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Inhibition of respiratory syncytial virus by nasally administered siRNA modified with F-ANA.

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Ce mémoire intitulé:

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RÉSUMÉ

Le virus respiratoire syncytial humain (VRS), un pneumovirus, est une cause importante de maladie des voies respiratoires inférieures. L'infection au VRS pendant la petite enfance est associée au développement de la bronchiolite et de l'asthme. Il n'existe aucun médicament ou vaccin qui prévienne l'infection à VRS. La seule thérapie antivirale disponible est la ribavirine, réservée aux personnes à haut risque de maladie grave. Des études récentes ont démontré que des 'small interfering RNAs' (siRNAs) dirigés contre les protéines virales P et NS1 inhibaient la réplication le VRS. Par ailleurs, on a aussi cherché à prolonger l'activité des siRNAs en leur apportant des modifications chimiques. Une de ces modifications est le F-ANA (acide 2'deoxy-2'-fluoro-β-D-arabinonucléique). Le présent travail étudie l'efficacité des siRNAs modifiés au F-ANA à inhiber la réplication du VRS. *In vitro*, les siRNAs F-ANA ciblant les protéines virales P et NS1 inhibent plus efficacement la réplication virale. *In vivo*, à plus forte concentration, les siRNAs modifiés ou non au F-ANA et la ribavirine inhibent la synthèse des protéines virales avec une efficacité comparable (inhibition de 44% ± 6 pour le siRNA non-modifié, 47% ± 8 pour le siRNA F-ANA et 41% ± 9 pour la ribavirine). Cependant, à faible dose, l'efficacité du siRNA F-ANA est 84% supérieure à celle du siRNA non-modifié, un taux qui surpasse l'efficacité de la ribavirine de 57%. Les modifications F-ANA ont donc le potentiel d'améliorer les performances des siRNAs en tant qu'agent prophylactiques ou thérapeutiques contre le VRS.

Mots clés : Virus respiratoire syncytial, agents antiviraux, ARN d'interférence, small interfering RNA, modifications avec F-ANA, phosphoprotéines virales, protéines non structurale virales, ribavirine, modèle de souris.

ABSTRACT

Human respiratory syncytial virus (hRSV), a pneumovirus, is the major cause of severe lower respiratory tract disease in young children, the elderly and the immunosuppressed. Additionally, hRSV infection during infancy has been associated with the development of bronchiolitis and asthma. Despite extensive research, no reliable drug or vaccine currently exists that can prevent RSV infection. The only antiviral therapy available is ribavirin, whose use is reserved for persons at high risk for severe disease. Recent studies have shown that small interfering RNAs (siRNAs), targeting viral P protein and also viral NS1 protein inhibited RSV growth in culture and *in vivo*. Recently, efforts have been made to enhance stability and duration of activity of siRNAs through chemical modifications. One such modification is F-ANA (2'deoxy-2'-fluoro-β-D-arabinonucleic acid). The efficacy of siRNAs modified with F-ANA at inhibiting hRSV replication was investigated both *in vitro* and *in vivo*. *In vitro* screening demonstrated greater efficacy of siRNA targeting viral P protein than NS1 protein. *In vivo*, both unmodified and F-ANA modified siRNAs targeting viral P gene inhibited hRSV protein levels equally well at high doses (44% ± 6 for unmodified siRNA and 47% ± 7 for F-ANA modified siRNA), comparable to 41% ± 8 inhibition achieved by ribavirin. At lower doses, the F-ANA modified siRNA demonstrated 84% increase in efficacy to inhibit hRSV replication compared to unmodified sequence, exceeding ribavirin by 57%. In summary, F-ANA modifications have the potential to optimize siRNAs as prophylactic and/or therapeutic agents against hRSV infection.

Key words: Respiratory syncytial virus, antiviral agents, RNA interference small interfering RNA, F-ANA modifications, viral phosphoroproteins, viral nonstructural proteins, ribavirin, mouse model.

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LIST OF ABBREVIATIONS

ASON	antisense oligonucleotide
BAL	bronchoalveolar lavage
bp	base pair
bRSV	bovine respiratory syncytial virus
BSA	bovine serum albumin
CTL	cytotoxic T cell
dNTP	desoxyribonucleoside triphosphates
dsRNA	double-stranded RNA
DTT	dithiothreitol
F-ANA	2'deoxy-2'-fluoro-β-D-arabinonucleic acid
FBS	fetal bovine serum
HRP	horseradish peroxidase
hRSV	human respiratory syncytial virus
IFN-	interferon
Ig	immunoglobulin
IL-	interleukin-
kDa	kilodalton
L	polymerase
Mi	mismatch
MIP-1α	macrophage inflammatory protein-1 alpha
miRNA	microRNA
mMIG	mouse monokine induced by gamma-interferon
MOI	multiplicity of infection
N	nucleoprotein
NS	nonstructural protein
nt	nucleotide
ORF-	open reading frame
P	phosphoprotein

PBS	phosphate-buffered saline
PBS-t	phosphate-buffered saline/0.05% Tween
PCR	polymerase chain reaction
RdRP	RNA-dependent RNA polymerase
PFU	plaque forming units
PI	post infection
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNase	ribonuclease
RNP	ribonucleoprotein
RPMI	Roswell Park Memorial Institute
RSV	respiratory syncytial virus
RT-PCR	reverse transcription PCR
SEM	standard error of mean
siRNA	small interfering RNA
TBS	tris-buffered saline
Th1	T helper 1
Th2	T helper 2
TLR	toll-like receptor
TMB	tetra methyl benzidine
TNF-α	tumor necrosis factor-alpha
UTR	untranslated region

To my beloved parents

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To my family and friends, especially Mike, I seriously do not know how I would have gotten this far without you. Thanks for all your love and understanding. You are my rock, my foundation and my inspiration in life.

CHAPTER I

GENERAL INTRODUCTION

Human Respiratory Syncytial virus (hRSV), an RNA virus of the Paramyxoviridae family, is recognized as the most frequent cause of severe lower respiratory tract disease in infants and young children worldwide [1]. hRSV infects approximately 50% of infants during the first year of life and almost all children by age of two. Lower respiratory tract infections, such as bronchiolitis and pneumonia, result in an estimated 51,000-82,000 hospitalizations annually among young children in the United States [2]. Bronchiolitis is associated with long-term impairment of pulmonary function and often hyperresponsiveness of the airways after an RSV-infection during infancy may presage the development of asthma later on in children and adults [3].

Acquired immunity provides neither an efficient protection against re-infections nor reduction in viral shedding after infection, thus acquired immunity is incomplete and not durable. Despite extensive research, no reliable drug or vaccine currently exists that can prevent RSV infection. Two forms of anti-RSV antibody prophylaxis are available on the market, RSV-IGIV and palivizumab, both of which are questionable in their cost-effectiveness [4, 5, 6]. Currently treatment is supportive and the sole antiviral therapy available is Ribavirin, whose use is reserved for persons at high risk for severe disease, and still remains controversial in efficacy and expensive [6]. In addition, due to its very labor-intensive administration methods and its potential hazardous effects on the caregivers [7], its use has been questioned on economic and safety grounds.

Recent studies have shown that small interfering RNA duplexes (siRNAs) targeting either the viral P protein [8], an essential subunit of the RSV replication machinery, or the viral NS1 protein [9], another essential component of viral replication, strongly inhibited all RSV growth in culture and also *in vivo*, when administered intra-nasally. The problem remains of enhancing siRNA stability so that it can find its way into cells. Interestingly, an increase in activity and an enhancement of serum stability of siRNAs has been observed when modified with F-ANA (2'deoxy-2'-fluoro-β-D-arabinonucleic acid) at specific regions of the sense or antisense strands in culture [10, 11], however has not been fully examined in an animal model. The efficacy of siRNA modified with F-ANA to

inhibit hRSV replication *in vitro* and also *in vivo* in a murine model of RSV infection was therefore investigated.

CHAPTER II

RESPIRATORY SYNCYTIAL VIRUS AND DISEASE

2.1 HISTORY

In 1956, a group of young chimpanzees in a colony outside Washington, DC (USA) were demonstrating respiratory symptoms of a cold-like illness [12]. Morris, Blount and Savage recovered the cytopathic agent from one of these chimpanzees, with an upper respiratory tract illness, which induced symptoms of coryza, runny nose, and malaise. They named this agent "chimpanzee coryza agent", which was responsible for an outbreak of severe coryza illness in adolescent monkeys in the previous year [12, 13]. Upon examination of the entire colony nearly all of the chimpanzees were found to be infected. Human contacts working with the chimpanzees were also shown to be infected and had exhibited mild upper respiratory tract illness and coryza, however at levels less severe than observed in the chimpanzees. Subsequent studies identified two major isolates of the virus recovered from other patients with upper respiratory tract illnesses [12]. In 1957, the initial name of "chimpanzee coryza agent" was changed to respiratory syncytial virus (RSV) because of its selectivity for the respiratory tract and the characteristic tendency of infected cells to fuse together and form giant cell syncytia in tissue culture [13, 14].

2.2 EPIDEMIOLOGY

Severe respiratory infection in infants and young children was first recognized over 150 years ago and termed "congestive catarrhal fever." Based on clinical descriptions published at the time, there is a high probability that the disease described can be attributed to RSV [15]. Following the discovery and identification of RSV in the late 1950s and early 1960s, epidemiological studies clearly established it as the most important cause of serious respiratory tract infection in infants and young children [16, 17]. Approximately 25 years later, RSV is recognized as the leading cause of infection of the lower respiratory tract in infants and young children [1]. It is the single most important cause of hospitalization during the first year of life [18], infecting around 50% of infants during the first year of life and almost all children by the age of two [19]. It is the leading cause of death due to viral illness in children younger than 1 year with a mortality rate in

this age group 10 times greater than that from influenza [20]. Unlike the common cold or flu, RSV continues to be the leading killer among infectious diseases with an annual death toll of about one million worldwide [21, 22]. Although RSV infection was reported in adults with pneumonia in the 1960s, it has only been during the past two decades that the potential for widespread occurrence with serious clinical impact in this population has been recognized [23, 24, 25, 26]. RSV has a global distribution and it is a seasonal infection, with peaks around winter and/or spring [27].

Two major antigenic subgroups of respiratory syncytial virus (designated A and B) have been described, differentiated mostly on the basis of reactivity patterns between monoclonal antibodies and the viral glycoprotein (G protein) [28, 29]. Both subgroups are infectious: one strain tends to dominate during an individual epidemic in an individual location, although at times both strains can be isolated from patients in the same area [30]. In addition, a number of studies suggest that Strain A may result in more virulent infections than Strain B [31], however there is great variability, possibly due to viral burden and individual host factors. Along with these 2 strains, several subtypes of each strain have been identified [32, 30]. In the United States and United Kingdom, Strain A is found more commonly, although it has been observed to alternate with Strain B in a somewhat irregular pattern from year to year [33]. Strain B, however, appears more commonly in epidemics in Europe [34].

2.3 VIRAL STRUCTURE, REPLICATION AND INFECTION

2.3.1 Virion structure

Respiratory syncytial viruses, both bovine (bRSV) and human (hRSV), are enveloped RNA viruses and members of the *Pneumovirus* subfamily, of the family *Paramyxoviridae* [35]. The *Paramyxoviridae* family includes two genera, *Morbillivirus* (measles virus) and the *paramyxoviruses* (containing, e.g., parainfluenza virus, types 1, 2, and 3, and mumps virus) [32]. The RSV virion is about 150-300 nm in diameter consisting of a nucleocapsid

core that is packaged within a lipid envelope [36] **(Figure 1)**. Each of the viral mRNAs encodes a major viral protein [37].

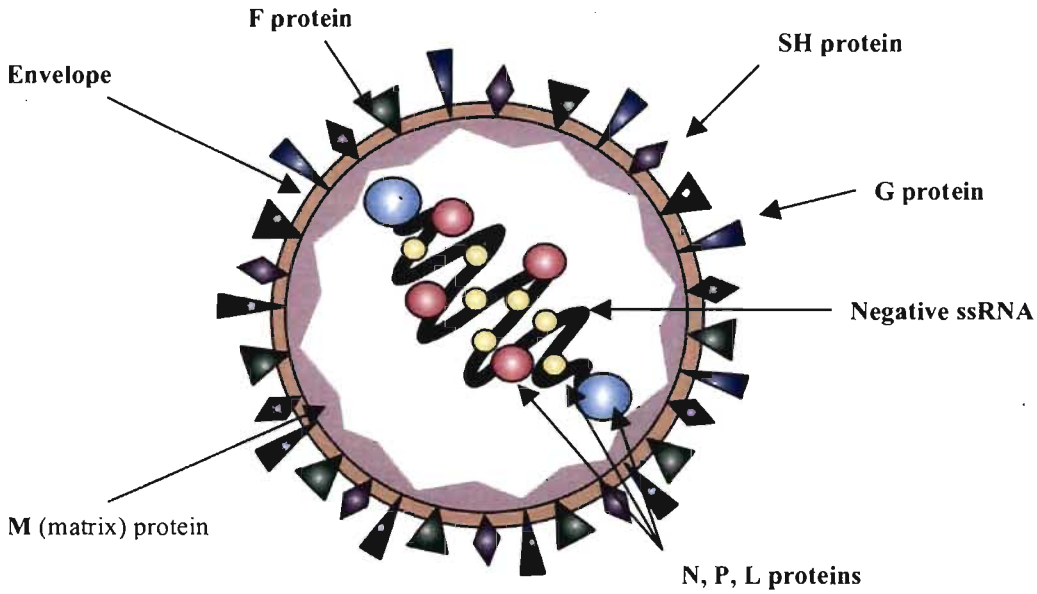


Figure 1. Respiratory syncytial virus structure. The RSV virion is about 200 nm in size and consists of a nucleocapsid within a lipid bilayer. The major nucleocapsid protein N, the phosphoprotein P, the large polymerase subunit L, the anti-termination factor M2 and a single-stranded RNA genome of negative polarity compose the RNP complex. The attachment protein G, the fusion protein F and the small hydrophobic protein SH are the three transmembrane surface glycoproteins present on the viral surface. The matrix protein M forms a scaffold on the inner layer of the envelope. Figure modified from (Hacking, 2002) [37].

The symmetrically helical nucleocapsid (RNP complex) is 12-15 nm in size and is composed of the nucleocapsid protein N, the phosphoprotein P, the large polymerase subunit L and the M2-1 protein. The envelope is a lipid bilayer derived from the host plasma membrane and containing on its surface virally encoded transmembrane glycoproteins (G) responsible for attachment, the fusion protein (F) and the small hydrophobic (SH) protein, whose function is unknown however speculated to also be involved in cell fusion [38]. Inside the lipid bilayer is a scaffold formed by the matrix protein (M), which connects the viral membrane with the RNP complex [30, 32] **(Figure 1)**.

2.3.2 Genome organization

The RSV genome is a 15,200 nucleotide-long non-segmented single-stranded negative-sense RNA containing 10 genes that are transcribed into 11 major sub-genomic mRNAs [39, 40, 41, 42] **(Figure 2)**. All mRNAs are capped at the 5'-end and polyadenylated at the 3'-end by the viral polymerase, however genomic and anti-genomic RNA are neither capped nor polyadenylated and are components of the nucleocapsids. Transcription and replication is initiated from the 3'-end in an obligatory sequential manner with only a fraction of the virally encoded RNA-dependent RNA polymerase (RdRP), minimally composed of the large protein (L) and the phosphoprotein (P), moving to the next gene. This mechanism creates a gradient of transcriptional attenuation with distance from the transcriptional start-site indicating the required relative abundance of the encoded proteins **(Figure 2)**. The overall strategy of RSV gene expression is common to all members of the negative-strand RNA virus super-family [43, 44]. The initial rounds of transcription, known as "primary" transcription, are carried out by the RdRP activity associated with the incoming viral genome. The transcribed mRNAs are translated into *de novo* viral proteins including more RdRP, which boosts new rounds of viral gene expression.

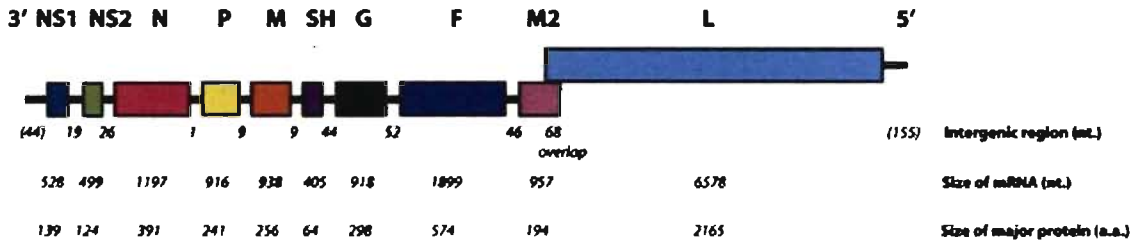


Figure 2. RSV genes and the sizes of their mRNA and encoded proteins. The RSV genome is a single stranded negative sense RNA of a 15,200 nucleotides that is transcribed into 11 major sub-genomic mRNAs. Transcription is initiated from the 3'-end with only a fraction of the polymerase moving on to the next gene. This mechanism creates a gradient of transcriptional attenuation with distance from the transcriptional start-site indicating the required relative abundance of the encoded proteins Figure modified from (Hacking, 2002) [37].

2.3.3 Viral proteins

Nine of the 11 major proteins, N, P, M, SH, G, F, M2, and L, are structural proteins (present in infected cells and virions) and surface glycoproteins [30, 32] **(Figure 2)**. Three of nine structural proteins are transmembrane surface glycoproteins (G, F, and SH), of 11-20 nm in size, embedded in the lipid bilayer derived from the host cell membrane, at intervals of 6-10 nm [37]. The viral nucleocapsid core is a symmetrical helix with a helical diameter of 12-15 nm [37, 38] and composed of N, P, L proteins (which together comprise the viral replicase) and M2 open reading frame 1 (M2 ORF-1) **(Table I)**. Finally, two proteins (NS1 and NS2) are nonstructural viral products that accumulate in infected cells but are present in only scarce amounts in mature virions [39, 45].

Genome	Proteins	Functions
3'		
NS1	NS1	Non-structural protein: Unknown function (Essential in replication?)
NS2	NS2	Non-structural protein: Unknown function (Anti-IFN α and β activity?)
N	N	Nucleocapsid protein: structural protein essential for transcriptional activity
P	P	Phosphoprotein protein: essential structural protein and cofactor of the polymerase
M	M	Matrix protein: viral assembly
SH	SH	Small hydrophobic protein: function unknown; not required for replication nor syncytium formation
G	G	Transmembrane protein: mediates viral attachment; membrane bound and secreted forms; neutralizing antigen
F	F	Fusion glycoprotein: mediates syncytial formation, penetration, neutralization and protection
M2	M2-1	M2-1 protein (upstream ORF): transcription and elongation factor
	M2-2	M2-2 protein (downstream ORF): regulation of transcription and RNA replication
L	L	Major polymerase subunit
5'		

Table I. The RSV genome, encoded viral proteins and functions. The RSV genome is a single stranded negative sense RNA of a 15,200 nucleotides that is transcribed into 11 major sub-genomic mRNAs. Each viral protein encoded by the genes play a key function in the replication and transcription of the virus. Nine are structural proteins and the remaining two are non-structural proteins.

The RSV F protein is a transmembrane glycoprotein essential in the role of viral entry, by mediating surface fusion of the virion envelope with the host's cellular plasma membrane and also in the formation of syncytium, late in the infection. The F protein (70 kDa) is a type I transmembrane glycoprotein with a cleaved N-terminal signal sequence and a transmembrane anchor near the C terminus, and is similar to that of the other Paramyxoviruses in structure and function [41, 46, 47, 48]. The F protein is synthesized as a precursor F_0 that is activated by cellular protease(s) and cleaved into two subunits, F_1 and F_2, linked by disulfide bonds, after the synthesis and modification by the addition of N-linked sugars [49]. The fusion protein is protein that induces in the human host the most important responses, including humoral and cytotoxic T-lymphocyte responses [50].

The RSV G protein is a heavily *N*- and *O*-glycosylated and large type II transmembrane glycoprotein, involved in mediating viral attachment but not essential for propagation [51]. The G protein (90 kDa) has a number of unusual structural features, including a large number of proline, serine and threonine residues and a significant content of *O*-linked oligosaccharides [52, 53] and it also as the lacks neuraminidase and hemagglutinin [36]. Both glycoproteins have been shown to be major antigenic determinants of the virus [36] especially the G protein, dividing the virus into the two major subgroups, A and B, as described above [54].

The small hydrophobic SH protein (7.5-30 kDa) is a short integral transmembrane protein and is present in glycosylated and non-glycosylated species in the form of oligomers. Its function is still unknown, however it has been established that it is not required for viral replication or syncytium formation *in vitro* [37], whereas attenuation has been achieved by targeting the SH protein in the lower respiratory tract *in vivo* [55].

The matrix protein M is a non-glycosylated protein that forms a scaffold on the inner side of the viral envelope. It plays an important role in virus assembly and budding by mediating the association between RNP complex and cell plasma membrane [56, 57]. In early infection, M protein localizes the nucleoprotein into the nucleus where it possibly inhibits host-cell transcription, and also inactivates transcription activity of the RNP

before packaging [57]. RSV M protein has also exhibited some RNA-binding capacity however the actual function of this interaction still remains unclear.

