JOURNAL OF VIROLOGY 78(11), 5554–5563.

Martinon-Torres, F., Rodriguez-Nunez, A. and Martinon-Sanchez, J.M. (2008) Nasal Continuous Positive Airway Pressure With Heliox Versus Air Oxygen in Infants With Acute Bronchiolitis: A Crossover Study. Pediatrics 121(5), e1190-1195.

Marty, A., Meanger, J., Mills, J., Shields, B. and Ghildyal, R. (2004) Association of matrix protein of respiratory syncytial virus with the host cell membrane of infected cells. Arch Virol. 149(1), 199-210. Epub 2003 Sep 19.

Matsumoto, A., Ichikawa, T., Nakao, K., Miyaaki, H., Hirano, K., Fujimito, M., Akiyama, M., Miuma, S., Ozawa, E., Shibata, H., Takeshita, S., Yamasaki, H., Ikeda, M., Kato, N. and Eguchi, K. (2009) Interferon-α-induced mTOR activation is an anti-hepatitis C virus signal via the phosphatidylinositol 3-kinase-Akt-independent pathway. J. Gastroenterology DOI 10.1007/s00535-009-0075-1.

McCullers, J.A. (2006) The Clinical Need for New Antiviral Drugs Directed against Influenza Virus. J. Infect. Dis. 193, 751–3.

McGivern, D.R., Collins, P.L. and Fearns, R. (2005) Identification of Internal Sequences in the 3' Leader Region of Human Respiratory Syncytial Virus That Enhance Transcription and Confer Replication Processivity. J. Virol. 79(4), 2449-2460.

Mortenson, M.M., Schlieman, M.G., Virudachalam, S. and Bold, R.J. (2004) Effects of the proteasome inhibitor bortezomib alone and in combination with chemotherapy in the A549 non-small-cell lung cancer cell line. Cancer Chemotherapy and Pharmacology 54(4), 343-353.

Moulton, H.M., Nelson, M.H., Hatlevig, S.A., Reddy, M.T. and Iversen, P.L. (2004) Cellular uptake of antisense morpholino oligomers conjugated to arginine-rich peptides. Bioconjug Chem 15, 290-9.

Müller, B. and Kräusslich, H.-G. (2009) Antiviral Strategies, pp. 1-24. Springer Berlin Heidelberg, New York, NY.

Nayak, E.K.-W.H.a.D.P. (2001) Role of ATP in Influenza Virus Budding. Virology 290, 329-341.

NCBI. (2004) Influenza Virus Resource. http://www.ncbi.nlm.nih.gov/genomes/FLU/(accessed on Oct 16, 2007).

Nelson, M.H., Stein, D.A., Kroeker, A.D., Hatlevig, S.A., Iversen, P.L. and Moulton, H.M. (2005) Arginine-rich peptide conjugation to morpholino oligomers: effects on antisense activity and specificity. Bioconjug Chem 16, 959-66.

Nencioni, A., Schwarzenberg, K., Brauer, K.M., Schmidt, S.M., Ballestrero, A., Grunebach, F. and Brossart, P. (2006) Proteasome inhibitor bortezomib modulates TLR4-induced dendritic cell activation. Blood 108(2), 551-558.

Neumann, G., Whitt, M.A. and Kawaoka, Y. (2002) A decade after the generation of a negative-sense RNA virus from cloned cDNA - what have we learned? J Gen Virol 83(11), 2635-2662.

Neznanov, N., Dragunsky, E.M., Chumakov, K.M., Neznanova, L., Wek, R.C., Gudkov, A.V. and Banerjee, A.K. (2008) Different Effect of Proteasome Inhibition on Vesicular Stomatitis Virus and Poliovirus Replication. PLoS ONE 3(4), e1887.

Nicholls, J.M., Chan, M.C.W., Chan, W.Y., Wong, H.K., Cheung, C.Y., Kwong, D.L.W., Wong, M.P., Chui, W.H., Poon, L.L.M., Tsao, S.W., Guan, Y. and Peiris, J.S.M. (2007) Tropism of avian influenza A (H5N1) in the upper and lower respiratory tract. Nature Med. 13(2), 147-149.

Nokes, D.J., Okiro, Emelda A., Ngama, M., Ochola, R., White, Lisa J., Scott, Paul D., English, M., Cane, Patricia A. and Medley, Graham F. (2008) Respiratory Syncytial Virus Infection and Disease in Infants and Young Children Observed from Birth in Kilifi District, Kenya. Clin. Infect. Dis. 46(1), 50-57.