The N (44 kDa), P (34 kDa), and L (~200 kDa) proteins, co-purified with the nucleocapsid, are all essential for RNA replication and transcription [37]. The major nucleocapsid protein is the nucleoprotein. It binds to genomic and antigenomic RNA conferring RNase resistance to the nucleocapsids. The exact positioning of the RNA relative to the N molecules is not yet determined [58]. It has also been suggested that the N protein itself might be a component of the promoter [59]. The phosphoprotein P is the major phosphorylated species, in which it acts as a chaperone for N protein and is essential together with N protein for encapsidation. The phosphoprotein P is also a polymerase cofactor which functions by converting initiated polymerase into stable complex and its phosphorylation of itself is imperative for its function [60]. Bitko *et al.* have demonstrated the importance of P protein in viral replication and propagation, through achieving attenuation of viral replication by inhibiting the viral P mRNA [8]. The large protein L is the major RNA-dependent RNA polymerase subunit and it is bound to its cofactors, the phosphoprotein P and M2-1 by the N protein [61]. The L protein together with the P protein forms the L-P complex and is proposed to be responsible for the recognition of the promoter, RNA synthesis, capping and methylation of the 5' termini of the mRNAs and polyadenylation of their 3' ends. Evidence has shown that the L protein contains the enzymic domains required for these processes and its multifunctional nature tallies with its size, with the L gene accounting for almost half of the RSV coding potential [62].

M2 ORF-1 and M2 ORF-2 are two open reading frame products of the M2 (22 kDa) protein that are important in the production of full-length mRNA by polymerase and in the regulation of transcription [41]. The M2-1 protein is a transcription elongation factor which complexes with N, P, and L to ensure efficient production of full-length mRNA [37]. The M2-1 protein is encoded by the upstream translational ORF of the M2 mRNA, whereas the M2-2 is a second, distinct protein also transcribed from the M2 gene, with defined properties in transcriptional regulation. The M2-2 protein overlaps with the first and encodes a putative negative regulatory activity on viral transcription and thus appears

to be an 11th RSV gene. The SH, M, and M2 proteins are all envelope-associated proteins [36].

The two non-structural proteins (~14-15 kDa), NS1 and NS2 proteins, are encoded by the first two genes in the viral genome and it is this presence that is perhaps what distinguishes the *Pneumovirus* genus from the rest of the *Paramyxoviridae* family. The proteins are so named because they are not packaged into the mature virion. Nonetheless, both genes are expressed in the infected cell and the mRNAs are relatively abundant due to the promoter-proximal (3' end of the negative-stranded genome) location of the genes [63]. The NS1 and NS2 proteins each contain the same found carboxy-terminal amino acids but otherwise are unrelated. While the function of both nonstructural proteins is still unknown, research has shown an anti-interferon α and β activity of these proteins [37, 64]. The NS1 protein has been suggested to be a potent inhibitor of intracellular RSV transcription and RNA replication [9, 65], while the NS2 protein decreased Stat2 levels in airway epithelial cells, and thus may modulate type I interferon signaling and gene expression [66]. Furthermore, the deletion of either NS1 or NS2 severely attenuated RSV infection both *in vivo* and *in vitro*, suggesting that the NS proteins play a vital role in viral replication [55, 67, 68, 69].

2.3.4 Viral replicative cycle

The viral cycle of RSV begins with the attachment and entry into the host cell, predominantly airway epithelial cells, which line the nose as well as the large and small airways. Binding and entry of RSV into these target cells is mediated by the interaction between the G and the F proteins with the host cell **(Figure 3)**. The specific cellular receptor(s) for the G protein has not yet been identified however cell surface glycosaminoglycans, such as heparin sulfate and chrondroitin sulfate B, have been shown to have an important function in *in vitro* infection through a putative heparin binding domain in the G protein [70]. Interestingly, recombinant RSV viruses with either mutated G protein or simply lacking the G protein are still infectious in cell cultures, whereas attenuation of infectivity is demonstrated *in vivo* [71, 72]. This observation suggests that the

G protein is non-essential for cell attachment but has other functions that may influence the efficiency of the process, as it contains a motif at 182-186 amino acids common to the CX3C chemokine fractalkine [53]. It has been shown that the G protein binds to the CX3C receptor (CX3CR1) and can facilitate RSV infection [73].

The F protein is absolutely required by RSV and can mediate attachment on its own. The fusion of the viral envelope or infected cell membranes with uninfected cell membranes and the formation of syncytium is an essential step in the virus life cycle [74]. Viral uptake appears to occur by fusion rather than endocytosis. Once incorporated into the target cell membrane, the nucleocapsid (RNP) is released into the cytoplasm, where the genome is transcribed and replicated **(Figure 3)**.

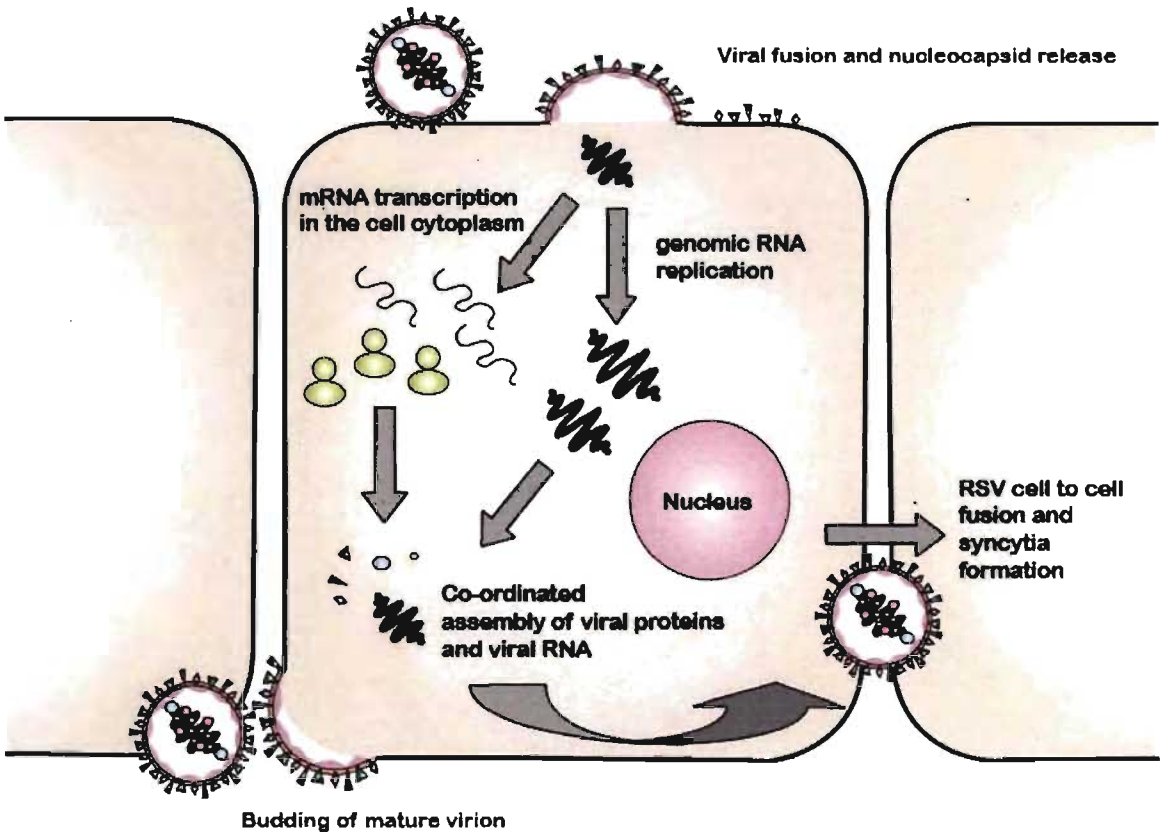


Figure 3. The schematic representation of the replicative cycle of RSV. The attachment protein G and fusion protein F mediate RSV entry and uptake respectively. After the viral envelope has been incorporated with the host cell membrane, the nucleocapsids (RNPs) are released into the cytoplasm, where the entire replication occurs. RNA transcription and replication is followed by encapsidation and budding on the cell surface of newly formed virions. Figure modified from (Hacking, 2002) [37].

The entire RSV replicative cycle takes place in the cytoplasm beginning with the transcription of the genome into ten monocistronic, 5'-capped, methylated and 3'-polyadenylated mRNAs by the viral RNA-dependent polymerase complex [61] **(Figure 3)**. RNA synthesis occurs in a sequential manner from the 3'-end of the genome with the polymerase complex terminating and reinitiating mRNA transcription at each junction. Re-initiating can be occasionally inefficient and this results in a gradient of mRNA decreasing proportionally to the distance of the gene from the 3'-end of the genome.

The polymerase complex is also responsible for the synthesis of antigenomic RNA, resulting in a complementary positive-sense copy of the viral genome. The antigenomic RNA represents a replicative intermediate that is less abundant in comparison to the genomic RNA. However, both RNAs are packed into virions in equal proportions. Antigenomic and genomic RNA synthesis correlates with protein translation suggesting a need for co-synthetic encapsidation. Where RSV differs from other Paramyxoviruses is in the switch between RSV transcription and RNA replication, as it seems to involve the M2-2 protein and is not dependent on the intracellular levels of N and P proteins [75]. Thus the M2-2 gene oversees this transition from transcription to production of genomic RNA. During the earlier stages of infection, low levels of M2-2 correlates with a high transcriptional rate, whereas afterwards, when the intracellular levels of M2-2 protein increase, transcription is inhibited in favor of replication [76]. Replication involves generation of a complete, positive-sense RNA complement of the genome, the antigenome, which in turn acts as a template for genome synthesis. The genomes are then incorporated into nucleocapsids as they are synthesized and recycled through the RNA-synthesis pathways, or are transported to the plasma membrane for assembly into virus particles **(Figure 3)**.

Assembly of the nucleocapsids is entirely intra-cytoplasmic. The N protein associates first with genomic and antigenomic RNA followed by P and L. Association of the nucleocapsids with the nascent envelope is mediated by the M protein, through a series of interactions by coordinating the assembly of the envelope proteins F and G, via their cytoplasmic domains, with the nucleocapsid proteins N P and M2-1 [77]. Once assembled,

nucleocapsids are transported close to the cell surface and bud at specific plasma membrane patches where glycoprotein G clusters. Budding appears to be the reverse of penetration and occurs *in vitro* on the apical cell surface **(Figure 3)**.

2.4 DISEASE AND CLINICAL RELEVANCE

2.4.1 RSV infection and transmission

The virus undergoes replication in the nasopharynx, and is then spread to the lungs via the respiratory epithelium or aspiration of secretions. Inoculation is via the upper respiratory tract and the virus is spread downwards to the lower respiratory tract along the respiratory mucosa through syncytial formation and epithelial cell sloughing [78]. Lower respiratory infection follows 1-3 days later [79]. There is however no systemic spread and the infection is confined to the respiratory mucosa [78].

Respiratory syncytial virus is very contagious and is thought to spread by large droplets of secretions (i.e. from sneezes) from an infected person. This occurs via direct contact with infectious secretions on environmental surfaces or through close contact with a person who has an active infection [80, 81, 82]. An example of this close contact may be hand-to-eye or hand-to-nasal-epithelium following hand contact with infectious secretions or even hand-to-hand transfer. However, studies have shown that no infection occurs if a person is at a distance of greater than six feet from an infected individual, thus the theory of small particle aerosol spread is rejected [83]. The virus is highly resistant and can survive on surfaces long after it has been in contact with an infected person. Nasal secretions on tissue or cloth are infectious for up to 30 min, whereas those on hard surfaces such as countertops, stethoscopes or silverware are infectious for at least 6-12 hours. After the initial contact, an incubation period of 2-8 days follows [15], in which the infected person will commonly exhibit mild to moderate nasal congestion and low-grade fever and a productive cough with mucous secretion. However, these symptoms may persist as an upper respiratory infection for several weeks and then resolve without

further incident, particularly in patients who have had a previous RSV infection [30].

Due to the extremely contagious nature of the virus, an infected person will remain contagious as long as the virus is being shed. Shedding of the virus begins within a day or so of infection, often before the onset of major signs of illness [81]. Shedding of the virus is highly variable and appears to be roughly paralleled with the age of the person, the severity of the infection, and whether the infected person is immunocompromised. Infected adults would typically shed the virus for 3-7 days following infection [84]. Since age seems to affect the shedding, infants would normally shed for up to 14 days in lighter infections, however infants less than 6 months of age with severe infections may shed for up to 3 to 4 weeks, thus increasing its contagious nature. Older children and adults may only shed the virus for 3 or 4 days [80, 85]. Immunocompromised individuals may shed for several months following an infection [86].

2.4.2 Clinical symptoms

RSV often causes an upper respiratory tract infection in infants and children. It is characterized by symptoms of rhinorrhea and cough. Lower respiratory tract infections are generally associated with expiratory wheezing (often referred to as bronchiolitis or wheezy bronchitis), pneumonia can occur with RSV infection and bacterial acute otitis media can follow [30, 36]. Also, approximately a third of RSV-infected children develop acute otitis media [87]. Acute otitis media is characterized by the presence of fluid, typically pus, in the middle ear with symptoms of pain, redness of the eardrum, and possible fever. RSV is the causative agent in about 50% of infant pneumonia and 10-30% of pediatric bronchitis [88].

The clinical profile of RSV infection differs according to age. In neonates, RSV infections differ from those in older children and apnoea (the cessation of breathing) may be the only symptom of infection. Almost all infants (6 weeks to 2 years of age) will be infected with RSV, the primary infection being often asymptomatic. However, RSV infection in infant will involve the lower respiratory tract and cause bronchiolitis and pneumonia. Although this occurs in a minority of infants, it becomes the number one

cause of hospitalization in this age group. In older children, infections are usually less severe. The mortality in healthy children is extremely low, but in immunocompromised patients (including children infected with human immunodeficiency virus, children with combined immunodeficiency syndrome, children undergoing chemotherapy for leukemia, and children who have undergone transplantation), in patients with cardiac abnormalities and in cystic fibrosis patients, RSV infection is often life threatening [88, 89, 90, 91]. In the elderly, RSV infection of the lower respiratory tract can manifest itself in the form of pneumonia [30, 36]. In adults and the elderly, infection of the upper respiratory tract manifests commonly with symptoms such as rhinorrhea, nasal congestion, pharyngitis, and cough [30, 36].

In addition, RSV infection may trigger acute respiratory distress syndrome with substantial morbidity and mortality [80]. RSV infections leading to ARDS appear to have long-term negative consequences similar to that of patients with other pulmonary diseases [30]. Infected patients hospitalized for RSV lower respiratory tract infection may develop recurrent wheezing during a 1-year follow up. The occurrence of wheezing was significantly higher in infants with airflow limitations than in those without airflow limitation during the acute infection [92, 93]. Although the persistence of wheezing has been established [94], the relationship between RSV and subsequent asthma and atopy is not nearly as clear [95]. A number of studies appear to have found at least a statistical connection between RSV infection and asthma in young children [3, 96, 97, 92].

2.5 THE IMMUNE RESPONSE TO RSV

The immune response to RSV infection is similar to that of any other infection, in that it is comprised of an innate response, and subsequently by activation of humoral and cell-mediated immunity. However, natural infection with RSV does not provide efficient protection against re-infections, indicating that the acquired immunity is incomplete and unstable. Ineffectiveness of the immune response against RSV infection has hindered the development of effective vaccines against both the bRSV and hRSV. The other major complication is that prior vaccination can actually enhance the severity of the disease

during subsequent natural infection [98, 99, 100, 101]. Humoral immunity plays a key role in limiting the severity of subsequent infections, however does not offer a complete protection against RSV infection.

2.5.1 The innate immune response to RSV

Respiratory epithelial cells, are not only the principle target for RSV infection but are also the first line of defense in the innate immune response to the virus, followed by the involvement of antigen presenting cells, namely macrophages and dendritic cells. Not only do respiratory epithelial cells release nitric oxide (NO) upon infection and produce opsonins and collectins (which are important in the clearance of the virus), but also secrete a range of inflammatory mediators, such as chemokines, leukotrienes and cytokines [37, 102, 103, 104], such as intracellular adhesion molecule (ICAM)-1 [105], interleukin (IL)-8 [106] and RANTES (Regulation upon activation normal T cell-expressed and secreted) [107]. The release of such inflammatory mediators initiates maturation and chemotaxis of macrophages and induces neutrophils, eosinophils, and CD4+ T helper cells. In addition to the action of epithelial cells, alveolar macrophages are very important in the innate immune defense against RSV as they may regulate the immune response [108], and when infected release pro-inflammatory cytokines [109], such as tumor necrosis factor (TNF) alpha, IL-10, IL-6 and IL-8 which are involved in the regulation of inflammation.

In both the macrophages and epithelial cells, RSV has been shown to induce the activation of nuclear factor kappa-B (NF-κB), which in turn stimulates transcription of genes affiliated with the antiviral response [110, 111]. NF-κB also plays a crucial role in the early stages of innate immune response, mainly via the Toll-like receptor (TLR) signaling pathway [112, 113]. TLRs are type 1 transmembrane proteins and are evolutionary conserved pattern recognition receptors, which respond to pathogen-associated molecular patterns. PAMPs include lipopolysaccharides, nonmethylated CpG DNA and dsRNA [114, 115, 116]. Cytokine and chemokine production in RSV-infected cells involve the Toll-like receptor-signaling pathway. Recently, glycoprotein F of RSV was shown to activate TLR4, possibly through their interaction with the surface molecule CD14, expressed primarily

on resting monocytes/macrophages [117, 118]. The role of several TLRs is still currently under extensive examination.

2.5.2 Humoral/antibody-mediated immunity to RSV

During the first 2 months of the life of a newborn, protection against infection is passively acquired via maternal immunoglobulins. Despite this form of "acquired" immunity, the presence of maternal antibodies decreases gradually during the first 6 months of life, leaving most infants un-protected against RSV between 2 and 4 months of age [119]. Thus the humoral response is stronger if primary infection with RSV occurs after 6 months of age. These responses are also enhanced after each subsequent episode of re-infection throughout life. However, not all humoral responses are favorable. For example RSV-specific IgE may also play a role in the increase of severity of the disease [120, 121, 122]. McIntosh and colleagues demonstrated that secretory antibodies (IgA) were defective in neutralizing the virus *in vitro*, which may explain the failure of immunity during the early stages of an infant's life [123]. Similarly, increased serum IgG titers do not entirely guarantee that a person will be protected from acquiring a new RSV infection or that a future disease will be mild. Thus humoral immunity does not appear to provide a complete protection against RSV infection and current evidence in the literature has focused on the importance of cell-mediated immune response in viral clearance.

2.5.3 Cell-mediated immunity to RSV

The activation of cellular immunity following an RSV infection is due mainly to epithelial cells and alveolar macrophages. Infants with a primary RSV infection often develop a cellular immune response within 10 days [124]. Human cytotoxic T cell lymphocytes (CTLs) recognize the N, SH, F, M, M2 and NS2 proteins but not the G protein [50]. In mouse models, specifically in BALB/c mice, the first cells to appear are the natural killer (NK) cells followed by CD8+ cytotoxic T cells, which may further modulate the immunity by secreting cytokines, especially IFN-γ [124, 125, 126]. These CTLs predominantly target the M2 protein followed by the F and N proteins [127, 128]. Both CD4+

and CD8+ T cells have been demonstrated to have antiviral activity on passive transfer to RSV infected mice and reduce pulmonary shedding of the virus. However both cell types also cause increased pulmonary damage [129].

The response to RSV by helper T (Th) cells has also been evaluated in the airways of RSV-infected patients [130, 131]. T cells produce pro-inflammatory mediators classified as type 1 (Th1) or type 2 (Th2) cytokines. Th1 cells secrete IL-2, IFNγ and lymphotoxin, whereas Th2 cells produce IL-4, IL-5, IL-6 and IL-13 [132]. Increase in disease severity is associated with enhanced T cell responses [133], as observed in the mouse model for RSV bronchiolitis, where prior sensitization with a formalin-inactivated RSV vaccine leads to a helper Th2-driven augmented disease, contrasting with the usual Th1 response seen in primary viral infections [134]. Individuals who have deficiencies in cell-mediated immune responsiveness have been shown to develop an unusual but severe form of RSV infection with prolonged proliferation of the virus in the respiratory tract and progressive pneumonia [135]. Of note, wheezing was not a major part of the clinical manifestations in these immunodeficient patients. These crucial data provide evidence that cell-mediated responses are important in terminating RSV infection and that cellular immunity may contribute to the development of wheezing after RSV infection and the severity of the disease. It is apparent that cell-mediated immunity is an important component in a patient's response to RSV infection, as well as in his/her recovery.

2.6 CURRENT TREATMENTS AND PROPHYLAXIS

Despite growing concerns over this problem, to this day, after almost five decades of effort, no effective and reliable vaccine or preventive antiviral against RSV exists [6, 21, 136]. Currently, treatment of RSV infection is supportive which involves ensuring adequate fluid intake, providing supplemental oxygen, and sometimes, administering bronchodilators. In some cases of severe illness and/or high-risk patients, the broad-spectrum antiviral drug ribavirin (Virazole) may be used in aerosolized form. Ribavirin, a synthetic guanosine analog with the empirical formula $C_8H_{12}N_4O_5$ **(Figure 4)**, was

approved and licensed in 1985 to be delivered via small-particle aerosol for use in hospitalized children in the United States [137]. The mechanism of action of its antiviral properties remains unknown, however it was shown that ribavirin inhibits of the synthesis of viral structural proteins, thereby slowing viral replication and also resulting in a reduced IgE response [138]. Although ribavirin is the only anti-viral preparation approved for RSV infections, the use of this teratogen is still controversial as it has not demonstrated significant reduction in either hospitalization or mortality rates [139, 140]. Furthermore, due to its very labor-intensive administration methods and its potential hazardous effects on the caregivers [7], its use has been questioned on economic and safety grounds.

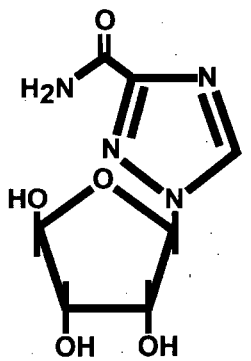


Figure 4. Structural formula of ribavirin. Ribavirin is a synthetic guanosine analog. It has an empirical formula of $C_8H_{12}N_4O_5$. Figure modified from (Domachowske, 1999) [35].

Prevention of RSV infection by passive administration of antibodies on the other hand is tremendously expensive thus is restricted solely to high-risk infants (defined by prematurity, immunocompromised status, or pre-existing pulmonary disease). There are currently two forms of anti-RSV antibody prophylaxis available on the market: RSV-IGIV and palivizumab. RSV-IGIV (RespiGam; MedImmune, Gaithersburg, MD) is produced with pooled serum from human donors with high titers of antibody against RSV [4]. It has been shown to significantly reduce hospitalization rates for high-risk infants when administered intravenously once a month at a dose of 750 mg/kg for each month of the peak RSV season. However due to the high cost and the complications associated with the intravenous administration, the cost-effectiveness of this product has become questionable [4, 5]. In 1998, MedImmune introduced palivizumab (Synagis), a humanized monoclonal antibody directed against the F protein. Palivizumab is administered

intramuscularly, at a dose of 15mg/kg, 50 fold lower than RSV-IGIV, yet appears to have similar efficacy in the reduction of RSV hospitalization rates. However, like RSV-IGIV, the cost for the injection of palivizumab is high. With this basis, revised guidelines for RSV prophylaxis limits its administration to infants with chronic lung disease, infants born earlier than 33 weeks gestational age, or later-term premature infants with at least one other risk factor [6].

Therapeutics	Function & Results
Oxygen	Used in cases where patients exhibit the typical signs of lower respiratory tract infection. The supplemental oxygen should be administered to maintain a saturation of >92%.
β2 agonist	Used to treat wheezing, frequently present in RSV infections. However, despite numerous clinical trials, the effectiveness of this therapy remains questionable.
Racemic Epinephrine	Given either via injection or nebulized to ameliorate the symptoms of RSV infection. Racemic administration may improve oxygenation, respiratory distress score, and pulmonary function measures in majority of the cases.
Aerosolized recombinant human DNase (Pulmozyme)	After DNase administration, chest radiograph scores improved significantly, however, other measures such as improvement in respiratory rate, wheezing, and retractions during hospitalization did not. Thus concluding that the effectiveness of this treatment is doubtful.
Inhaled and systemic corticosteroids	Prescribed for the treatment of bronchiolitis, both during the acute phase and during the period of recurrent wheezing that follows RSV infection in many infants. Studies have shown that the anti-inflammatory action of the corticosteroids should provide effective therapy for infections, however its efficacy for RSV remains still unclear.
Nasopharyngeal suctioning	Defined as extending a suction catheter up through the nose, passing the tip to the hypopharynx, and then applying suction as the catheter is withdrawn. An exceptionally effective palliative measure for infants with RSV by clearing the secretions present in the nose, pharynx and lower airways to improve the resistance of breathing and provide some symptom relief.
Helium-Oxygen gas mixtures (Heliox)	Functions by decreasing the work of breathing and by improving gas exchange in obstructive conditions (i.e. croup and chronic obstructive pulmonary disease). Studies have shown that this may be useful in avoiding respiratory failure and intubation in RSV-infected infants.
Inhaled Nitric Oxide	Currently approved by the United States Food and Drug Administration for the treatment of persistent pulmonary hypertension of newborns only. Given to treat severe RSV pneumonia and bronchopulmonary dysplasia and may improve oxygenation and respiratory system resistance.
Extracorporeal membrane oxygenation	A suitable option for patients with severe RSV disease who cannot be supported on mechanical ventilation.
Ribavirin	A synthetic nucleoside (guanosine analog) and the sole anti-viral preparation currently approved for the treatment of RSV infections. While the mechanism of action of its antiviral properties remains unknown, it may act by inhibiting RSV replication during the active replication phase of the virus.

Table II. Current therapies for RSV infections and their functions. Despite almost five decades of research, no effective and reliable vaccine or preventive antiviral against RSV exists. Currently, treatment

of RSV infection is supportive, such as the administration of supplemental oxygen, β2 agonist, racemic epinephrine, aerosolized recombinant human DNase, inhaled and systemic corticosteroids, helium-oxygen gas mixtures, inhaled nitric oxide, suctioning of the nasopharyngeal regions, and oxygenation of the extracorporeal membrane oxygenation. The sole antiviral drug available is Ribavirin, whose use is reserved for persons at high risk for severe disease [95].

Different approaches have been developed for RSV infections **(Table II),** however no effective treatment beyond palliative measures has yet appeared [95]. With very few therapies available for RSV infection, more research is needed to find effective measures to prevent or treat RSV infection. Although the disease is self-limited by the body's own immune response, the persons most susceptible to severe infection which include immunocompromised patients, infants, elderly, and bone marrow transplanted patients are those individuals who are the least capable of coping with an infection. Ultimately, prophylaxis is the most effective way to handle the infection and a novel technology termed RNA interference may be the answer to treat an infection during epidemics or once established.

CHAPTER III

RNA INTERFERENCE AND siRNAS FOR THE THERAPY OF RSV INFECTION

3.1 RNA INTERFERENCE

The discovery of the structure of DNA by Watson and Crick in 1953 is considered to be one of the most important scientific breakthroughs in the last century [141]. Through their model of the DNA structure, genetic information was described as being coded along the polymeric DNA molecule composed of only four types of monomeric units, which were organized into genes, and that DNA molecules are the basis of heredity [142, 143]. Genes were described as controlling the synthesis of various types of RNA, most of which were involved in the process of protein synthesis [144].

Since all genomes of complex organisms are potential targets of invasion by viruses and transposable elements, gene therapy seems to hold tremendous potential in the treatment of the many genetic and acquired diseases. Although there are many nucleic acid drugs currently on the market and in production, recently a growing interest has been placed on RNA interference (RNAi) as a potential therapeutic agent for silencing numerous viral infectious (genes required for viral replication) and cancer genes (oncogenes).

3.1.1 Nucleic acid drugs

The majority of currently employed pharmacological approaches for modulation of gene function rely upon the interaction of low molecular weight chemical compounds, such as nucleic acids, targeting proteins so as to down-regulate the function of the proteins themselves. Nucleic acids may be defined as the macromolecules in which the nucleotides remain linked to each other by phosphodiester bonds between the 3'- and 5'-positions of the sugars [145]. These macromolecules control metabolism throughout the life of a cell by directing and regulating protein and enzyme synthesis and are key elements in transferring genetic information from one offspring to another. These characteristics can be taken advantage when developing drugs targeting cancer or infectious diseases.

There are five major classes of nucleic acid drugs which are categorized based on their target site and mechanism of action: aptamers, antisense oligonucleotides (ASONs), ribozyme nucleic acids, antigene nucleic acids, and RNA interference **(Table I)**.

Nucleic acid drugs	Properties & function
Aptamers	Single or double stranded nucleic acids. Selected and amplified oligonucleotides that have been isolated from random pools of synthetic oligonucleotides, based on their ability to bind with high affinity to biological target molecules. Bind to proteins involved in the regulation and expression of genes dependent upon activity of protein. Stable under various buffer conditions. Resistant to harsh treatment such as physical or chemical denaturation with no loss of activity.
Ribozyme nucleic acids (Catalytic RNAs)	RNAs that catalytically cleave covalent bonds in a target RNA. The catalytic site is the result of conformation adopted by the RNA-RNA complex in the presence of divalent cations. Fairly unstable against nucleolytic degradation, leading to conformational changes that abolishes the catalytic activity. Challenges in their delivery to target cells.
Antigene nucleic acid compounds	Nucleic acids that bind to single stranded or double stranded DNA. Binding occurs at a replication or transcription bubble, thus interfering with the transcription process.
Antisense oligonucleotides (ASONs)	An oligonucleotide with a sequence complementary to the target gene mRNA. Functions by binding to the mRNA of the target gene and blocking translation of the message into protein. The antisense may also bind to DNA in the nucleus, blocking transcription or to the transcript during its processing and transport from the nucleus to the cytoplasm. Designed to prevent or at least reduce the expression of a specific target gene. Unmodified antisense oligonucleotides are often unstable and rapidly degraded by nucleases.
RNA interference	Initiated by long double-stranded RNA molecules, which are processed into smaller sized RNAs by the enzyme Dicer. The small RNAs then degrade the target RNA with a nuclease, by incorporating itself into the RNA-induced silencing complex (RISC), a protein-RNA complex. Challenges in the stability and delivery to target cells.

Table I. The five major classes of nucleic acid drugs. Nucleic acid-based molecular therapeutics, such as aptamers, ribozyme nucleic acids, antigene nucleic acid compounds, antisense oligonucleotides, and RNA interference, can down regulate the expression of certain target genes. However, issues with potency, specificity, and delivery in most classes of nucleic acid drugs, scientists have turned to RNAi for means of solving these obstacles [145].

Among the promising therapeutic approaches, antisense oligonucleotides have been vigorously pursued as a therapeutic strategy for over 20 years. The progress, however, has been particularly slow because of problems with delivery to the target cells, stability, specificity and toxicity. Antisense oligonucleotides are short (~20 base) nucleic acids, designed to be complementary to the target RNA sequence in order to bind to the target and to limit the consequence of RNA by obstructing the production of proteins [146]. This

gene selectivity enables targeted drug design and therefore the production of more effective and less toxic therapeutics. However, recent discoveries of RNA interference have had immediate and widespread impact [147, 148]. Once evidence that double-stranded short synthetic RNAs can inhibit gene expression in cultured mammalian cells, a surge of experimentation resulted, affecting virtually every field of biological science. Similarities exist between RNAi and antisense oligonucleotides [146] such as their length, their complementarity to target the mRNA and their methods of synthesis, however there are advantages to RNAi over ASONs that will be described in greater length in the subsequent sections.

3.1.2 Discovery of RNA interference

RNA interference is a naturally occurring, sequence-specific mechanism for gene silencing. It is thought to silence viruses and rogue genetic elements that make double-stranded RNA (dsRNA) intermediates (types of RNA but not usually produced by cells). Napoli and Jorgensen were the first to observe the mechanism of gene silencing in plants in 1990. Two years later, Romano and Macino reported a similar phenomenon in *Neurospora crassa* [149]. Then in 1997, evidence that RNA could elicit gene silencing in animal cells came from the work by Guo and Kemphues, who demonstrated that sense RNA was equally effective in reducing gene expression in the nematode *Caenorhabditis elegans* as was an antisense RNA [150]. At the time, these observations were puzzling, however they were readily understood when Fire and Mello's work was published in 1998. Fire and Mello detailed the phenomenon of RNAi and discovered that very small amounts of dsRNA (the combination of sense and antisense RNAs) was ten-fold more effective than either RNA strand alone [147]. This observation of interference was further explored by several leading groups including Baulcombe, in plants as part of post-transcriptional gene silencing [151] and summarized by Sharp [152]. It was these findings that led to the realization that RNA was responsible for the "homology-dependent gene silencing" phenomena observed a decade ago and initiated the cascade of research on RNAi in a variety of organisms including plants, fungi, flies, worms and also complex mammals [147, 152, 153, 154, 151, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166].

3.1.3 Types of RNA interference

RNA interference is initiated when dsRNA is processed into small RNAs of 21-23 nucleotides (nt) in length by the intracellular RNase III enzyme Dicer [167]. These small RNAs are then incorporated into the RNA-induced silencing complex (RISC) within the cytoplasm of cells, which acts as a guide to the complementary nucleic-acid targets resulting in cleavage of the target RNA **(Figure 1)**. Two relatively well-defined classes of small RNAs are involved in RNA silencing: microRNAs (miRNAs) and small interfering RNAs (siRNAs) (**Table II**, shaded in yellow). Because the active forms of miRNA and siRNA are sometimes biochemically or functionally indistinguishable, they are classified based on their origins, their evolutionary conservation, and the types of genes they silence.

Type of RNAi	Characteristics and properties
MicroRNA (miRNA)	A class of 19–25-nt single-stranded RNAs. Encoded in the genomes of most multicellular organisms studied. Some are evolutionarily conserved and are developmentally regulated. Undergoes a two-step cleavage of hairpin precursors by two RNAse III enzymes, Drosha and Dicer. Elicit translational repression and mRNA cleavage. [168]
Small interfering RNA (siRNA)	A class of double-stranded RNAs of 21–24 nt in length. Generated from the cleavage of long dsRNAs by Dicer. Gene silencing by mRNA cleavage, by histone and/or DNA modification, or by histone methylation leading to DNA elimination. [169 170 171 172 173]
Tiny non-coding RNA (tncRNA)	A newly discovered class of short, 20–22-nucleotide RNAs. Encoded in the genome of *C. elegans*. Not evolutionarily conserved, but some are developmentally regulated. Produced by Dicer from unidentified precursor. Function still unknown. [174]
Small modulatory RNA (smRNA)	A short ~20 nucleotide dsRNA, identified in 2004 in mice. Allows the expression of neuron-specific genes only in adult neurons. [175]

Table II. Various types of RNA interference and their main characteristics. Modified from Novina (2004) and Kim (2005) [176 177].

MiRNAs, previously named small temporal RNAs (stRNAs), were first discovered in *Caenorhabditis elegans* in 1993 [178, 179, 180, 181]. MiRNAs are endogenous RNAs, generated from the non-protein-coding dsRNA region of the hairpin-shaped precursors while siRNAs are derived from long dsRNAs. Whereas miRNAs derive from loci that are unique for a diverse range of other recognized genes, endogenous siRNAs are often

occurring and originate from transposons [170, 182, 183, 184], and viruses that produce dsRNA during replication [173], as well as other types of bi-directionally transcribed repetitive sequences [185, 186] and genes [173, 174]. While miRNA sequences are nearly always conserved among related organisms, endogenous siRNA sequences are rarely conserved. Furthermore, miRNAs "hetero-silence" genes that are very different from their own origination, whereas, endogenous siRNAs "auto-silence" genes at the same locus, or very similar loci, from which they originate.

3.2 SMALL INTERFERING RNA

The discovery of siRNA was first observed in 1999 by Hamilton and Baulcombe [151] followed by the demonstration that synthetic siRNAs induced RNAi in mammalian cells by Tuschl *et al* in 2001 [187], leading to a rise in interest in employing RNAi for biomedical research and drug development.

3.2.1 Silencing pathway

The siRNA pathway begins with the cleavage of long dsRNAs by Dicer enzyme complex, a member of the RNase III superfamily of endonucleases, into small fragments of 21-24 nt in length with 3'-overhangs [151, 166, 188, 189, 190] **(Figure 1)**. A multi-enzyme complex, which includes the RISC and Argonaute-2 (AGO2, also known as EIF2C2), a protein that can bind small RNAs [191] and in some cases execute target-mRNA cleavage [192 193], bind to the siRNA duplex and discards the passenger (sense) strand to form an activated complex containing the guide (antisense) strand. The siRNAs guide this AGO2-RISC nuclease complex in search of complementary target mRNA sequences. Once the antisense or guide strand is of perfect or near perfect complementarity to the target mRNA resulting from the specificity dictated by base-pairing interaction between guide and target RNA strands [166, 189, 194], the complex then cleaves the mRNA strand (carried out by the catalytic domain of AGO2) between the nucleotides that are complementary to nucleotides 10 and 11 of the guide strand relative to the 5'-end **(Figure 1)**. Cleavage of

the target mRNA results in the prevention the synthesis of proteins and reduction in the level of the proteins.

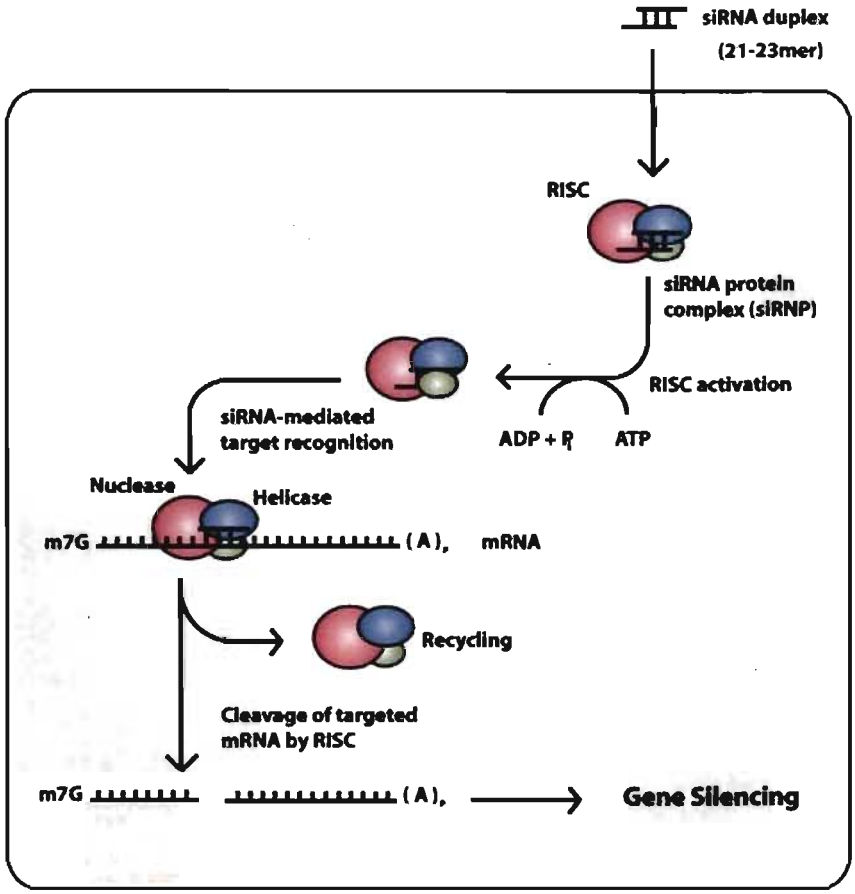


Figure 1. Silencing pathway of synthetic siRNA. Once inside the cell, the siRNA is bound by proteins of the RNA-induced silencing complex (RISC) [195] [148] [196] to form the siRNA protein complex (siRNP). The siRNA then guides the RISC proteins in search of RNA sequences that are complementary to one of the two strands of the siRNA duplex in the genome. The sense or passenger strand dissociates from the complex, while the antisense or guide strand is matched with its complementary RNA target. Recognition of mRNA by the antisense strand of the siRNA results in the cleavage and destruction of the target mRNA, prevention of protein synthesis, and a reduction in the level of protein inside cells. Figure modified from (Novina 2004) [176]

While double-stranded RNAs larger than 21-23 nt in length may be appropriately Diced, it has been demonstrated that dsRNA longer than 30 nt triggers a non-specific interferon pathway through interaction with the protein kinase PKR [197] and 2',5'-oligoadenylate synthetase [198]) rather than RNA interference. Therefore chemically synthesized siRNA duplexes are generally ~21 nt in length such that they bypass Dicer and can induce RNAi by incorporating directly onto the AGO2-RISC complex [199, 200, 201] **(Figure 1)**, thereby potentially eliminating the PKR/interferon response (see below) [187, 202].

3.2.2 Non-specific off-target effects

Despite having developed an understanding of the key players and mechanisms involved in RNAi, a significant hurdle remains in the development of RNAi into a research and therapeutic tool, mainly the non-specific off-target effects. Two types of off-target effects need to be avoided or minimized: silencing of genes sharing partial homology to the siRNA (off-targeting), and immune stimulation stemming from the recognition of certain siRNAs by the innate immune system.

Although siRNA-mediated mRNA down-regulation was initially reported to be highly specific [203, 204, 205], siRNAs can also recognize and interfere with the expression of certain mRNAs that share partial homology with the target mRNA. Therefore it was not surprising to find that many of the off-target genes that have reduced mRNA levels contained regions that are complementary to one of the two strands in the siRNA duplex using *in vitro* transcriptional profiling [206, 207, 208]. Complementarity between the 5'-end of the guide strand and the mRNA is key to off-target silencing with the critical nucleotides being in positions two to eight (from the 5'-end of the guide strand) [209, 210].

It was also noted early on that siRNAs seemed highly specific, as a single mutation in the target site could completely interfere with the silencing [187]. Furthermore, as the first microarray experiments demonstrated excellent specificity for siRNAs [203, 204] it was not noticed that mutations in the target region corresponding to the central region of several siRNAs did not always abolish RNAi [211]. Several later reports demonstrated that a single nucleotide mismatch between the siRNA strand and the target mRNA greatly decreased the rate of target mRNA cleavage [212, 213, 214]. It was then that Jackson *et al.* revealed that siRNAs with only partial complementarity to mRNAs could also elicit gene silencing [206]. These effects were observed both for sense and antisense siRNAs. These silencing effects were evidently not related to the nonspecific interferon effect, as was observed for both siRNAs [215] and short hairpin RNAs [216], but dependent on the sequence of the siRNA. This type of mRNA regulation was then coined the term "off-target" effects and is

typically associated with less than two-fold mRNA down-regulation [205, 206, 207, 209, 210, 217, 218, 219, 220, 221].

Other reports have shown that siRNAs may indeed induce sequence-specific off-target effects as described, however if they contain certain sequence motifs and structural features, nonspecific activation of the innate immune system, including induction of interferon pathway proteins, may be the result [222, 223]. As mentioned previously, although dsRNA of 21-23 nt can be expected to avoid inducing the interferon response, it has been shown that at high concentrations, even these smaller siRNAs may be able to activate this pathway, resulting in global translational blockade and cell death. Perhaps of greater concern is the potential to activate TLRs, especially the dsRNA receptor TLR7 in plasmacytoid dendritic cells, which in turn leads to the production of type I interferons and pro-inflammatory cytokines, and induce NF-κB activation [223]. Interferon induction by some siRNAs occurs principally through TLR7, which is largely found in endosomes of immune antigen-presenting cells. Fortunately, TLR7 binding is highly sequence specific, favoring GU-rich sequence and can be fairly easily avoided by selecting and designing sequences that are not recognized by this receptor.

3.2.3 SiRNA selection and design

A synthetic siRNA consists of a 19 base-pair double-stranded region that is complementary to the gene of interest, contains 5' phosphate and 3' hydroxyl termini, and possesses two single-stranded nucleotides on the 3' ends [187] **(Figure 2)**.