Nowis, D., McConnell, E.J., Dierlam, L., Palamarchuk, A., Lass, A. and Wójcik, C. (2007) TNF potentiates anticancer activity of bortezomib (Velcade®) through reduced expression of proteasome subunits and dysregulation of unfolded protein response. International Journal of Cancer 121(2), 431-441.

Ott, D.E., Coren, L.V., Chertova, E.N., Gagliardi, T.D. and Schubert, U. (2000) Ubiquitination of HIV-1 and MuLV Gag. Virology. 278(1), 111-21.

Palese, P. and Compans, R.W. (1976) Inhibition of Influenza Virus Replication in Tissue Culture by 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA): Mechanism of Action. J. Gen. Virol. 33(1), 159-163.

Park, A.W. and Glass, K. (2007) Dynamic patterns of avian and human influenza in east and southeast Asia. The Lancet Infectious Diseases 7(8), 543-548.
Parveen, S., Broor, S., Kapoor, S.K., Fowler, K. and Sullender, W.M. (2006) Genetic diversity among respiratory syncytial viruses that have caused repeated infections in children from rural India. J. Med. Virol. 78(5), 659-665.
Pastey, M.K. and Samal, S.K. (1997) Analysis of bovine respiratory syncytial virus envelope glycoproteins in cell fusion. J Gen Virol 78, 1885-1889.
Patnaik, A., Chau, V., Li, F., Montelaro, R.C. and Wills, J.W. (2002) Budding of Equine Infectious Anemia Virus Is Insensitive to Proteasome Inhibitors. JOURNAL OF VIROLOGY 76(6), 2641–2647.
Patnaik, A., Chau, V. and Wills, J.W. (2000) Ubiquitin is part of the retrovirus budding machinery. PNAS 97(24), 13069–13074.
Peltola, Ville T., Murti, K.G. and McCullers, Jonathan A. (2005) Influenza Virus Neuraminidase Contributes to Secondary Bacterial Pneumonia. J. Infect. Dis. 192(2), 249-257.
Pinto, R.A., Arredondo, S.M., Bono, M.R., Gaggero, A.A. and Diaz, P.V. (2006) T Helper 1/T Helper 2 Cytokine Imbalance in Respiratory Syncytial Virus Infection Is Associated With Increased Endogenous Plasma Cortisol. Pediatrics 117(5), e878-886.
Portela, A. and Digard, P. (2002) The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication. J. Gen. Virol. 83(4), 723-734.
Precious, B., Childs, K., Fitzpatrick-Swallow, V., Goodbourn, S. and Randall, R.E. (2005a) Simian Virus 5 V Protein Acts as an Adaptor, Linking DDB1 to STAT2, To Facilitate the Ubiquitination of STAT1. J. Virol. 79(21), 13434-13441.
Precious, B., Young, D.F., Andrejeva, L., Goodbourn, S. and Randall, R.E. (2005b) In vitro and in vivo specificity of ubiquitination and degradation of STAT1 and STAT2 by the V proteins of the paramyxoviruses simian virus 5 and human parainfluenza virus type 2. J Gen Virol 86(1), 151-158.
Ramaswamy, M., Shi, L., Monick, M.M., Hunninghake, G.W. and Look, D.C. (2004) Specific Inhibition of Type I Interferon Signal Transduction by Respiratory Syncytial Virus. Am. J. Respir. Cell Mol. Biol. 30(6), 893-900.
Ramaswamy, M., Shi, L., Varga, S.M., Barik, S., Behlke, M.A. and Look, D.C. (2006) Respiratory syncytial virus nonstructural protein 2 specifically inhibits type I interferon signal transduction. Virology 344(2), 328-339.
Rameix-Welti, M.A., Agou, F., Buchy, P., Mardy, S., Aubin, J.T., Veron, M., Werf, S.v.d. and Naffakh, N. (2006) Natural Variation Can Significantly Alter the Sensitivity of Influenza A (H5N1) Viruses to Oseltamivir. ANTIMICROBIAL AGENTS AND CHEMOTHERAPY 50(11), 3809–3815.
Robb, N.C., Smith, M., Vreede, F.T. and Fodor, E. (2009) NS2/NEP protein regulates transcription and replication of the influenza virus RNA genome. J. Gen. Virol. 90(6), 1398-1407.
Rodriguez, L., Cuesta, I., Asenjo, A. and Villanueva, N. (2004) Human respiratory syncytial virus matrix protein is an RNA-binding protein: binding properties, location and identity of the RNA contact residues. J Gen Virol. 85(Pt 3), 709-19.