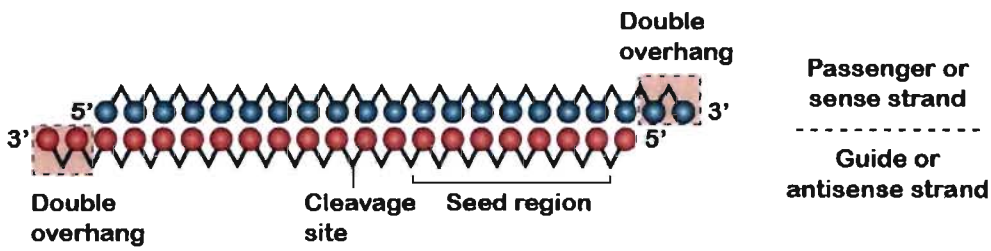


Figure 2. Schematic illustration of important features of siRNA structure. A basic siRNA is composed of two base pair overhangs, a seed region and an mRNA cleavage site. Figure modified from (de Fourgerolles, 2007) [224].

Although there are currently no reliable methods to identify the ideal sequence for a siRNA, a number of general guidelines have been suggested by Tuschl *et al* [225] and also various company synthesis protocols, such as Dharmacon, QIAGEN, and Ambion. Selection of a target mRNA region should be within 50 to 100 nucleotides downstream of the start codon. A 23-nt long sequence should be selected from the mRNA conforming to the consensus 5'-AA[N19]UU-3' or 5'-NA[N19]NN-3', where N is any nucleotide. Sequences with GC content between 30% and 70% would be ideal, however highly G-rich areas should be avoided. Also to be avoided are regions that are likely to bind to regulatory proteins, such as 5'-UTR, 3'-UTR and regions close to the start codon [225]. Although one can follow these guidelines it is still necessary to test several siRNAs, targeting distinct regions within the gene of interest, because there is great variability in the capacity of an individual siRNA to induce silencing [226, 227]. The search for more accurate, efficient siRNA selection has resulted in a number of new developments, such as their mechanism of delivery and modifications of the chemical structure of siRNAs.

3.3 DELIVERY AND MODIFICATIONS OF siRNAS

SiRNA represents a powerful tool for specific gene silencing and many are being pursued as potential means to inhibit expression of clinically relevant genes in a wide array of therapeutic applications. Thus far, *in vivo* gene silencing approaches are very limited in the mammalian system. Nonetheless, a number of potential candidate genes, especially in viral infections, cancers and inherited genetic disorders but also in chronic inflammatory diseases such as autoimmune arthritis, have been defined and successfully targeted *in vitro* [228, 229, 230]. SiRNAs, as with other oligonucleotide-based therapies, face some key hurdles including delivery, cellular uptake and biostability. Initial works with siRNAs were undertaken with unmodified, natural molecules. It soon became clear, however that native oligonucleotides were subject to relatively rapid degradation and thus require the need for better modes of delivery and chemical modifications for *in vivo* applications

3.3.1 Delivery of siRNAs *in vitro* and *in vivo*

Naked siRNAs are degraded by the activity of RNase nucleases in human plasma with a half-life of minutes [231, 232]. Although direct injection of naked siRNA (unmodified or chemically modified) has proven efficacious in multiple contexts of ocular, respiratory and central nervous system disease [8, 233, 234, 235, 236, 237, 238, 239], they are rapidly excreted through the urine when administered into the blood stream. SiRNAs when formulated into different carrier systems for *in vivo* delivery they may be protected from nuclease digestion and last longer than naked siRNAs. Effective delivery is the most challenging obstacle in the development of RNAi as a broad therapeutic platform. Animal studies using siRNA have either used no additional formulation (naked siRNA) or have delivered siRNA formulated as conjugates, liposome/lipoplexes or as complexes with peptides, polymers or antibodies. Different siRNA delivery approaches have been utilized by many scientists and were able to achieve RNAi-mediated silencing both *in vitro* and *in vivo* **(Figure 3)**.

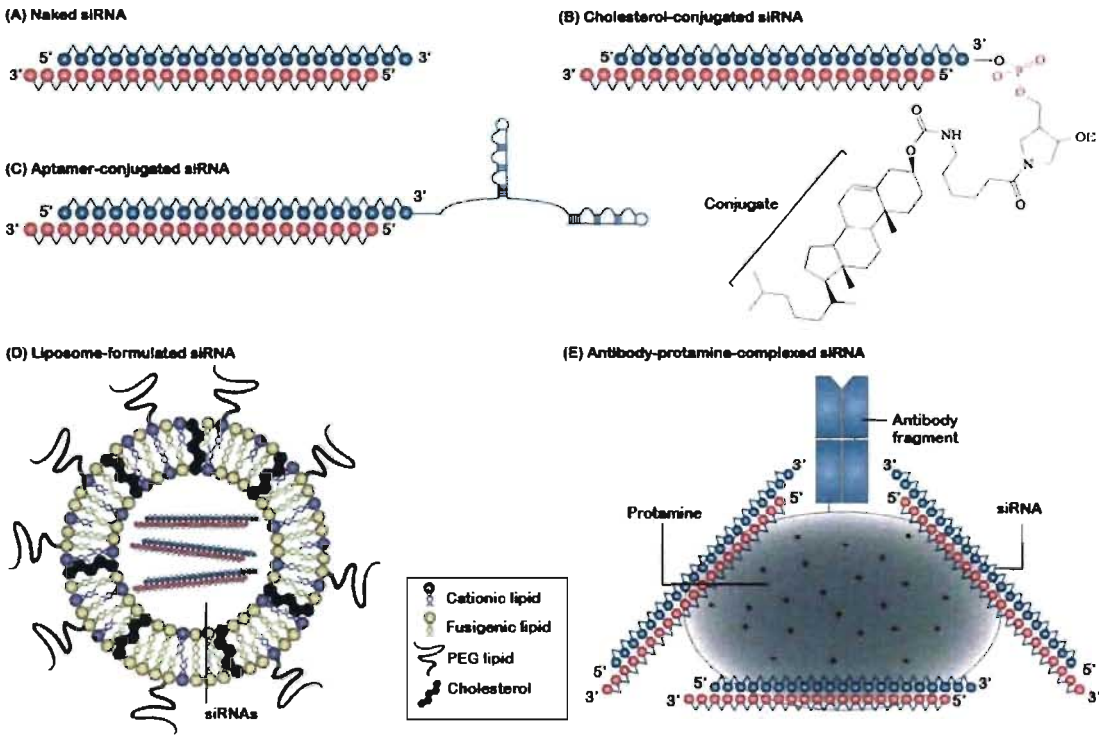


Figure 3. Delivery of small interfering RNAs. Different strategies have been used to deliver and achieve RNAi-mediated silencing *in vivo* (**A**) Direct injection of naked siRNA (unmodified or chemically modified.

(B) Direct conjugation of siRNA to a natural ligand such as cholesterol. **(C)** Aptamer-siRNA conjugates. **(D)** Liposome-formulated delivery of siRNA. **(E)** Antibody-protamine fusion proteins have been used to non-covalently bind siRNAs through charge interactions. The passenger strand and the guide strand are represented in blue and orange, respectively. Figure modified from (de Fougerolles, 2007) [224].

Improved nuclease-stability is essential for siRNA duplexes that are exposed to nuclease-rich environments (such as blood) and are formulated using excipients that they themselves do not confer additional nuclease protection. As might be expected in these situations, nuclease-stabilized siRNAs show improved pharmacokinetic properties *in vivo* [240]. In other situations, when delivering siRNA directly to less nuclease-rich sites (such as the lung) or when delivering siRNA in conjunction with delivery agents such as liposomes **(Figure 3)**, the degree of nuclease stabilization can be reduced significantly. Although the ability of an siRNA duplex to reach its target cell intact is vitally important, since it needs to overcome the blood vessel endothelial wall and multiple tissue barriers, whether nuclease protection confers a measurable benefit once an siRNA is inside the cell remains to be determined. Finally, once the siRNA reaches the target cells, cellular uptake of those siRNAs and intracellular RNAi activity require efficient endocytosis and intact double-stranded siRNAs, respectively. Therefore various delivery methods have been developed to counter this problem of biodistribution **(Figure 3)**, however a broader and simpler approach would be shifting focus from systemic delivery to local.

Local delivery involves administration of the siRNAs to a smaller and potentially more protected area within the body and is a good strategy because it simplifies distribution, lowers the likelihood of adverse side effects, and reduces the amount and cost of the siRNA dose. An example of this approach would be airway delivery of siRNA for respiratory diseases. Intra-nasal administration of cationic liposome-formulated siRNA specifically targeting the influenza virus RNA genome into mouse lung infected with the influenza virus significantly reduced lung virus titer in infected mice and protected animals from lethal challenge [241]. However, *in vivo* delivery of siRNA with cationic polymer carriers (i.e. PEI) is often associated with severe toxicity in the host and possibly the induction of nonspecific interferon response through the Toll-like receptor pathway, as previously discussed. Therefore, pulmonary siRNA delivery might require formulations without cationic carriers. Naked delivery of siRNA intra-nasally into the

lungs has been successfully employed by Bitko to inhibit respiratory syncytial viral protein P in lungs of mice, reducing the viral titers by up to 80% [8, 9].

Local delivery has important implications for the role of chemical modifications to siRNA. However if the siRNA is systemically administered, there is a greater need for modifications because the obstacles in bio-distribution and the requirements for stability are much greater. Nevertheless, although chemical modifications might be less essential for local delivery than systemic delivery, it is still probable that they can significantly improve the properties of drug candidates and this possibility should be explored during development.

3.3.2 Modifications of siRNAs

As previously mentioned, naked siRNAs are degraded in human plasma with a half-life of minutes [231, 232]. Therefore to convert siRNAs into optimized drugs, prolonging the siRNA half-life, enhancing their stability, increasing their affinity with RNA and bio-distribution without jeopardizing biological activity is crucial [242, 243, 244, 245, 246, 247, 248] and can be accomplished by chemical modification. Many different types of chemical modifications have been developed to improve the *in vivo* properties of nucleic acids [249, 250, 251]. Each modification offers a different potential for tailoring the properties of siRNAs. They can be used alone or in combination and the number of modified nucleotides can vary relative to the number that remain as unmodified RNA. The modifications also differ in how they are tolerated by the RISC complex. Some modifications can be introduced at almost all bases of both RNA strands, whereas other modifications cannot be placed at some positions without interfering with efficacy. The modifications generally involve alterations to the ribonucleotide ribose ring or to the oligonucleotide phosphate backbone **(Figure 4)**.

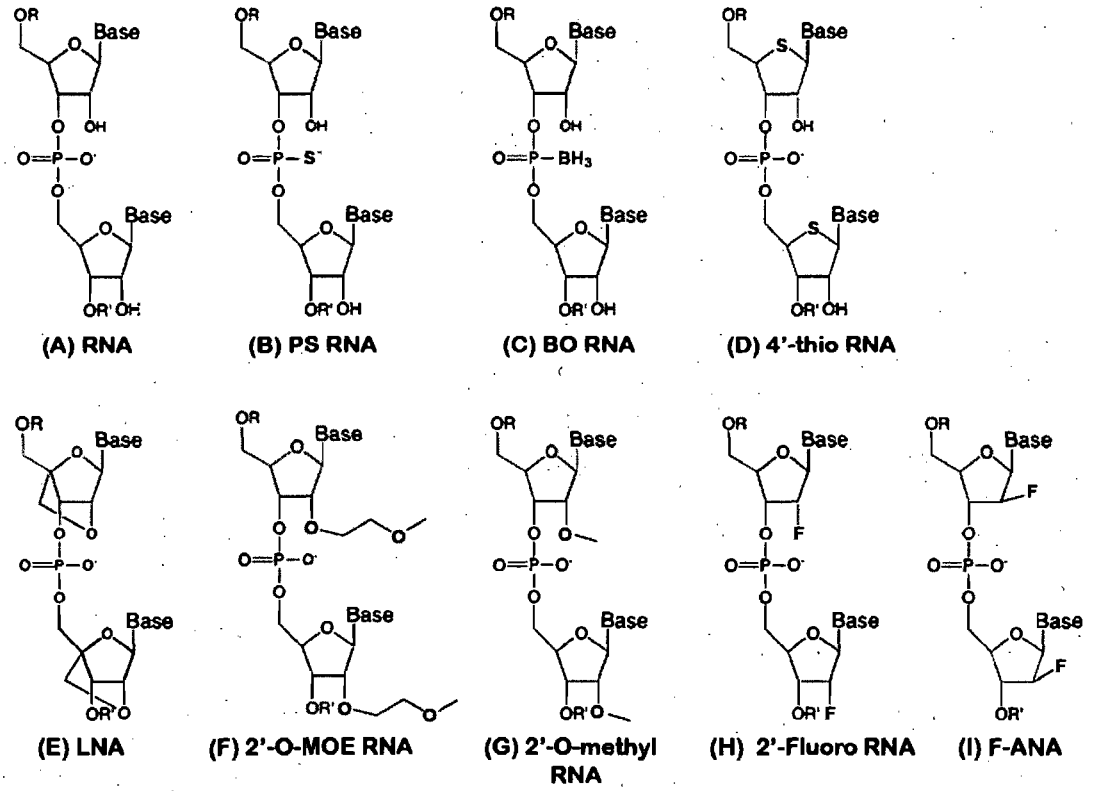


Figure 4. Structure of RNA and the nucleic acid analogs and chemical modifications. Modifications identified in blue. **(A)** Native RNA; **(B)** Phosphorothioate (PS) backbone linkage; **(C)** Boranophosphate (BO) linkage; **(D)** 4'-thio modified RNA; **(E)** Locked nucleic acid (LNA); 2'modified RNA: **(F)** 2'-*O*-methoxyethyl (2'-MOE) RNA; **(G)** 2'-*O*-methyl RNA; **(H)** 2'-Fluoro RNA; and **(I)** 2'-deoxy-2'-fluoro-β-D-arabinonucleic acid (F-ANA). Figure modified from (Corey, 2007) [252].

The starting point for these efforts has been chemistries that are already in use with antisense oligonucleotide and aptamer therapeutics. For instance, the introduction of a phosphorothioate (PS) backbone linkage (instead of phosphodiester (PO)) at the 3'-end protects against exonuclease degradation and improves the half-life of the oligonucleotides in circulation by increasing their binding to serum protein [253, 254]. A phosphorothioate linkage is created by replacing a non-bridging oxygen atom on the backbone phosphate between ribonucleotides with a sulfur atom [255] **(Figure 4B)**. PS linkages have shown to retain their ability to silence target sequences when introduced into siRNAs according to several published studies [232, 240, 256, 257, 258, 259, 260, 261]. Another alternative for modifying the phosphate backbone of an oligonucleotide is by replacing one of the non-bridging oxygen atoms with a boron atom to create a boranophosphate

(BO) linkage **(Figure 4C)**. Although this modification has attracted relatively little study, preliminary research reported an improvement in gene silencing activity at lower concentrations relative to either PS siRNAs or native siRNAs and an enhancement in their resistance to degradation by nucleases [262].

Various modifications can also be introduced to the ribonucleotide ribose ring, such as replacing the oxygen that is attached to the 4' carbon of the ring with a sulfur atom generating a 4'-thio modified RNA **(Figure 4D)**. By introducing this sulfur atom into the ribose ring within both sense and antisense strands of the siRNAs, their resistance to digestion by nucleases was greatly enhanced [263, 264] and so was their potencies [264]. Another modification to the ribose ring is by inserting a methylene bridge between the 2' and 4' carbons of the ribose ring [265, 266] and creating locked nucleic acids (LNA) **(Figure 4E)**. This methylene linkage "locks" the ribose ring into a conformation close to that formed by RNA after hybridization. By introducing this constraint, it increases the thermal stability of the siRNA duplexes and also resistance to digestion by nucleases [243].

Many RNA analogs have been developed that include substitutions for the hydroxyl group on the 2' carbon atom of the ribose ring. These RNA analogs include 2'-*O*-methoxyethyl (2'-MOE) RNA **(Figure 4F)** [267, 268], 2'-*O*-methyl RNA **(Figure 4G)** [269] and 2'-fluoro RNA **(Figure 4H)** [270]. Enhanced affinity of the RNA oligomers for complementary RNA or DNA sequences and increased resistance to nuclease digestion has been reported with these RNA analogs [217, 232, 240, 243, 256, 257, 258, 259, 260, 263, 264, 271, 272, 273, 274, 275, 261]

Lastly, a relatively newer class of 2' modification is 2'-deoxy-2'-fluoro-β-D-arabinonucleic acid (F-ANA). F-ANA nucleotides are based on the sugar arabinose rather than ribose and have the stereochemistry at the 2'-position inverted relative to that found in 2'-*O*-methyl RNA and 2'-fluoro RNA **(Figure 4I)**. The characteristics and properties of F-ANA modifications will be discussed in the following section.

3.4 F-ANA MODIFICATIONS OF OLIGONUCLEOTIDES

Arabinonucleic acid (ANA) is a 2'-stereoisomer of RNA with the sugar ribose replaced by arabinose [276]. When the ANA is further modified by substituting the C2'-OH group with a fluorine atom, 2'-deoxy-2'-fluoro-β-D-arabinonucleic acid (F-ANA) is created **(Figure 5)** [277, 278].

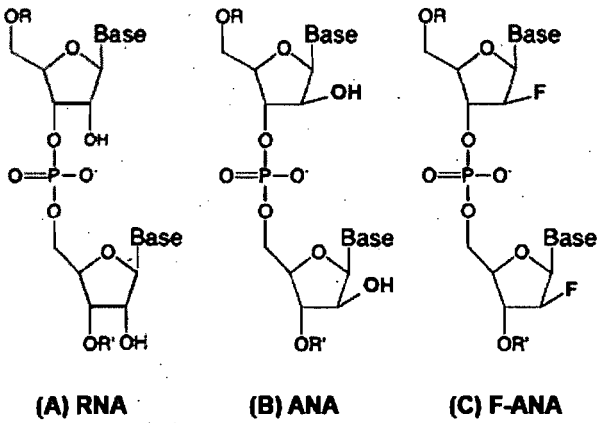


Figure 5 Chemical structure of Ribonucleic and arabinonucleic acids. Inversion of configuration at the 2'-position of **(A)** RNA and 2'-F-RNA gives the corresponding epimeric arabinonucleic acids, **(B)** ANA and **(C)** F-ANA. Figure modified from (Corey, 2007) [252] and (Kalota, 2006) [277].

ANA and the corresponding F-ANA were the first uniformly 2'-sugar-modified oligonucleotides reported to induce RNase H cleavage of a bound RNA molecule [279], due to their structural similarities to normal DNA/RNA duplexes [280, 281]. Also, the 2'F-substituent in F-ANA/RNA hybrid projects into the major groove of the helix, such that it does not significantly interfere with the binding and subsequence catalysis of RNA by RNase H [282, 283]. By replacing the 2'-OH group with a smaller 2'-F atom, the resulting compound has a significantly enhanced binding affinity to the target mRNA compared with native or PS-modified DNA [284, 279]. Upon the incorporation of the "up" fluorine atom, the sugar becomes 'rigid' as a result of a strong effect between the 2'-F atom and the oxygen ring, causing the equilibrium to favor this newly formed conformation [285] **(Figure 5)**.

Various studies were conducted to compare the gene silencing efficiency of F-ANA modified siRNA and native siRNA molecules. Dowler *et al* had observed that the efficacy and stability of their siRNA could be greatly improved by strategic modifications with F-ANA, particularly when made in the sense strand together with modification of the 3'-overhanging end of the antisense strand, *in vitro* **(Figure 6)** [10].

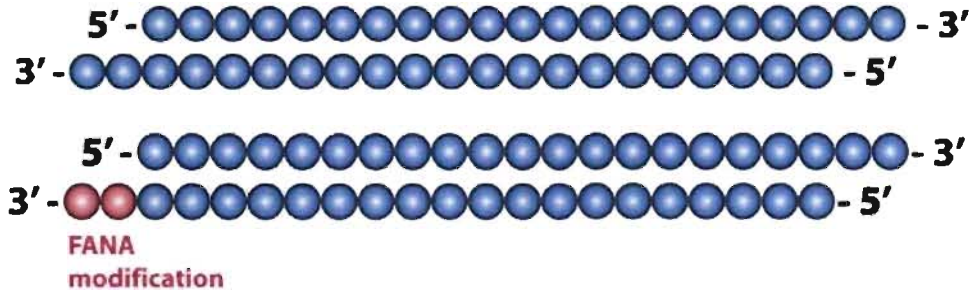


Figure 6. Example of the modifications of siRNA with F-ANA chemistries along either or both the sense and antisense strands. (A) Native 21-nt siRNA; **(B)** Two F-ANA modifications at the 3'-end of the siRNA.

Recent studies have shown that siRNAs with completely F-ANA-substituted sense strands are 4-fold more potent than analogous unmodified RNAs and have a longer half-life in serum [10, 286]. F-ANA substitutions can also be tolerated for gene silencing when placed at the 3' and 5' positions of the antisense strand or throughout the sense strand. In addition F-ANA substitution enhanced the resistance of the siRNA duplex to degradation in serum containing medium, a property that may impact upon the future therapeutic development of chemically modified siRNAs. However, their improvements capabilities *in vivo* are still unknown, and will be explored in further on in this document.

3.5 APPLICATIONS OF siRNA IN RSV DISEASE

As previously mentioned, hRSV is the leading cause of severe lower respiratory tract disease in infants and young children throughout the world. hRSV disease in the young has been linked to the possible development of asthma later on. A lot of research has been conducted on finding prophylaxis and vaccine but has been thus far quite

unsuccessful. However a newcomer, siRNA, has great potential in this domain. Many researchers have shown to inhibit various viruses with the use of siRNAs. Bitko and Zhang were able to establish inhibition of the hRSV in their mouse models [8, 9]. Bitko's research focused on the inhibition of the polymerase P gene of the hRSV, responsible for the viral replication and propagation. They infected with 10^7 PFU and transfected siRNAs intra-nasally at 70 μg intra-nasally per mouse and were able to obtain up to 99% inhibition of viral load in their results. Zhang and colleagues were also able to attenuate the virus by targeting the viral nonstructural NS1 gene, speculated to also play a vital role in the replication of the virus. Zhang et al observed a substantial knock down of NS1 gene expression with 10 g of siRNA plasmid with a nanochitosan polymer when administering intra-nasally in mice infected with $5x10^6$ to $1x10^7$ PFU of hRSV.