Saito, R., Li, D., Suzuki, Y., Sato, I., Masaki, H., Nishimura, H., Kawashima, T., Shirahige, Y., Shimomura, C., Asoh, N., Degawa, S., Ishikawa, H., Sato, M., Shobugawa, Y. and Suzuki, H. (2007) High Prevalence of Amantadine-Resistance Influenza A (H3N2) in Six Prefectures, Japan, in the 2005–2006 Season. J.Med. Virol. 79, 1569–1576.

Satterly, N., Tsai, P.-L., van Deursen, J., Nussenzveig, D.R., Wang, Y., Faria, P.A., Levay, A., Levy, D.E. and Fontoura, B.M.A. (2007) Influenza virus targets the mRNA export machinery and the nuclear pore complex. PNAS 104(6), 1853-1858.

Schlender, J., Bossert, B., Buchholz, U. and Conzelmann, K.-K. (2000) Bovine Respiratory Syncytial Virus Nonstructural Proteins NS1 and NS2 Cooperatively Antagonize Alpha/Beta Interferon-Induced Antiviral Response. J. Virol. 74(18), 8234-8242.

Schmitt, A.P., Leser, G.P., Morita, E., Sundquist, W.I. and Lamb, a.R.A. (2005) Evidence for a New Viral Late-Domain Core Sequence, FPIV, Necessary for Budding of a Paramyxovirus. JOURNAL OF VIROLOGY 79(5), 2988-2997.

Shields, B., Mills, J., Ghildyal, R., Gooley, P. and Meanger, J. (2003) Multiple heparin binding domains of respiratory syncytial virus G mediate binding to mammalian cells. Archives of Virology 148(10), 1987-2003.

Shinya, K., Ebina, M., Yamada, S., Ono, M., Kasai, N. and Kawaoka, Y. (2006) Influenza virus receptors in the human airway. Nature 440, 435-436.

Sidwell, R.W., Barnard, D.L., Day, C.W., Smee, D.F., Bailey, K.W., Wong, M.-H., Morrey, J.D. and Furuta, Y. (2007) Efficacy of Orally Administered T-705 on Lethal Avian Influenza A (H5N1) Virus Infections in Mice. Antimcrob. Agents Chemother. 51(3), 845–851.

Sidwell, R.W., Smee, D.F., Huffman, J.H., Barnard, D.L., Bailey, K.W., Morrey, J.D. and Babu, Y.S. (2001) In Vivo Influenza Virus-Inhibitory Effects of the Cyclopentane Neuraminidase Inhibitor RWJ-270201. Antimcrob. Agents Chemother. 45(3), 749-757.

Sigurs, N., Gustafsson, P.M., Bjarnason, R., Lundberg, F., Schmidt, S., Sigurbergsson, F. and Kjellman, B. (2005) Severe Respiratory Syncytial Virus Bronchiolitis in Infancy and Asthma and Allergy at Age 13. Am. J. Respir. Crit. Care Med. 171(2), 137-141.

Simmons, C.P., Bernasconi, N.L., Jr., A.L.S., Mills, K., Ward, J.M., Chau, N.V.V., Hien, T.T., Sallusto, F., Ha, D.Q., Farrar, J., Jong, M.D.d., Lanzavecchia, A. and Subbarao, K. (2007) Prophylactic and Therapeutic Efficacy of Human Monoclonal Antibodies against H5N1 Influenza. PLoS Medicine 4(5), 928-936.

Spann, K.M., Tran, K.-C., Chi, B., Rabin, R.L. and Collins, P.L. (2004) Suppression of the Induction of Alpha, Beta, and Gamma Interferons by the NS1 and NS2 Proteins of Human Respiratory Syncytial Virus in Human Epithelial Cells and Macrophages. JOURNAL OF VIROLOGY 78(8), 4363–4369.

Stein, D., Foster, E., Huang, S.B., Weller, D. and Summerton, J. (1997) A specificity comparison of four antisense types: morpholino, 2'-O-methyl RNA, DNA, and phosphorothioate DNA. Antisense Nucleic Acid Drug Dev 7, 151-7.