These findings combined with those from Dowler and Ferrari on F-ANA modifications of siRNA and ASONs, prompted us to investigate whether siRNAs modified with F-ANA would inhibit viral propagation and replication equally as well as or if so, even better than unmodified siRNAs. In this research project, we investigated siRNAs targeting both P and NS1 genes, from the best siRNA sequences selected from Bitko and Zhang's research.

CHAPTER IV

MATERIALS AND METHODS

4.1 CELL CULTURE AND MAINTENANCE

A549 cells were maintained in Ham's F12K with 2 mM L-glutamine and supplemented with 10% of fetal bovine serum (FBS). HEp-2 cells were maintained in Minimal Essential Media with 2mM L-glutamine and Earle's BSS and with 100 U/mL penicillin, 100mg/mL streptomycin, 0.1 mM non-essential amino acids and 1mM sodium pyruvate then supplemented with 10% of fetal bovine serum. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. A549 (lung epithelial) and HEp-2 (larynx epithelial) cell lines were obtained from the American Type Culture Collection (ATCC).

4.2 VIRUS PROPAGATION AND PURIFICATION

Human RSV type A2, obtained from ATCC (#VR-1540), were grown on HEp-2 monolayers and purified by plaque purification. HEp-2 cells are the recommended host cells for RSV propagation [287, 288, 289] (ATCC product description sheet for product VR-1540). HEp-2 cells were seeded in tissue culture flasks and grown to ~80% confluency after an overnight incubation. The cells were then rinsed with 1x Phosphate-buffered saline (PBS), so as to remove traces of FBS, as it may interfere with virus absorption, and then exposed to 0.1 plaque forming units (PFU)/cell for 2 hours at 37°C, with agitation of the flasks every 15 minutes. Viruses were propagated at low multiplicity of infection (0.1 MOI or less) to favor the amplification of the wild-type version of the virus rather than mutant viruses as wild-type viruses are expected to be more efficient at completing the viral life cycle. Following the incubation period, the cells were washed once more with 1xPBS and then replaced with fresh infection medium (HEp-2 culture media with only 2% FBS and no antibiotics). The cells were kept at 37°C, 5% CO_2 and the infection was monitored daily for syncytia formation and cytopathology by examining the cells microscopically, until greater than 75% of the cells were involved in syncytium formation and cytopathology. The cells and the cellular debris were then resuspended vigorously and centrifuged for 10 minutes at 1,000 *g* to remove the debris. As sugars, more specifically sucrose [290, 291, 292], stabilize RSV virus particles the supernatant was

adjusted to 10% sucrose final with 50% sterile sucrose solution (EM Sciences) and stored at -80°C after flash freezing the aliquots in liquid nitrogen. This freezing method ensures no loss of titer over a six-month freezing period [292].

With increasing virus propagations, the likelihood of generating mutant virus particles incapable of replicating themselves but able to bind target cells is greater. These mutant viruses can interfere with cell attachment of fully competent virus particles [293], which can lead to a decrease of the titer of the virus preparations over time. To avoid this situation, plaque purification was performed to isolate efficiently replicating virus clones. HEp-2 cells were seeded in tissue culture plates to obtain a ~80% confluency after an overnight incubation at 37°C, 5% CO_2. Cells were rinsed with 1xPBS and incubated for 2 hours with hRSV in infection media at various PFUs/cell at 37°C, 5% CO_2 with intermittent shaking. Cells were rinsed once again with 1xPBS, overlaid with 0.5% noble agar (Bishop Canada Inc.) in EMEM with 2% FBS and allowed to incubate at 37°C, 5% CO_2 for 4 days. On the day of cloning, 20 well-isolated large virus clones were selected. These virus clones should be wild-type infectious hRSV particles but the possibility of isolating a super infectious hRSV mutant cannot be excluded. In order to minimize the likelihood and impact of such an occurrence, these high titer clones were combined with infection media and incubated at room temperature for 4 hours. The selected cloned viruses were pooled, infected onto pre-seeded HEp-2 cells and adjusted to 10% sucrose final with a 50% sterile sucrose solution in water for storage at -80°C after flash freezing in liquid nitrogen. The final preparations had titers in the range of 10^6-10^7 PFU/mL.

As a control, hRSV was inactivated by treating hRSV to UV irradiation using the UltraLum UV cross-linking apparatus for 20 minutes at 0.12 Joules/cm^2 on ice.

4.3 SiRNA AND siF-ANA SEQUENCES AND ANNEALING

Zhang *et al* and Bitko *et al* both published siRNA sequences that exhibited inhibitory effects both *in vitro* and *in vivo* [8, 9]. The nucleotide sequence for each siRNA selected from their research is as follows:

Name	Target	siRNA sequence
siRSV-P1	RSV-P	5'-CGAUAAUAUAACUGCAAGAdTdT-3' 3'-dTdTGCUAUUAUAUUGACGUUCU-5'
siRSV-P2	RSV-P	5'-CCCUACACCAAGUGAUAAUdTdT-3' 3'-dTdTGGGAUGUGGUUCACUAUUA-5'
siRSV-NS1	RSV-NS1	5'-GGCAGCAAUUCAUUGAGUAdTdT-3' 3'-dTdTCCGUCGUUAAGUAACUCAU-5'
siRSV-NS1a	RSV-NS1a	5'-GUGUGCCCUGAUAACAAUAdTdT-3' 3'-dTdTCACACGGGACUAUUGUUAU-5'
siRSV-P1-Mi	RSV-P	5'-CGAUAAUACGACUGAAAUGdTdT-3' 3'-dTdTGCUAUUAUGCUGACUUUAC-5'
siRSV-P2-Mi	RSV-P	5'-CCCUACACCGAGUAAUAUAdTdT-3' 3'-dTdTGGGAUGUGGCUCAUUAUAU-5'
siRSV-NS1-Mi	RSV-NS1	5'-GGCAGCAAAUCAUAUAGGUdTdT-3' 3'-dTdTCCGUCGUUUAGUAUAUCCA-5'
siRSV-NS1a-Mi	RSV-NS1a	5'-GUGUGCCCGUAUACAAAAUdTdT-3' 3'-dTdTCACACGGGCAUAUGAUUUA-5'

Table I. Sequences of unmodified siRNAs. SiRSV-P1 and siRSV-P2 were designed and tested by Bitko *et al* against the hRSV P protein [8] (shaded in yellow). The siRNA sequences were based on actual sequencing of the viral strains in their laboratory. SiRSV-NS1 and siRSV-NS1a were designed according to the sequences by Zhang *et al* to target hRSV NS1 protein [9](shaded in green). Mismatches (-Mi) were created with scrambling at various positions along the sequence. Sequences were purchased from University Core DNA Services from University of Calgary.

siF-ANAs were designed with modifications of the siRNA by replacing certain RNA by their 2'fluoroarabinosides. **The RNAs that are replaced by F-ANAs were written in bold:**

Name	Target	siRNA sequence
siRSV-P2 F3/O	RSV-P	5'-**CCCTACACCAAGTGATAATTT**-3' 3'-dtdtGGGAUGUGGUUCACUAUUA-5'
siRSV-P2 O/F4	RSV-P	5'-CCCUACACCAAGUGAUAAUdtdt-3' 3'-**TT**GGGAUGUGGUUCACUAUUA-5'
siRSV-P2 F3/F4	RSV-P	5'-**CCCTACACCAAGTGATAATTT**-3' 3'-**TT**GGGAUGUGGUUCACUAUUA-5'
siRSV-NS1 F3/O	RSV-NS1	5'-**GGCAGCAATTCATTGAGTATT**-3' 3'-d**T**d**T**CCGUCGUUAAGUAACUCAU-5'
siRSV-NS1 O/F4	RSV-NS1	5'-GGCAGCAAUUCAUUGAGUAd**T**d**T**-3' 3'-**TT**CCGUCGUUAAGUAACUCAU-5'
siRSV-NS1 F3/F4	RSV-NS1	5'-**GGCAGCAATTCATTGAGTATT**-3' 3'-**TT**CCGUCGUUAAGUAACUCAU-5'
siRSV-P2-Mi F3/O	RSV-P	5'-**CCCTACACCGAGTAATATATT**-3' 3'-d**T**dTGCUAUUAUGCUGACUUUAC-5'
siRSV-P2-Mi O/F4	RSV-P	5'-CGAUAAUACGACUGAAAUGdTdT-3' 3'-**TT**GGGAUGUGGCUCAUUAUAU-5'
siRSV-P2-Mi F3/F4	RSV-P	5'-**CCCTACACCGAGTAATATATT**-3' 3'-**TT**GGGAUGUGGCUCAUUAUAU-5'
siRSV-NS1-Mi F3/O	RSV-NS1	5'-**GGCAGCAAATCATATAGGTTT**-3' 3'-dTdTCCGUCGUUUAGUAUAUCCA-5'
siRSV-NS1-Mi O/F4	RSV-NS1	5'-GGCAGCAAAUCAUAUAGGUdTdT-3' 3'-TTCCGUCGUUUAGUAUAUCCA-5'
siRSV-NS1-Mi F3/F4	RSV-NS1	5'-**GGCAGCAAATCATATAGGTTT**-3' 3'-**TT**CCGUCGUUUAGUAUAUCCA-5'

Table II. Sequences of siRNAs modified with F-ANA. F-ANA modifications were made for siRSV-P2 (shaded in yellow) and siRSV-NS1 (shaded in green) at various positions along the siRNA strands, **denoted in bold**. Mismatches (-Mi) were created with scrambling at various positions along the sequence. Sequences were purchased from University Core DNA Services from University of Calgary.

Mismatches, denoted with a –Mi, were created with modifications at 3 to 6 positions in each strand for every siRNA and siF-ANA as controls.

All oligonucleotides were obtained commercially from the University of Calgary DNA Synthesis Laboratory (Calgary, AB). The siRNAs and siF-ANAs were purchased from the center already gel-purified, thus purity was not defined. The single stranded siRNAs or siF-ANAs were resuspended in RNAse-free H_2O, dosed by Spectrophotometry and adjusted to a final concentration of either 50 or 150 μM. Equal molarity of each complementary strands were combined with siRNA annealing buffer (Tris 250 mM, NaCl 500 mM in RNAse-Free H_2O with pH 7.5-8.0) then incubated at 90°C for 1-5 minutes before being allowed to gradually cool to below room temperature. Final concentrations of the annealed complex were either 20 or 60 μM.

4.4 SiRNA TRANSFECTION AND VIRUS INFECTION *IN VITRO*

SiRSV-P1, siRSV-P2, siRSV-NS1, siRSV-NS1a and controls (their respective mismatches) were diluted to equal concentration of 0.1μg/uL and then further diluted in Opti-MEM to have final quantities of 0.05, 0.1, 0.2 and 0.4 μg per reaction. Lipofectamine 2000 (Invitrogen, Burling, ON) reagent was diluted to a DNA : lipid ratio of 1:3 and incubated at room temperature for 5 minutes. Equal volumes of siRNA and reagent were combined and incubated for 20 minutes at room temperature to allow complex formation. A549 cells were then transfected drop-wise with this complex. Following an overnight incubation at 37°C, 5% CO_2, the transfected cells were rinsed with 1xPBS and then infected with hRSV at an MOI of 1 (i.e. 1 PFU/cell) in A549 infection medium (similar to A549 culture media with 2% FBS). Virus was incubated with the cells for 2 hours at 37°C, 5% CO_2 with intermittent shaking, after which the media was removed and replaced with fresh infection medium for overnight incubation.

4.5 IMMUNOSTAINING PLAQUE ASSAY

Four days following infection, supernatant from infected A549 cells were harvested and serially diluted tenfold with A549 infection medium. HEp-2 cells, seeded from previous

day, were rinsed with 1xPBS to remove traces of FBS and the diluted A549 supernatants were loaded onto the cells and allowed to adsorb for 2 hours at 37°C, 5% CO_2 with intermittent shaking. Once the adsorption time of the virus has been completed, sterile noble agar mixture (as previously described) was overlaid onto the cells and allowed to solidify at room temperature. Cells were incubated at 37°C, 5% CO_2 for 4 days.

On day of staining, the agar overlay was removed by aspiration and a fixing solution containing 4% formaldehyde (Sigma-Aldrich) in 1xPBS was added for 15-30 minutes to the cells. The fixing solution was removed by washing with 1xPBS and then a blocking solution (5% bovine serum albumin (BSA) fraction V in 1xPBS containing 0.1% Tween 20 (PBS-t, purchased from Sigma)) was added for 2 hours to block non-specific absorption of other proteins. Following the incubation period, the blocking solution was removed and the cells were incubated for 1 hour at room temperature with biotin-labeled goat anti-RSV antibody (Virostat) diluted 1:480 in 5% BSA-1xPBS-t. Excess goat antibody was removed by performing three 5-minute incubations at room temperature with 1xPBS. The cells were then incubated for 1 hour at room temperature with a 1:300 dilution of streptavidin-alkaline phosphatase (Calbiochem) in 5% BSA 1xPBS-t. Excess streptavidin remnants were removed by washing twice with 1xTBS (Tris-buffer saline) for 15 minutes at room temperature. For the color reaction, BCIP/NBT solution (Sigma-Aldrich) was added to the cells and incubated at room temperature in the dark until desired coloration was achieved.

4.6 ANIMALS

The study was approved by the Animal Ethics Committee of Mispro Biotech Services (Montreal, QC). Female BALB/c mice 6 to 8 weeks of age and weighing 14 to 18 g were purchased from Charles River Laboratories. Mice were maintained in Mispro's animal facilities in filter cages in a separate room. Mice were anesthetized via intraperitoneal administration of 0.15 mL of xylazine-ketamine mix (100 mg/kg and 10 mg/kg

respectively) prior to administration of siRNA or virus. At time of sacrifice, mice were administered 0.2 mL sodium pentobarbital (45mg/kg) followed by exsanguination.

4.7 NASAL ADMINISTRATION OF siRNA AND hRSV

For nasal delivery of siRNA, various amounts of siRNA were diluted in Opti-MEM to obtain a final volume of 50 µl. hRSV was diluted in 10% sucrose such that 50µl contained $5x10^5$ PFUs. Sham infection was performed with the same volume of vehicle. While under anesthesia, the mice were inoculated intra-nasally, with the use of a micropipette, into a single nostril with selected siRNA and then followed by an infection into same nostril after a short period of rest for the animals. On day 3, 6 and 10 post-infection, the mice were sacrificed as described above then BAL was performed and whole lungs were harvested. As a positive control, hRSV-infected mice were given 50 mg/kg/day of ribavirin subcutaneously daily. On day 6 post-infection, the mice were sacrificed, BAL was performed and whole lungs were collected for further analyses.

4.8 BRONCHOALVEOLAR LAVAGE, WHOLE LUNG COLLECTION AND CELL COUNT

Bronchoalveolar lavage (BAL) fluid was collected by perfusing the bronchi and washing the lungs 5 times with 0.8 mL cold normal saline. Total volume recovered was 3.5-3.8 mL per mouse. BAL fluid was centrifuged at 1600 rpm for 7 minutes at 4°C, and then supernatant was aliquoted and stored at -20°C until determination of cytokine and chemokine measurements such mouse monokine induced by gamma interferon (mMIG) and tumor necrosis factor-alpha (TNF-α) by enzyme-linked immunosorbent assay (ELISA) kits along with hRSV protein levels). The remaining BAL cells were resuspended in filtered RPMI-20%FBS and the viability of the cells was established by ViaCount (GUAVA). For subsequent differential cell analysis by microscopy, BAL cells

were cytospun onto slides using the Cytospin 3 (Shannon) and stained with Hema-3 stain (Fisher). Lungs were collected by opening the chest cavity and retrieving the whole lung that was then cut into 0.5 cm pieces. Lung specimens were immediately snap frozen and stored at -80°C for later examination of target mRNA levels by real-time RT-PCR.

4.9 ANALYSIS OF INTRACELLULAR CYTOKINE PRODUCTION IN CELL SUPERNATANT AND BAL

Interleukin-8 (IL-8), mMIG, TNF-alpha ELISAs were carried out according to manufacturer's protocol from the BD OptEIA™ Set Human IL-8 (BD Biosciences, Mississauga, ON), Mouse CXCL9/MIG DuoSet kit (R&D) and Mouse TNF-alpha CytoSets anibody pair system (BioSource) respectively.

4.10 PROTEIN EXPRESSION ANALYSIS BY hRSV PROTEIN ELISA

ELISA plate wells were first coated with a coating antibody solution composed of unconjugated polyclonal goat anti-RSV antibody (Virostat, Portland, ME) diluted 1:1,000 in 1xPBS and incubated for 90 minutes at 37°C followed by washing 3 times with 1xPBS-t. Wells were then blocked with 5% BSA in 1xPBS (i.e. assay diluent) and incubated at room temperature for one hour after which wells were washed another three times with PBS-t. Serial dilutions of RSV for the standards and the diluted supernatant or BALF were dispensed into the wells and incubated overnight at room temperature. On the following day, signal detection began with first washing the wells with PBS-t 3 times then the addition of detection antibody solution (i.e. biotin conjugated polyclonal goat anti-RSV antibody (Virostat, Portland, ME) diluted 1:20,000 in assay diluent). Wells were incubated with the detection antibody solution at 37°C for 75 minutes then were washed 3 times with PBS-t. Streptavidin horseradish peroxidase (HRP) diluted at 0.2 µg/mL was applied to the wells and incubated for 45mins at room temperature followed by three washes with PBS-t. Tetra Methyl Benzidine (TMB) (Neogen) substrate solution

was added and the color reaction was allowed to develop in the dark. The color reaction was stopped with the addition of 2N H_2SO_4 and the signal was read within 30 minutes using the ELISA Microplate reader at 450 nm subtracting the signal detected at 540 nm.

4.11 DETECTION OF VIRAL mRNA IN CELL LYSATES AND LUNG HOMOGENATES

Total RNAs were extracted directly by Qiagen RNeasy Kit (Qiagen, Mississauga, ON) from cell lysates in RLT buffer with DNAse treatment carried out on column according to manufacturer's protocol, in RNeasy Mini Handbook. Whole lungs harvested from mice were homogenized with either the Polytron PT 1200 (Kinematics, Switzerland) or the Precellys 24 (Bertin, France) in RLT buffer then RNA was extracted from the supernatant using Qiagen RNeasy Kit.

Quantification of RNA was performed using the Ribogreen fluorescent assay according to its product information sheet from Invitrogen-Molecular Probes. Final concentration of 1 μg/μl of total RNA was used to prepare cDNA. Reverse transcription was performed by adding a mixture of 10mM dNTPs, Random Hexamers in RNAse-free H_2O with the RNA samples, and heated at 65°C for 5 minutes. The reaction was put on ice for at least 1 minute to allow the temperature to decrease to 25°C (since Random primers were used). A second mixture composed of 5X buffer, 0.1M DTT, SSII-RT in RNase-free H_2O is added to the reaction for a 20 μl total and allowed to equilibrate for 10 minutes at 25°C followed by 1 hour at 50°C until the first strand cDNA had been synthesized.

Gene expression of target RSV P and NS1 mRNAs was analyzed by real-time PCR with Roche Lightcycler®2.0 instrument using the LightCycler® DNA Master SYBR Green I kit (Roche, Laval, QC). 17 μl of 5X Roche SYBR Green mix, 25 mM RSV primers (both forward and reverse) in RNase-free H_2O was added to 3μl of diluted cDNA samples, and allowed to cycle at 57°C for 40 cycles. Primers for RSV P and NS1 mRNA (TibBioMol)

were based on RSV (*Acc#* M74568) according to Bitko *et al* and Zhang *et al* 's published research [8, 9] **(Table III)**.

Target		Primer sequence
RSV-P	forward	5'-CCCTTTTCTAAACTATACAAAGAAACC-3'
	reverse	5'-AGCAGATGTAGGTCCTGCACTTG-3'
RSV-NS1	forward	5'-ATGGGGTGCAATTCATTGAG-3'
	reverse	5'-CAGGGCACACTTCACTGCT-3'

Table III. Primer sequences. Targeted against RSV P and NS1 genes.

Ppib and β2m were determined to be the optimal housekeeping genes for *in vitro* and *in vivo* experiments, respectively and the levels of RSV-P and RSV-NS1 in samples were expressed as a ratio to the housekeeping genes. A calibrator and a negative control (i.e. RNase-free H_2O) were included in every PCR run.

4.12 STATISTICAL ANALYSIS

All results are expressed as mean values for the groups analyzed ± 1 standard error of the mean (SEM) (error bars in graphs). Groups were compared to either their respective mismatches or to the hRSV-only control by Mann-Whitney test Differences were considered to be significant at $P < 0.05$.

CHAPTER V

RESULTS

5.1 ESTABLISHMENT AND VALIDATION OF MEASUREMENT METHODS

5.1.1. Establishing and maintenance of hRSV culture in human epithelial HEp-2 cells.

HEp-2 cells are host cells for RSV propagation as recommended by the ATCC and in numerous studies [288] [287] [289]. Our initial experiments were aimed to not only demonstrate that in our hands hRSV was able to properly propagate on HEp-2 monolayers but also that immunostaining was a sensitive and straightforward method of counting virus plaques to determine titers (i.e. the "gold-standard"). In the presence of hRSV on the HEp-2 cells, immunostaining produces a dark purple precipitate (shown as a dark gray dot in the black and white **Figure 1**), whereas areas without syncytia formations are not detected by the assay.

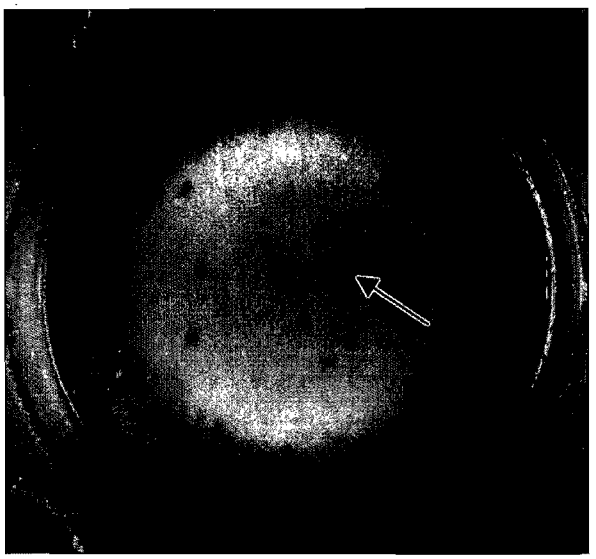
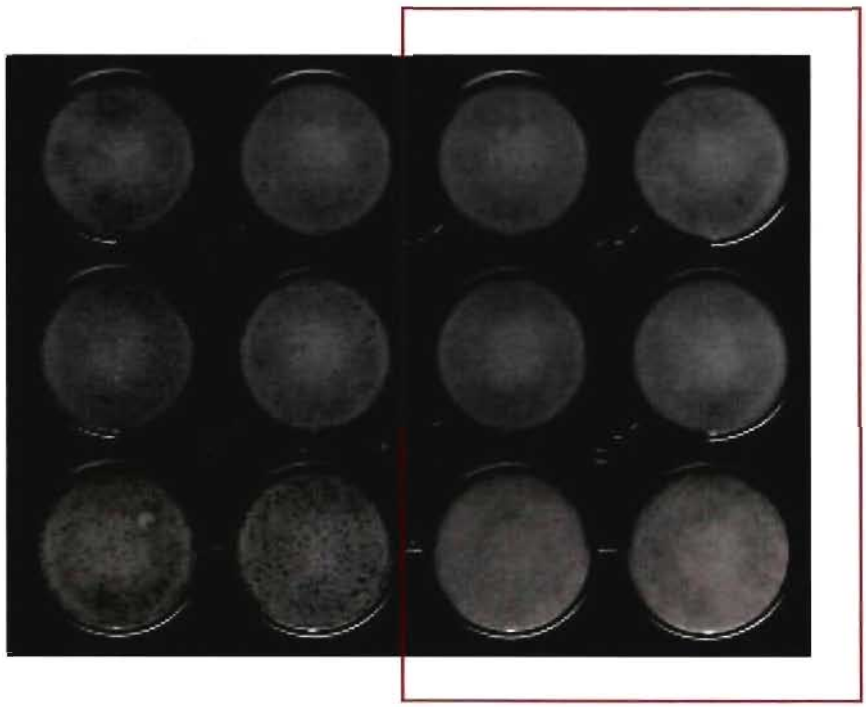


Figure 1. Detection of hRSV on HEp-2 monolayer by method of immunostaining. Confluent monolayers of HEp-2 cells were infected with hRSV strain A2. On day 4 post-infection (PI), the cells were fixed then immunostained with a combination of goat anti-RSV antibodies linked to biotin and streptavidin conjugated to alkaline phosphatase. The virus plaques were visualized after the cells were incubated with BCIP/NBT. An hRSV plaque detected is shown with arrow. Figure is representative of a single well. Average titer of hRSV measured from duplicates was found to be 1.99×10^7 PFU/mL.

However, because the hRSV preparation was made in house and may contain other molecules secreted during the infection process, UV-inactivated hRSV was used as a control. When the virus was treated for 20 minutes at 0.12 J/cm^2, the UV rays completely abrogated viral replication **(Figure 2 B)**. No hRSV plaques were detected on the HEp-2 cells of the UV-treated virus preparation, while plaques were clearly visible when the virus preparation did not undergo an UV irradiation **(Figure 2 A)** to yield an estimated titer of 2.7×10^8 PFU/mL.



(A) **(B)**

Figure 2. Propagation of UV-treated hRSV. (A) As a control, half of the viral preparation did not receive any irradiation and was incubated on ice. **(B)** The other half was subjected to a 20 min 0.12 J/cm^2 UV treatment on ice using the UV cross-linker apparatus (framed in red). Then, the titer of both aliquots was determined by plaque assay. The UV irradiated aliquot was assayed undiluted and at a 1:10 and 1:100 dilution. The aliquot that was not UV irradiated was assayed at the following dilutions: 10^{-3}, 10^{-4} and 10^{-5}. Each dilution was performed in duplicate.

These findings clearly demonstrated the usefulness of the immunostaining assay and as such this assay was used in subsequent studies.

5.1.2 Inhibition of RSV infection with ribavirin

Although the recommended cell host for hRSV growth is HEp-2 cells, the human lung epithelial cell line A549 have also been used for infection and has been optimized in our laboratory for certain transfections with siRNAs. Therefore, in order to use this cell line for transfection assays to inhibit viral propagation, we first had to determine the experimental conditions necessary to detect viral replication. Stability was also investigated to determine the optimal storage conditions for re-suspended ribavirin, as no information on re-suspended ribavirin was available. A549 cells were exposed to hRSV as described in the methods and the immunostaining technique was used to determine viral propagation in this cell type. Experiments included treatment conditions where A549 cells were treated with the anti-viral ribavirin, stored either at 4°C or -80°C, for 2 hours prior to viral exposure and maintained in ribavirin-containing medium after virus absorption. When treating infected cells with increasing doses of Ribavirin, a dose-dependent response was observed in both the titer levels **(Figure 3)** and the protein level **(Figure 4)** with almost complete inhibition of RSV replication at the highest dose, under both storage conditions. However, at the 100 μM dose, the ribavirin that was kept at 4°C appeared slightly more efficient (Figure 3). As for the lower doses, 50 μM and 10 μM, the titer decrease with ribavirin was similar between 4°C and -80°C, thus both storage conditions seem equivalent. Since ribavirin was stable after undergoing one freeze thaw cycle and the long-term stability of ribavirin at 4°C was unknown, re-suspended ribavirin stored at -80oC was used to carry out all future experiments utilizing ribavirin.

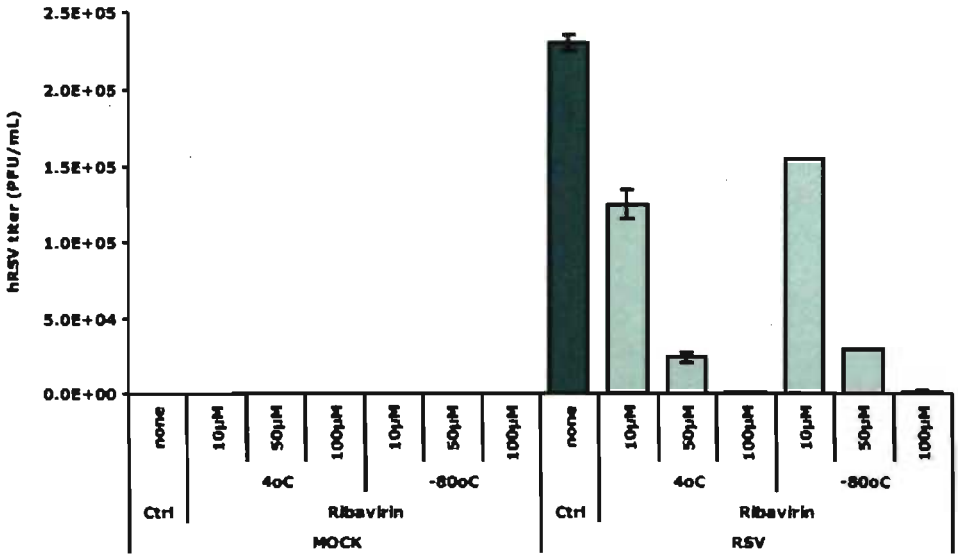


Figure 3. hRSV titer levels detected in A549 cells treated with ribavirin. A549 cells were treated for 2hrs with ribavirin (10 µM, 50 µM or 100 µM final), stored either at 4°C or -80°C, in duplicates. Following this treatment, the cells were infected at an MOI of 1 with hRSV strain A2 or MOCK infected with the same volume of vehicle only. After a 2hr absorption, the virus inoculum was removed and fresh infection media containing ribavirin to 10 µM, 50 µM or 100 µM final was laid over the A549 cells. On day 3 PI, the supernatants from the infected A549 cells were recovered and analyzed by immunostaining plaque assay. hRSV titer expressed as mean ± SEM.

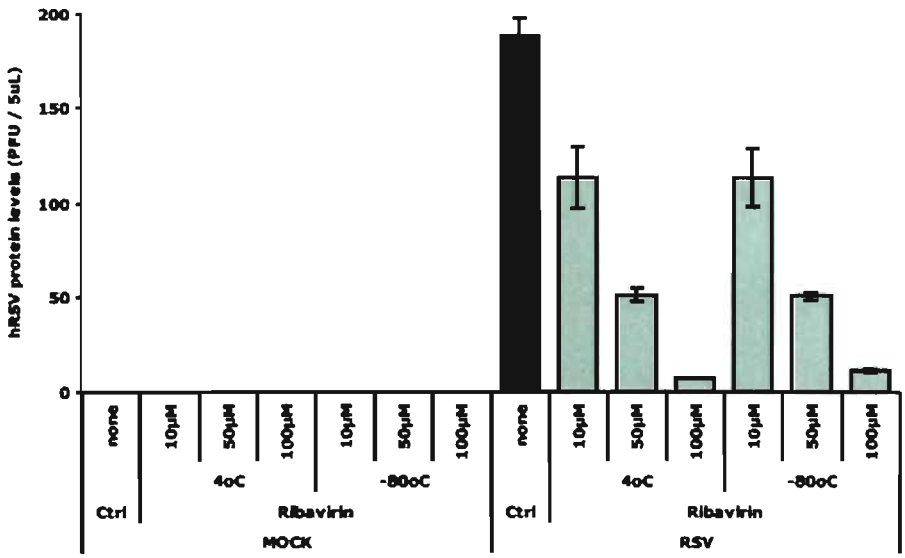


Figure 4. hRSV protein levels detected in A549 cells treated with ribavirin. A549 cells were treated for 2hrs with ribavirin (10 µM, 50 µM or 100 µM final), stored either at 4°C or -80°C, in duplicates. Following this treatment, the cells were infected at an MOI of 1 with hRSV strain A2 or MOCK infected with the same volume of vehicle only. After a 2hr absorption, the virus inoculum was removed and fresh infection media containing ribavirin to 10 µM, 50 µM or 100 µM final was laid over the A549 cells. On day 3 PI, the supernatants from the infected A549 cells were recovered and analyzed with hRSV ELISA. hRSV titer expressed as mean ± SEM.

5.1.3 Establishing infection of hRSV in A549 cells.

As stated in the previous section, both HEp-2 and A549 cells have been able to efficiently propagate the virus, however as the main focus of this project is the inhibition of the virus with siRNAs, and the cell line optimized for siRNA transfection was A549 cells, we determined whether the viral growth in A549 cells paralleled the growth seen in the ideal HEp-2 cells. Monolayers of both cell lines were infected with increasing MOIs and immunostaining plaque assay was utilized on the infected supernatant 3 days post-infection. Upon visual inspection, A549 cells were not as susceptible as HEp-2 cells to hRSV infection since the infection-mediated morphological changes and cell death were delayed in A549 cells relative to HEp-2 cells. However, only in A549 cells, and not in HEp-2 cells did the viral titer vary in a dose-dependent fashion **(Table I and Figure 5)**. In A549 cells, the titer increased by 10-fold for every10-fold increase in the MOI used to initially infect the cells, whereas in HEp-2 cells, there was a 10 fold increase in the titer of the cells infected with an MOI of 1 and 10 but the titer was of the same magnitude when comparing the cells infected with an MOI of 0.1 and 1.

Experimental Condition			PFU/mL	SEM
A549	MOCK	MOCK	0	0
	RSV	MOI of 0.1	2.76×10^4	1.26×10^3
		MOI of 1	3.30×10^5	3.52×10^4
		MOI of 10	3.46×10^6	1.91×10^5
HEp-2	MOCK	MOCK	0	0
	RSV	MOI of 0.1	4.56×10^5	1.01×10^4
		MOI of 1	5.99×10^5	4.86×10^4
		MOI of 10	5.81×10^6	1.14×10^5

Table I. Titer measured by plaque assay on day 3 PI from supernatant. A549 and HEp-2 cells were either mock infected or infected with hRSV at an MOI of 0.1, 1 and 10 in triplicates. On day 3 post-infection, the supernatant was used to determine the virus titer by immunostaining plaque assay.

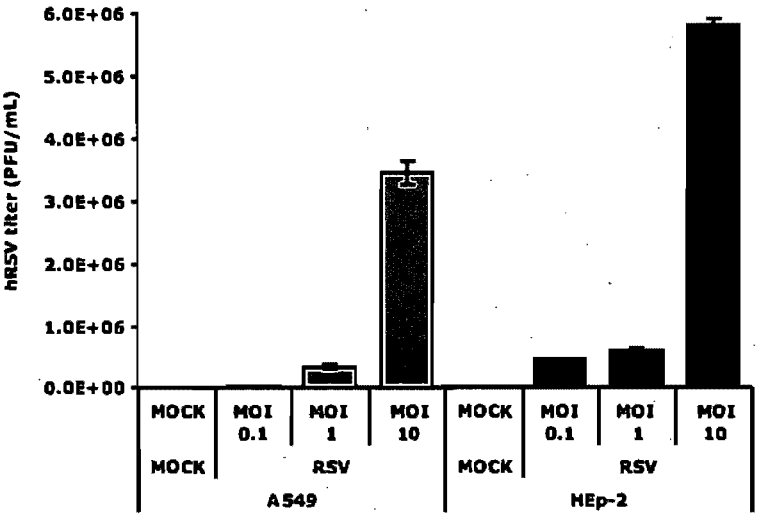


Figure 5. Viral titer measured by plaque assay on day 3 PI from supernatant. A549 and HEp-2 cells were either mock infected or infected with hRSV at an MOI of 0.1, 1 and 10 in triplicates. On day 3 post-infection, the supernatant was used to determine the virus titer by immunostaining plaque assay. Serial dilutions were performed in quadruplicates and means were calculated with 10^{-3} the plaque counts ± SEM.

We therefore concluded that A549 cells were a good cellular system to investigate hRSV replication.

5.1.4 Determination of readout methods for measuring infection.

Although viral load is most accurately assessed by immunostaining plaque assay, this procedure is highly time-consuming and in order to rapidly screen molecules that interfere with hRSV infection, a faster readout monitoring hRSV virus production was required. As such we investigated alternative readouts including assessment of viral protein levels by hRSV ELISA, secretion of cytokine (IL-8 and MIP-1α) as assessed by ELISA, and viral mRNA levels by real-time RT-PCR.

Developing an ELISA to monitor viral protein production offers several advantages as it is faster than plaque assay, allows high throughput as it can be performed using a small amount of material on 96-well assay plates. First however, we needed to determine whether viral protein detection would correlate to the level of virus replication.

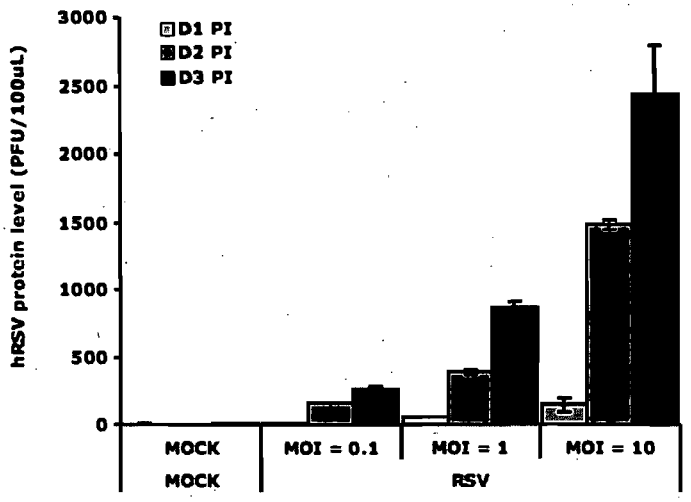


Figure 6. hRSV protein levels detected with hRSV ELISA over time. Supernatant from A549 cells infected at an MOI of 0, 0.1, 1 and 10 with hRSV were harvested on day 1, 2 and 3 post-infection. The samples were diluted 1:20 in assay diluent which was found to be the most appropriate for signal detection. Unconjugated goat polyclonal anti-RSV antibodies (coating antibody; Virostat) was diluted 1:1000, biotin-conjugated polyclonal goat anti-RSV antibodies (detection antibody; Virostat) was diluted at 1:20,000 and Streptavidin-HRP (Biosource) was diluted at 0.2 ug/mL in 5%BSA PBS-t. hRSV protein levels expressed as mean of duplicates ± SEM.

Using the ELISA method, hRSV protein was not detected in mock-infected cells over time, whereas hRSV infected cells exhibited an increasing dose-dependent response with each additional day **(Figure 6)**. The ELISA was optimized under conditions such that A549 cells were infected with low MOIs between 1 and 5 and samples harvested either on day 2 or 3 post-infection.

The response of cells to hRSV infection is also of great importance and can be examined by measuring specific cytokines secreted by the host cells as an indirect of method of quantifying the viral propagation. Different groups have shown that RSV-infection of A549 cells can induce IL-8 production [294, 295, 296]. One group in particular has shown that for A549 cells, there is an hRSV dose-dependent release of IL-8 [295]. Thus, IL-8 was verified as a quantitative readout method of hRSV infection in A549 cells **(Figure 7)**. In A549 cells, we observed that IL-8 release increased over time and with increasing MOI.

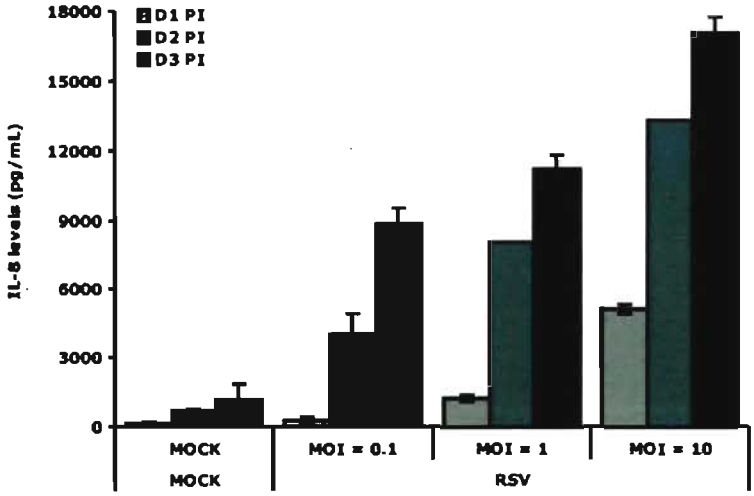


Figure 7. IL-8 secretion levels detected by IL-8 ELISA over time. A549 cells were either mock infected or infected with hRSV at an MOI of 0.1, 1 and 10 in duplicates. On days 1, 2 and 3 post-infection, the supernatants were collected for IL-8 ELISA (BD Biosciences). Data expressed as mean ± SEM.

In comparison to IL-8, another cytokine, MIP-1α was not detected in mock-infected cells over time **(Figure 7)**. Assaying for MIP-1α by ELISA therefore yielded results that are easier to interpret without the potential for conflicting background cytokine levels. Assaying MIP-1α release via ELISA is a reliable readout for hRSV replication. This system will be most sensitive at detecting molecules interfering with hRSV replication in supernatants collected on day 2 post-infection from A549 cells infected at an MOI of 1.

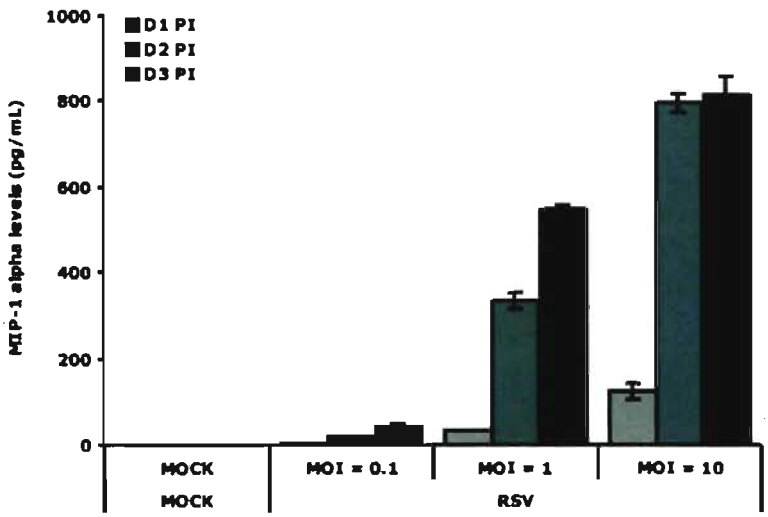


Figure 8. MIP-1α secretion levels detected by MIP-1α ELISA over time. Supernatants from A549 cells infected at an MOI of 0, 0.1, 1 or 10 were collected on day 1, 2 and 3 post-infection. The MIP-1α content of these supernatants was determined by ELISA (R&D). Data expressed as mean of duplicates ± SEM.

Measuring cytokine secretion levels by the infected cells is an indirect method of measuring viral propagation. In order to directly determine viral replication, examination at the mRNA level was also considered (**Figure 9**). hRSV viral mRNA levels in RSV-infected A549 cell lysates were assessed via real-time RT-PCR. Optimization of real-time RT-PCR method is crucial and determination of the primers for reverse transcription and housekeeping gene plays an important role. Two reverse transcription primers, Oligo dT and Random Hexamers, were examined in which no difference was observed between the levels detected, therefore either primer had the capacity for transcription. Potential housekeeping genes Ppib, ribosomal 18S and β-actin were all tested, however Ppib showed the least amount of variability between samples thus it was determined to be the optimal housekeeping gene for *in vitro* screening of hRSV P mRNA. Under such conditions, hRSV P mRNA was detectable by real-time PCR 24 hours post-infection. The optimal infection load appeared yet again to be at MOI of 1 (**Figure 9**). A dose-response was observed from MOI of 0.1 to 1 however; at MOI of 10 there is a decrease in the amount of hRSV P mRNA. This may possibly be explained by cell death caused by an overabundance of virus. Nonetheless, viral mRNA can be readily detected by real-time PCR at very low MOIs and was included as a reliable readout for hRSV propagation.