Strauss, J. and Strauss, E. (2002) Viruses and Human Disease, pp. 123-170. Academic Press, London, UK.

Stuchell, M.D., Garrus, J.E., Muller, B., Stray, K.M., Ghaffarian, S., McKinnon, R., Krausslich, H.-G., Morham, S.G. and Sundquist, W.I. (2004) The Human Endosomal Sorting Complex Required for Transport (ESCRT-I) and Its Role in HIV-1 Budding. J. Biol. Chem. 279(34), 36059-36071.

Summerton, J., Weller, D. and (1997) Morpholino antisense oligomers: design, preparation, and properties. Antisense Nucleic Acid Drug Dev 7, 187-95.

Takeuchi, K. and Lamb, R.A. (1994) Influenza virus M2 protein ion channel activity stabilizes the native form of fowl plague virus hemagglutinin during intracellular transport. J. Virol. 68(2), 911-919.

Taubenberger, J. and Morens, D. (2006) 1918 Influenza: the mother of all pandemics. Emerg Infect Dis 12(1), 15-22.

Taylor, G.M., Hanson, P.I. and Kielian, M. (2007) Ubiquitin Depletion and Dominant-Negative VPS4 Inhibit Rhabdovirus Budding without Affecting Alphavirus Budding. J. Virol. 81(24), 13631-13639.

Teicher, B.A., Ara, G., Herbst, R., Palombella, V.J. and Adams, J. (1999) The Proteasome Inhibitor PS-341 in Cancer Therapy. Clin Cancer Res 5(9), 2638-2645.

Thomas, K.W., Monick, M.M., Staber, J.M., Yarovinsky, T., Carter, A.B. and Hunninghake, G.W. (2002) Respiratory Syncytial Virus Inhibits Apoptosis and Induces NF-kappa B Activity through a Phosphatidylinositol 3-Kinase-dependent Pathway. J. Biol. Chem. 277(1), 492-501.

Thompson, W.W., Shay, D.K., Weintraub, E., Brammer, L., Cox, N., Anderson, L.J. and Fukuda, K. (2003) Mortality Associated With Influenza and Respiratory Syncytial Virus in the United States. JAMA 289(2), 179-186.

Tolley, K.P., Marriott, A.C., Simpson, A., Plows, D.J., Matthews, D.A., Longhurst, S.J., Evans, J.E., Johnson, J.L., Cane, P.A., Randolph, V.B., Easton, A.J. and Pringle, C.R. (1996) Identification of mutations contributing to the reduced virulence of a modified strain of respiratory syncytial virus. Vaccine 14(17-18), 1637-1646.

Tompkins, S.M., Lo, C.-Y., Tumpey, T.M. and Epstein, S.L. (2004) Protection against lethal influenza virus challenge by RNA interference in vivo. PNAS 101(23), 8682–8686.

Tremayne, J. (2006) Canine flu confirmed in 22states DVM Newsmagazine, Sept 21, 2007.

Ulane, C.M. and Horvath, C.M. (2002) Paramyxoviruses SV5 and HPIV2 Assemble STAT Protein Ubiquitin Ligase Complexes from Cellular Components. Virology 304(2), 160-166.

Utley, T.J., Ducharme, N.A., Varthakavi, V., Shepherd, B.E., Santangelo, P.J., Lindquist, M.E., Goldenring, J.R. and Crowe, J.E. (2008) Respiratory syncytial virus uses a Vps4-independent budding mechanism controlled by Rab11-FIP2. Proceedings of the National Academy of Sciences 105(29), 10209-10214.

Vagnozzi, A., Stein, D.A., Iversen, P.L. and E, R. (2007) Inhibition of foot-and-mouth disease virus in cell cultures with antisense morpholino oligomers. J Virol 81(21), 11669-80