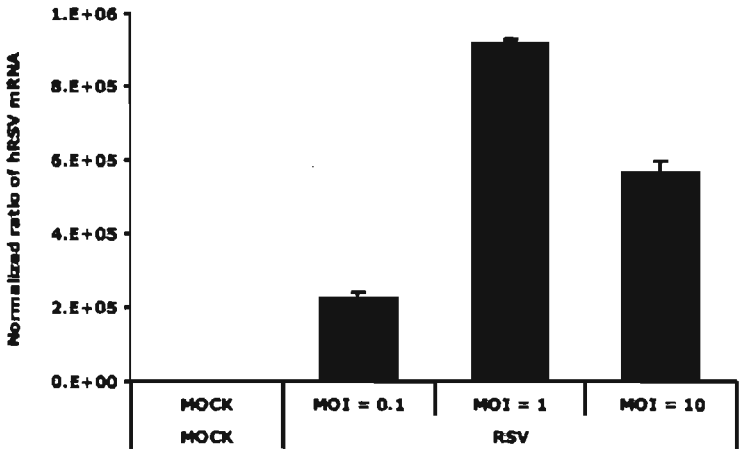


Figure 9. mRNA levels detected by real-time RT-PCR. Cell lysates were harvested from A549 cells infected with hRSV at various MOIs 24 hrs post-infection, in duplicates, and RNA was isolated with RNeasy kit. Reverse transcription was performed to make cDNA by Random Hexamers. Viral P gene was amplified by real-time PCR with the Qiagen kit using Ppib as the housekeeping reference gene. CP values were normalized against the housekeeping gene and expressed as mean ± SEM.

In summary, A549 cells provided a suitable and valid cellular system to investigate hRSV replication since they exhibit a dose-dependent virus production, which can be monitored by assessment of either viral protein levels, IL-8 and MIP-1α release, and also viral mRNA levels.

5.2 CHOICE OF APPROPRIATE siRNAS:

5.2.1 Designing siRNAs and their mismatches

According to research published by Bitko and Zhang, inhibition of viral replication can be achieved following intranasal administration of siRNAs [8, 9]. As one of our goals was to determine the effect of chemical modifications of siRNA, we opted to select two siRNAs targeting the hRSV P protein and two against the NS1 protein for study. These siRNAs were synthesized by University Core DNA Services (University of Calgary) with 3' dT overhangs, initially as unmodified siRNAs **(Table II)**.

siRNA name	Sequence
siRSV-P1	5'-CGAUAAUAUAACUGCAAGAdTdT-3'
siRSV-P2	5'-CCCUACACCAAGUGAUAAUdTdT-3'
siRSV-NS1	5'-GGCAGCAAUUCAUUGAGUAdTdT-3'
siRSV-NS1a	5'-GUGUGCCCUGAUAACAAUAdTdT-3'
siRSV-P1-Mi	5'-CGAUAAUACGACUGAAAUGdTdT-3'
siRSV-P2-Mi	5'-CCCUACACCGAGUAAUAUAdTdT-3'
siRSV-NS1-Mi	5'-GGCAGCAAAUCAUAUAGGUdTdT-3'
siRSV-NS1a-Mi	5'-GUGUGCCCGUAUACAAAAUdTdT-3'

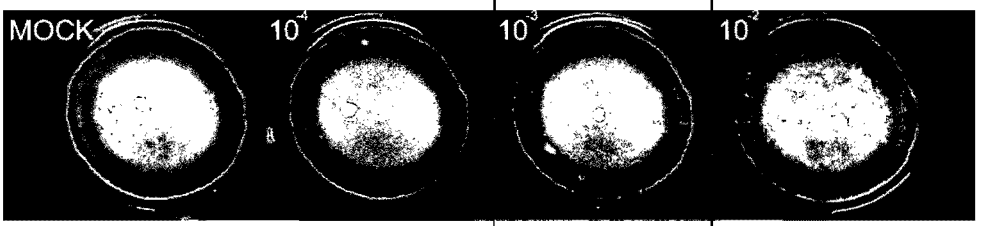
Table II. SiRNA name and sequences. siRSV-P1 and siRSV-P2 were designed and tested by Bitko *et al* against the hRSV P protein [8] (shaded in yellow). The siRNA sequences were based on actual sequencing of the viral strains in their laboratory. SiRSV-NS1 and siRSV-NS1a were designed according to the sequences by Zhang *et al.* to target hRSV NS1 protein[9](shaded in green). Mismatches (-Mi) were created with scrambling at various positions along the sequence. Sequences were purchased from University Core DNA Services from University of Calgary.

5.2.2 *In vitro* screening of all four siRNAs:

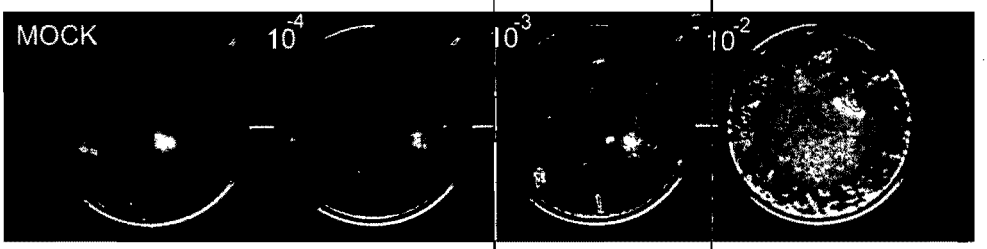
With the infection and readout methods optimized, the four unmodified siRNAs (**Table II**, shaded in yellow and green) were screened for in vitro efficacy to inhibit RSV propagation. SiRSV-P1, -P2, -NS1 and –NS1a were transfected in A549 cells followed by an hRSV infection 24-hours later at an MOI of 1. Supernatant and cell lysates were harvested on different days post-infection according their respective readout methods.

(A) Controls

Mock transfected + Mock infected

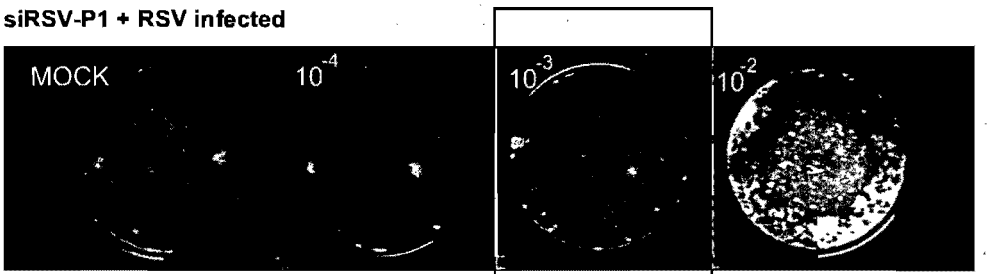


Mock transfected + RSV infected

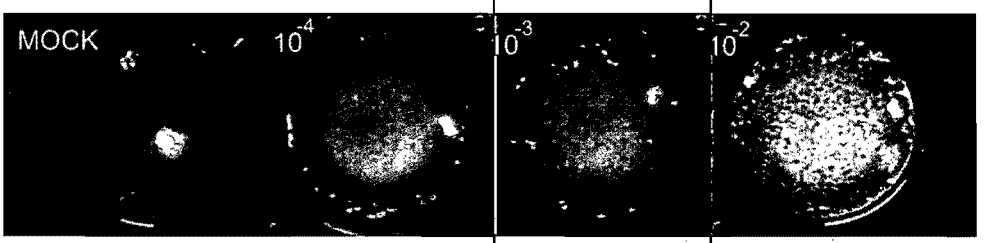


(B) Transfection with siRSV-P1

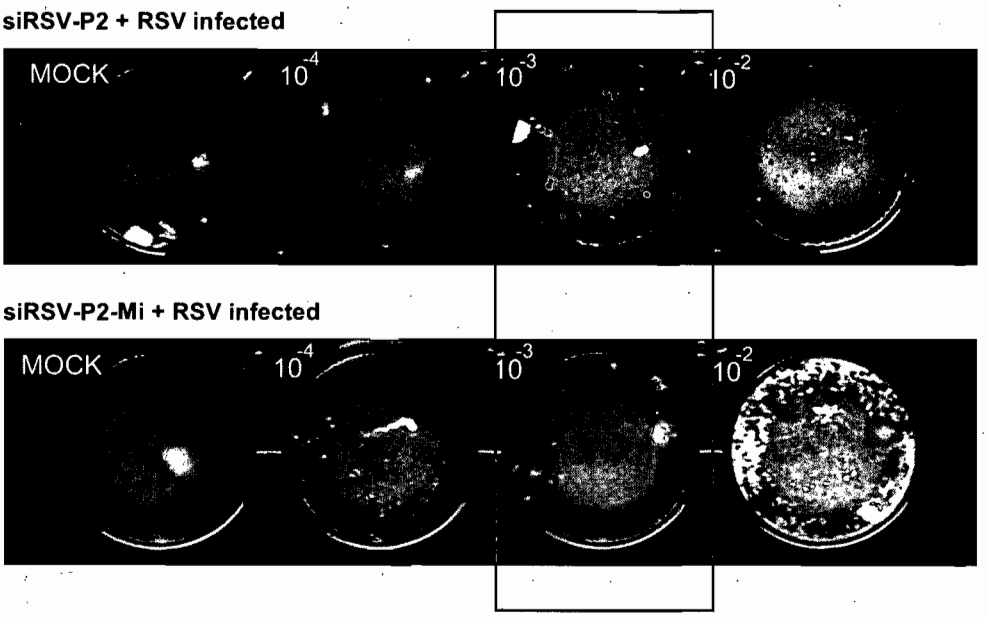
siRSV-P1 + RSV infected



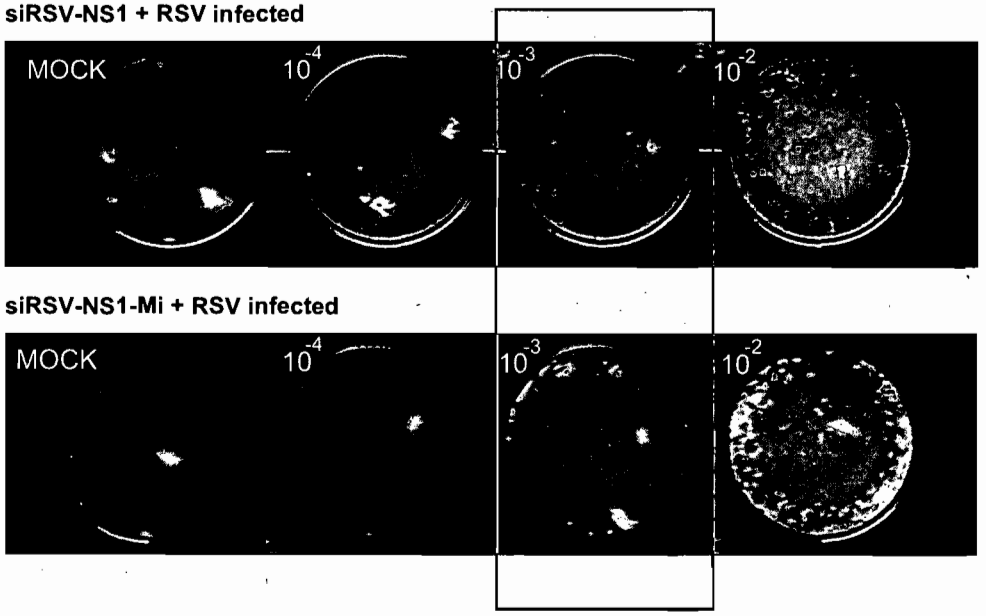
siRSV-P1-Mi + RSV infected



(C) Transfection with siRSV-P2



(D) Transfection with siRSV-NS1



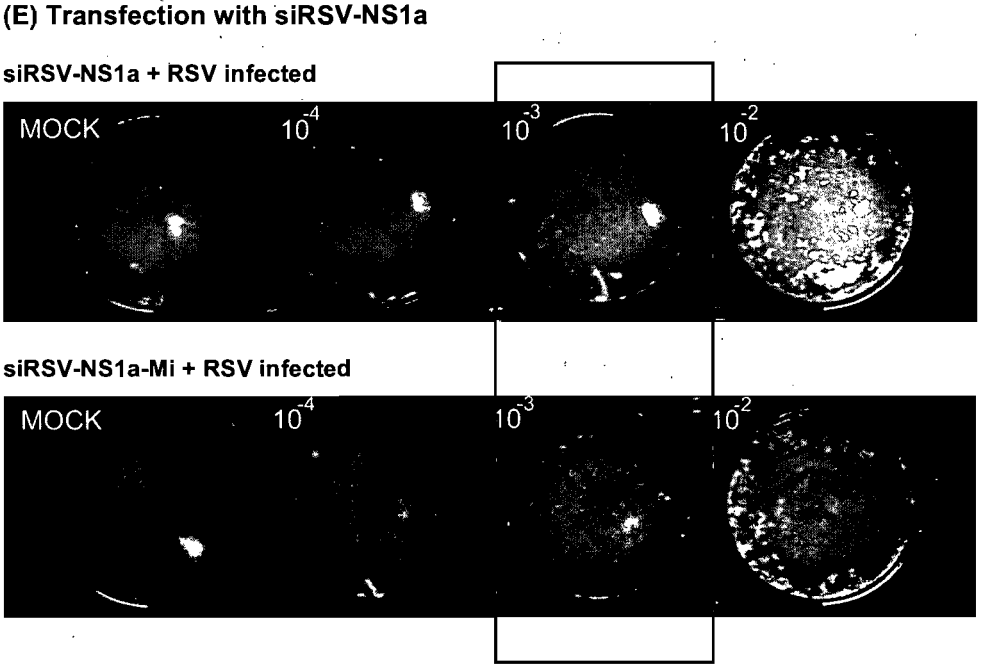


Figure 10. Inhibition of hRSV titer by immunostaining plaque assay. A549 cells were transfected with siRSV-P1, -P2, -NS1, -NS1a and their respective mismatches at 0.4 μg with Lipofectamine 2000 at a ratio of 1:3, in duplicates, followed by an overnight incubation at 37°C, 5%CO_2. Cells were then infected with hRSV at MOI of 1 and allowed to incubate for 2 more days in which serial dilutions of the supernatant was overlayed on HEp-2 cells. Immunostaining was performed on day 4 post-infection. **(A)** Negative and positive controls, **(B)** siRSV-P1 and –Mi, **(C)** siRSV-P2 and –Mi, **(D)** siRSV-NS1 and –Mi and **(E)** siRSV-NS1a and –Mi. Plates performed in duplicate. hRSV titer was determined with the 10^{-3} dilution (outlined in red).

Immunostaining plaque assay was performed in order to obtain a precise determination of the inhibition of viral load in hRSV-infected cells by any of the four siRNAs, despite the time-consumption involved with this assay. As controls, A549 cells were mock transfected followed by either infection with hRSV or mock infection with vehicle. SiRSV-P1, siRSV-P2, siRSV-NS1 and siRSV-NS1a were individually transfected over an overlay of A549 cells followed by an infection with hRSV at MOI of 1 24 hours following transfection. Cell supernatant were harvested on day 2 post-infection, dispensed over HEp-2 cells overlayed with agar and immunostained 4 days post-infection. Titer was only determined for dilutions with a good number of discrete countable plaques. Ideally, the number of plaques per well should be between 20 and 80 for a 12-well plate to establish the virus titer. Thus the 10^{-3} dilution was used for plaque counting **(Figure 10)**. Two siRNAs, siRSV-P2 and siRSV-NS1 appeared to have almost completely inhibited viral growth (90%) compared to their respective mismatches, while

siRSV-P1 had very mild inhibitory effect of 13% and siRSV-NS1a did not appear to inhibit the virus whatsoever.

Efficacy of siRSV-P1, siRSV-P2, siRSV-NS1 and siRSV-NS1a to inhibit RSV infection of A549 cells was also assessed by determining hRSV protein levels **(Figure 11)**, and secretion of MIP-1α **(Figure 12)** and IL-8 **(Figure 13)** by these cells. When measuring hRSV proteins, siRSV-P1, siRSV-P2 and also siRSV-NS1a inhibited at either all or some doses, however siRSV-P2 was the sole siRNA among the four siRNAs that exhibited a non-significant dose-response inhibition **(Figure 20)**. At the lowest 0.1 μg dose a 35% ± 1% inhibition was observed which increased to 55% ± 3% at the highest dose of 0.4 μg.

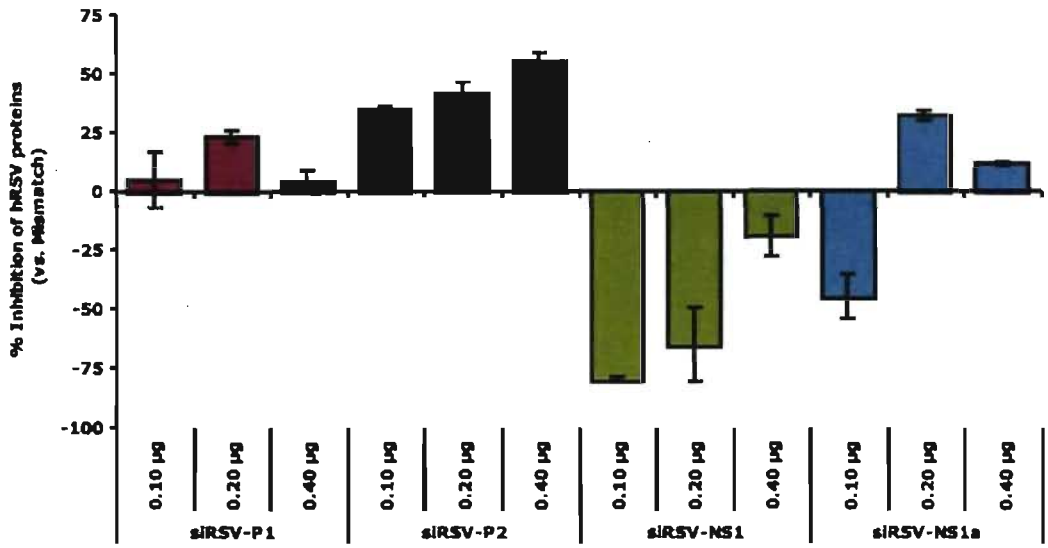


Figure 11. % Inhibition of hRSV protein in hRSV infected A549 cells. A549 cells were transfected with 0.1 μg, 0.2 μg, and 0.4 μg of siRSV-P1, siRSV-P2, siRSV-NS1, siRSV-NS1a and their mismatches with Lipofectamine2000 transfection reagent ratio "1:3" in quadruplicates. A set of untransfected cell cultures was used as control group. One day post-transfection, cells were then infected with hRSV at MOI of 1 and kept incubated at 37°C, 5%CO_2 overnight. Supernatants were harvested 24-hrs post-infection and hRSV protein levels were assessed by hRSV ELISA, expressed as mean ± SEM.

Two siRNAs, siRSV-P2 and siRSV-NS1 inhibited MIP-1α levels **(Figure 12)**, however the efficacy of siRSV-P2 was maintained throughout all three doses tested. Significant inhibitions for siRSV-P2 ranged from 48% ± 1% at the lowest dose to 58% to 73% at the

highest dose. Whereas for siRSV-NS1, only at the high dose of 0.4 μg was there an inhibitory effect of 40%.

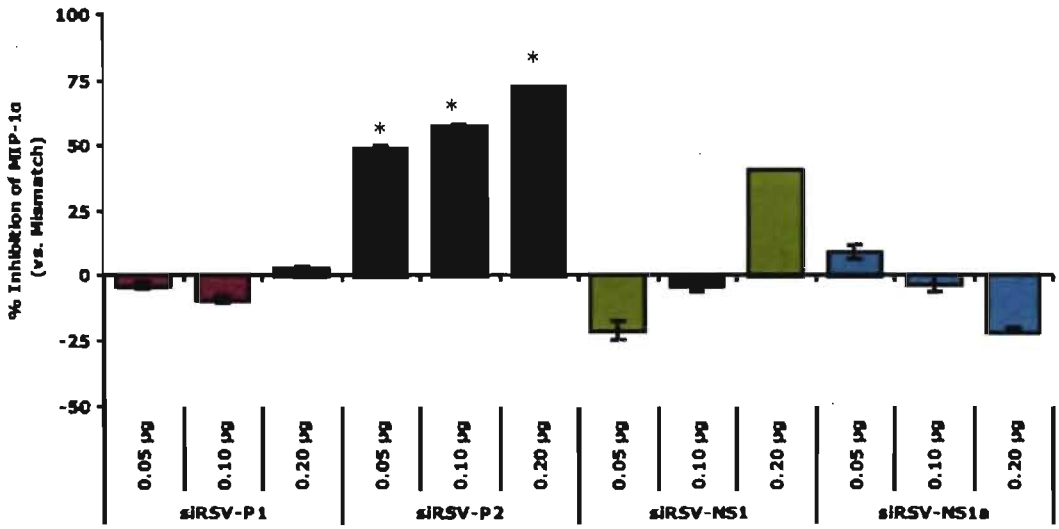


Figure 12. % Inhibition of MIP-1α levels in hRSV infected A549 cells. A549 cells were transfected with 0.1 μg, 0.2 μg , and 0.4 μg of siRSV-P1, siRSV-P2, siRSV-NS1, siRSV-NS1a and their mismatches with Lipofectamine2000 transfection reagent ratio "1:3" in quadruplicates. A set of untransfected cell cultures was used as control group. One day post-transfection, cells were then infected with hRSV at MOI of 1 and kept incubated at 37°C, 5%CO_2 overnight. Supernatants were harvested 24-hrs post-infection and MIP-1α levels secreted were determined by MIP-1α ELISA. Data expressed as mean ± SEM. Asterisks indicate significant inhibition ($P < 0.05$).