van den Born, E., Stein, D.A., Iversen, P.L. and Snijder, E.J. (2005) Antiviral activity of morpholino oligomers designed to block various aspects of equine arteritis virus amplification in cell culture. J Gen Virol 86(Pt 11), 3081-90.
van Riel, D., Munster, V.J., Wit, E.d., Rimmelzwaan, G.F., Fouchier, R.A.M., Osterhaus, A.D.M.E. and Kuiken, T. (2006) H5N1 Virus Attachment to Lower Respiratory Tract. Science 312 399.
Walsh, E.E. and Hruska, J. (1983) Monoclonal antibodies to respiratory syncytial virus proteins: identification of the fusion protein. J. Virol. 47(1), 171-177.
Waris, M.E., Tsou, C., Erdman, D.D., Zaki, S.R. and Anderson, L.J. (1996) Respiratory synctial virus infection in BALB/c mice previously immunized with formalin-inactivated virus induces enhanced pulmonary inflammatory response with a predominant Th2-like cytokine pattern. J. Virol. 70(5), 2852-2860.
Watanabe, H., Tanaka, Y., Shimazu, Y., Sugahara, F., Kuwayama, M., Hiramatsu, A., Kiyotani, K., Yoshida, T. and Sakaguchi, T. (2005) Cell-Specific Inhibition of Paramyxovirus Maturation by Proteasome Inhibitors. MICROBIOLOGY and IMMUNOLOGY 49(9), 835-844.
Wharton, S.A., Belshe, R.B., Skehel, J.J. and Hay, A.J. (1994) Role of virion M2 protein in influenza virus uncoating: specific reduction in the rate of membrane fusion between virus and liposomes by amantadine. J. Gen. Virol. 75(4), 945-948.
WHO. (2009a) Acute Respiratory Infections,
. Vol. (May 26, 2009.
WHO. (2009b) Confirmed human cases of avain influenza A (H5N1). Vol. (May 26, 2009.
Yang, C.H., Murti, A., Pfeffer, S.R., Kim, J.G., Donner, D.B. and Pfeffer, L.M. (2001) Interferon alpha /beta Promotes Cell Survival by Activating Nuclear Factor kappa B through Phosphatidylinositol 3-Kinase and Akt. J. Biol. Chem. 276(17), 13756-13761.
Yerlikaya, A., Kimball, S.R. and Stanley, B.A. (2008) Phosphorylation of eIF2alpha in Response to 26S Proteasome Inhibition is Mediated by the Heme-regulated Inhibitor (HRI) Kinase. Biochem J 21, 21.
Yoon, K.-J., Cooper, V.L., Schwartz, K.J., Harmon, K.M., Kim, W.-I., Janke, B.H., Strohbehn, J., Butts, D. and Troutman, J. (2005) Influenza Virus Infection in Racing Greyhounds. Emerg. Infect. Dis. 11(12), 1974-1976.
Yuan, J., Stein, D.A., Lim, T., Qiu, D., Coughlin, S., Liu, Z., Wang, Y., Blouch, R., Moulton, H.M., Iversen, P.L. and Yang, D. (2006) Inhibition of coxsackievirus B3 in cell cultures and in mice by peptide-conjugated morpholino oligomers targeting the internal ribosome entry site. J Virol 80, 11510-9.
Yueh, A., Leung, J., Bhattacharyya, S., Perrone, L.A., de los Santos, K., Pu, S.Y. and Goff, S.P. (2006) Interaction of moloney murine leukemia virus capsid with Ubc9 and PIASy mediates SUMO-1 addition required early in infection. J Virol. 80(1), 342-52.
Zhadina, M., McClure, M.O., Johnson, M.C. and Bieniasz, P.D. (2007) Ubiquitin-dependent virus particle budding without viral protein ubiquitination. PNAS 104(50), 20031-20036.

Zhang, Y.J., Stein, D.A., Fan, S.M., Wang, K.Y., Kroeker, A.D., Meng, X.J., Iversen, P.L. and Matson, D.O. (2006) Suppression of porcine reproductive and respiratory syndrome virus replication by morpholino antisene oligomers. Vet Micro 117, 117-29.

Zheng, H., Lee, H.A., Palese, P. and Garcia-Sastre, A. (1999) Influenza A Virus RNA Polymerase Has the Ability To Stutter at the Polyadenylation Site of a Viral RNA Template during RNA Replication. J. Virol. 73(6), 5240-5243.

Zhirnov, O.P., Ovcharenko, A.V. and Bukrinskaya, A.G. (1982) Proteolytic Activation of Influenza WSN Virus in Cultured Cells is Performed by Homologous Plasma Enzymes. J. Gen. Virol. 63(2), 469-474.

Zimmer, G., Rohn, M., McGregor, G.P., Schemann, M., Conzelmann, K.-K. and Herrler, G. (2003) Virokinin, a Bioactive Peptide of the Tachykinin Family, Is Released from the Fusion Protein of Bovine Respiratory Syncytial Virus. J. Biol. Chem. 278(47), 46854-46861.