IL-8 secretions were inhibited solely by siRSV-P2 while the other three siRNAs appeared to stimulate viral production of IL-8 **(Figure 13)**. Inhibition was observed in a dose-dependent fashion, with a 21% ± 5% significant inhibition at 0.2μg and a 40% ± 9% inhibition at 0.4 μg.

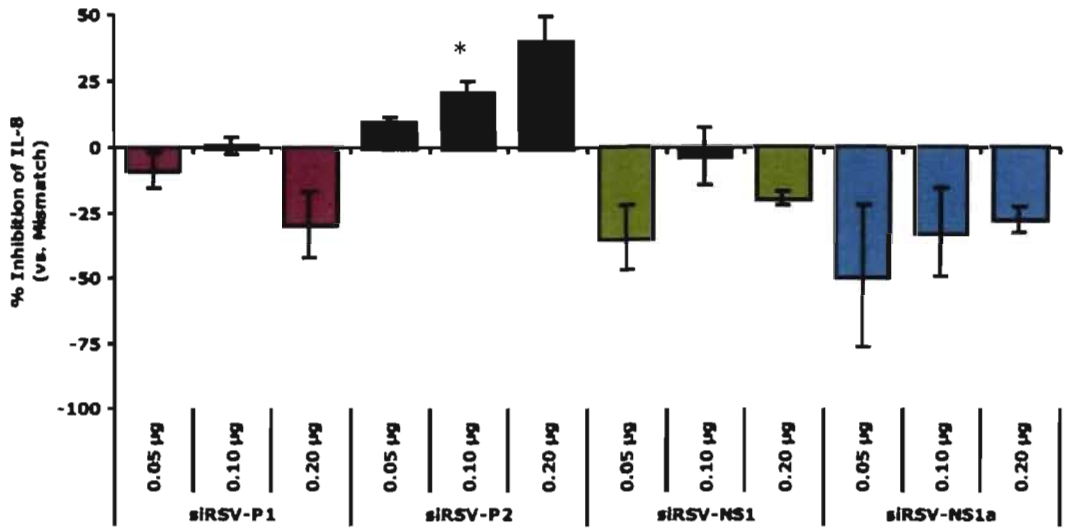


Figure 13. % Inhibition of IL-8 in hRSV infected A549 cells. A549 cells were transfected with 0.1 µg, 0.2 µg, and 0.4 µg of siRSV-P1, siRSV-P2, siRSV-NS1, siRSV-NS1a and their mismatches with Lipofectamine2000 transfection reagent ratio "1:3" in quadruplicates. A set of untransfected cell cultures was used as control group. One day post-transfection, cells were then infected with hRSV at MOI of 1 and kept incubated at 37°C, 5%CO_2 overnight. Supernatants were harvested 24-hrs post-infection and secreted IL-8 levels were assessed by IL-8 ELISA, expressed as mean ± SEM. Asterisks indicate significant inhibition ($P < 0.05$).

When assessing the results from all three readouts in conjunction, the siRSV-P2 consistently demonstrated inhibition (when compared to its mismatch), whereas the other three siRNAs were less consistent in their efficacy as they either stimulated or inhibited very weakly. In all the readouts, a dose-dependent inhibition appeared for the siRSV-P2. These results, in addition to the evidence obtained from the immunostaining plaque assay, indicated that siRSV-P2 was our most promising candidate siRNA for further examination.

5.2.3 *In vitro* screening of siRSV-P1 and -P2:

Among the four siRNAs, siRSV-NS1 and –NS1a did not seem to have a consistent inhibitory effect thus we focused on an examination of the efficacy of transfection of siRNAs targeting the hRSV P protein. A549 cells were transfected with siRSV-P1 and siRSV-P2 at a range of doses starting as low as 0.05 µg, followed by an hRSV infection the following day. Cell lysates and supernatants were harvested for viral P mRNA and viral protein assessment, respectively. As expected siRSV-P2 proved to be the most

effective siRNA at attaining target knock down of the hRSV P mRNA **(Figure 14)**. In this study, a low dose of 0.05 μg was included to observe a possible inhibitory effect. Surprisingly, at such low dose, a 42% ± 4% inhibition was observed. At the high dose of 0.4 μg, 89% ± 1% of the viral P mRNA has been effectively inhibited. These findings were supported by the results from the hRSV ELISA **(Figure 15)**.

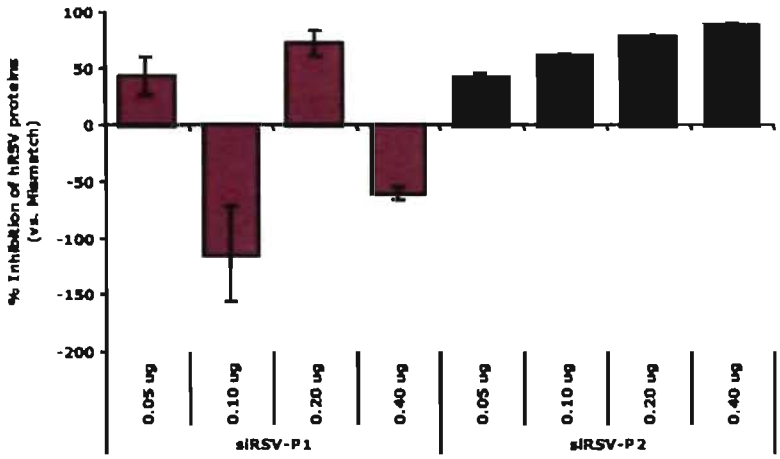


Figure 14. % Inhibition of hRSV P mRNA in hRSV infected A549 cells. A549 cells were transfected with 0.05 μg, 0.1 μg, 0.2 μg, and 0.4 μg of siRSV-P1, siRSV-P2, and their mismatches with Lipofectamine2000 transfection reagent ratio "1:3" in duplicates. A set of untransfected cell cultures was used as control group. One day post-transfection, cells were then infected with hRSV at MOI of 1 and kept incubated at 37°C, 5%CO_2 overnight. Cell lysates were harvested 24-hrs post-infection for RNA extraction followed by reverse transcription to be assayed by real-time RT-PCR. Data was normalized over the Ppibg housekeeping gene. % Inhibition was determined in comparison to each mismatch, respectively and expressed as mean ± SEM.

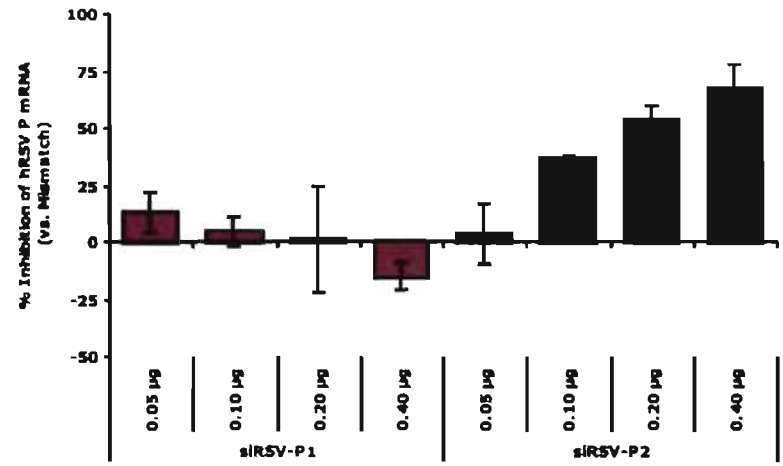


Figure 15. % Inhibition of hRSV proteins in hRSV infected A549 cells. A549 cells were transfected with 0.05 μg, 0.1 μg, 0.2 μg, and 0.4 μg of siRSV-P1, siRSV-P2, and their mismatches with Lipofectamine2000 transfection reagent ratio "1:3" in duplicates. A set of untransfected cell cultures was used as control group. One day post-transfection, cells were then infected with hRSV at MOI of 1 and kept

incubated at 37°C, 5%CO_2 overnight. Supernatants were harvested 24-hrs post-infection to be assessed by hRSV ELISA. % Inhibition was determined in comparison to each mismatch, respectively and expressed as mean ± SEM.

As shown in **Figure 15**, siRSV-P2 inhibited viral proteins in a dose-dependent manner. Even at the lowest dose of 0.05 μg a slight 4% inhibition was observed, however with an SEM of 13% these data cannot be of any real significance **(Figure 15)**. Nevertheless, at the highest dose, 68% of hRSV proteins were been inhibited.

Therefore, comparing these siRNAs, it was clearly visible that siRSV-P2 consistently inhibited viral propagation in a dose-dependent fashion.

5.2.4 *In vitro* screening of siRSV-P2 and all its F-ANA combinations:

In order to improve the stability and duration of activity of our siRNAs both *in vitro* and also *in vivo*, chemical modifications have been selected to overcome those hurdles. With siRSV-P2 being the most efficient at inhibiting viral growth in all aspects, we decided to either completely replace the sense strand with F-ANA or only modify the two positions closest to the 3' end in the antisense strand **(Table III)**, as demonstrated previously *in vitro* efficacy work done by Dowler *et al* [10]. F-ANA modifications were synthesized from University Core DNA Services (University of Calgary).

siF-ANA name	Sequence
siRSV-P2 F3	5'-**CCCTACACCAAGTGATAATTT**-3'
siRSV-P2 F4	5'-AUUAUCACUUGGUGUAGGG**TT**-3'
siRSV-P2-Mi F3	5'-**CCCTACACCGAGTAATATATT**-3'
siRSV-P2-Mi F4	5'-UAUAUUACUCGGUGUAGGG**TT**-3'

Table III. F-ANA modified siRNA names and sequences. F-ANA modifications were made for siRSV-P2 at various positions along the siRNA strands (shaded in yellow), **denoted in bold**. Mismatches (-Mi) were created with scrambling at various positions along the sequence. Sequences were purchased from University Core DNA Services from University of Calgary.

Cell lysates and supernatants from siRNA-transfected hRSV-infected A549 cells were harvested for evaluation for inhibition of hRSV replication. All siRNAs, unmodified and F-ANA modified, appeared to inhibit viral P mRNA at doses higher than 0.1 μg **(Figure 16)**. At the lowest dose of 0.05 μg, only siRSV-P2 F3/F4 lost its inhibitory effects. Interestingly, siRSV-P2 O/F4 inhibited 30% better than the unmodified siRNA at the lower doses while maintaining a similar inhibitory effect of approximately 92% at the highest dose.

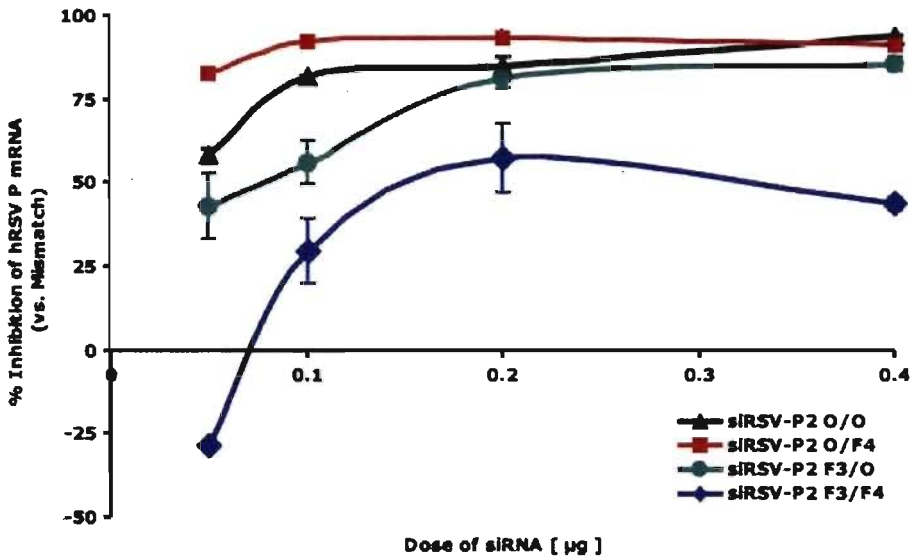


Figure 16. % Inhibition of hRSV P mRNA in hRSV infected A549 cells. A549 cells were transfected with 0.05 μg, 0.1 μg, 0.2 μg, and 0.4 μg siRSV-P2 O/O, O/F4, F3/O F3/F4, and their mismatches with Lipofectamine2000 transfection reagent ratio "1:3" in duplicates. A set of untransfected cell cultures was used as control group. One day post-transfection, cells were then infected with hRSV at MOI of 1 and kept incubated at 37°C, 5%CO_2 overnight. Cell lysates were harvested 24-hrs post-infection for RNA extraction followed by reverse transcription to be assayed by real-time RT-PCR. Data was normalized over the Ppib housekeeping gene. % Inhibition was determined in comparison to each mismatch, respectively and expressed as mean ± SEM.

Inhibition of hRSV proteins however did not parallel the inhibition of viral P mRNA **(Figure 17)**. Using this readout, siRSV-P2 O/O and O/F4 were still inhibitory as well as the F3/F4 modification, consistent with the findings by Dowler, where increased potency was observed when modifications at the 3'-end were made, with minor loss when the entire sense strand was converted in addition [10]. Unmodified siRSV-P2 appeared to

inhibit 38.8% greater than the O/F4 at 0.1 μg with an 85% ± 1% inhibition, however efficacy of the unmodified siRNA quickly decreased at 0.05 μg dose. At this low dose, both O/O and O/F4 inhibit at similar levels however O/F4 with a slight increment of an advantage, whereas F3/O was not inhibitory.

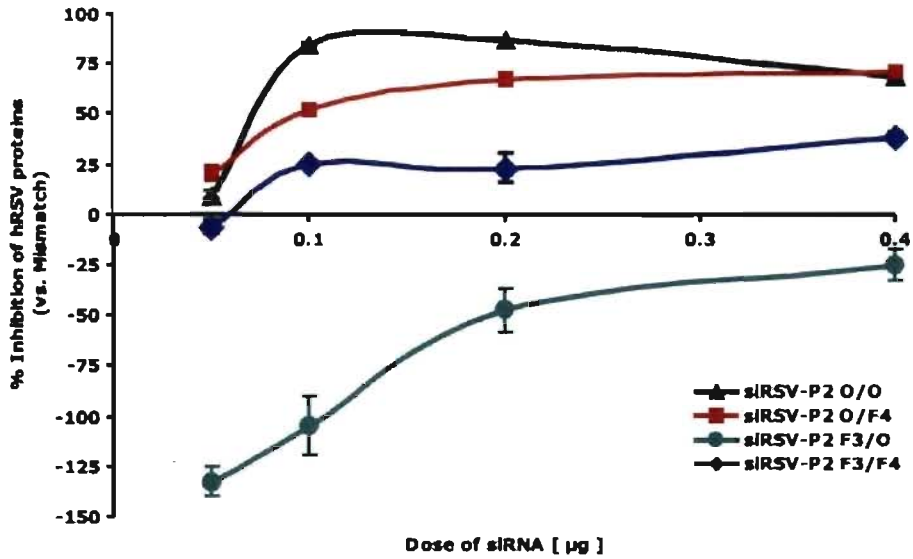


Figure 17. % Inhibition of hRSV proteins in hRSV infected A549 cells. A549 cells were transfected with 0.05 μg, 0.1 μg, 0.2 μg, and 0.4 μg of siRSV-P2 O/O, O/F4, F3/O, F3/F4 and their mismatches with Lipofectamine2000 transfection reagent ratio "1:3" in quadruplicates. A set of untransfected cell cultures was used as control group. One day post-transfection, cells were then infected with hRSV at MOI of 1 and kept incubated at 37°C, 5%CO_2 overnight. Supernatants were harvested 24-hrs post-infection to be assessed by hRSV ELISA. % Inhibition was determined in comparison to each mismatch, respectively and expressed as mean ± SEM.

Inhibition of MIP-1α secretion by the hRSV-infected A549 cells with siRSV-P2 O/O, O/F4, F3/O and F3/F4 is summarized in **Figure 18**. There is a distinct segregation between the siRNAs that inhibited cytokine secretion as compared to those that had stimulatory effects. SiRSV-P2 F3/O and F3/F4 clearly did not inhibit the MIP-1α levels whatsoever, while both O/O and O/F4 inhibited at almost the same levels. Unmodified siRSV-P2 at the highest dose (0.4 μg) managed to almost completely inhibit MIP-1α secretions by 96%.

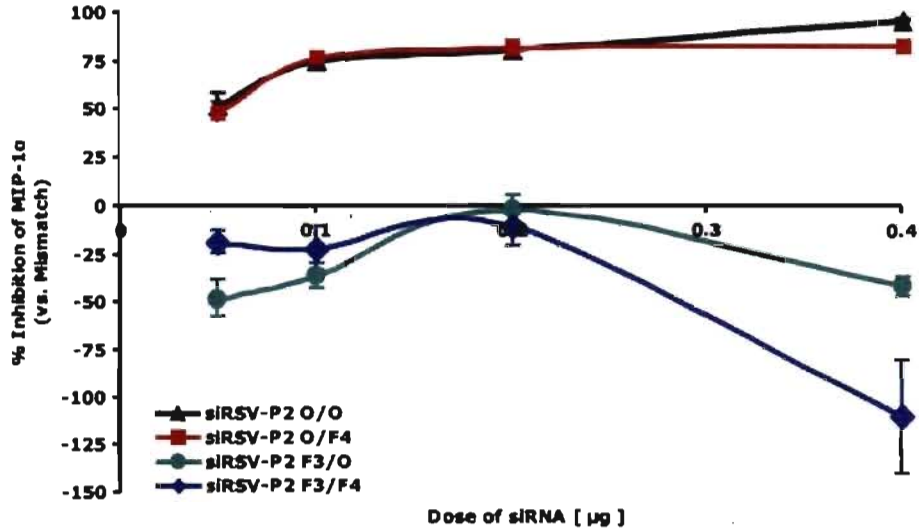


Figure 18. % Inhibition of MIP-1α in hRSV infected A549 cells. A549 cells were transfected with 0.1 μg, 0.2 μg, and 0.4 μg of siRSV-P2 O/O, O/F4, F3/O, F3/F4 and their mismatches with Lipofectamine2000 transfection reagent ratio "1:3" in triplicates. A set of untransfected cell cultures was used as control group. One day post-transfection, cells were then infected with hRSV at M.O.I of 1 and kept incubated at 37°C, 5%CO_2 overnight. Supernatants were harvested 24-hrs post-infection and MIP-1α levels secreted were determined by MIP-1α ELISA. % Inhibition was determined in comparison to each mismatch, respectively and expressed as mean ± SEM.

When examining all three readouts together, a pattern emerges. Unmodified siRSV-P2 is indeed the best choice of siRNA amongst the initial four siRNA as it reliably inhibited viral replication as determined in all readouts. F-ANA modifications of siRSV-P2 F3/O and F3/F4 did not appear to have much inhibitory effect while O/F4 looked promising as it demonstrated better efficacy at inhibiting target mRNA than the unmodified siRSV-P2 at lower doses, although inhibited levels of viral proteins and MIP-1α similar to the unmodified version. Our next step was to determine the efficacy of these siRNAs in an animal model of hRSV infection.

5.3 VALIDATION OF *IN VIVO* MODEL.

As the results from our *in vitro* studies suggested that the siRNAs, siRSV-P2 O/O and O/F4 exhibited the greatest efficacy in reducing levels of RSV replication we next explored the efficacy of these two siRNAs in an animal model of hRSV infection.

Humans are the natural host for hRSV and an exact representation of the disease cannot be identically reproduced in any other species, however efficient hRSV replication has been shown to take place in BALB/c mice [297]. We first had to assess the course and robustness of the infection in BALB/c mice using the hRSV inoculum we prepared in house.

5.3.1 Developing an *in vivo* mouse hRSV model.

To determine the optimal viral load in the hRSV murine model, mice were infected with both a high and low titer of hRSV preparations. Preliminary studies were performed by infecting BALB/c mice with a single dose of hRSV delivered intranasally (in 50 µl) and physiological symptoms were monitored daily. Viral load and immune response were assessed on days 3, 6 and 10 post-infection **(Figure 19)**.

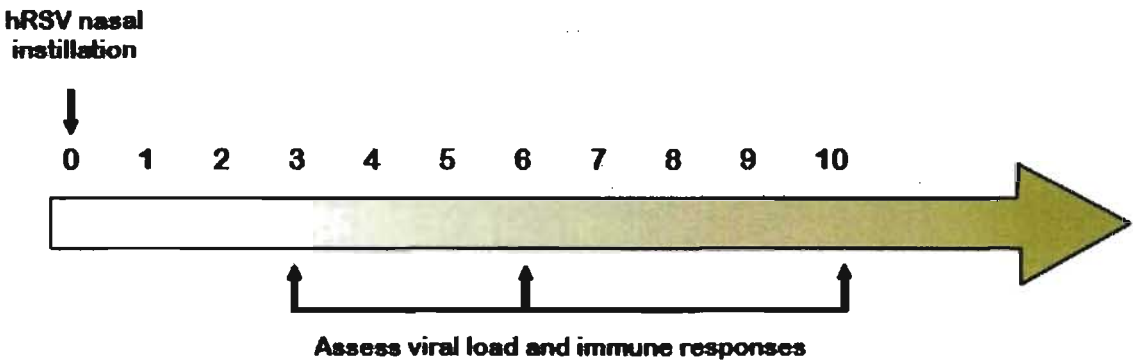


Figure 19. Timeline of hRSV infection. BALB/c mice were anesthetized with a mixture of ketamine-xylazine then infected with 50 µL of vehicle, UV-irradiated hRSV at 1×10^6 PFU, and active hRSV at 1×10^5 PFU or 1×10^6 PFU by nasal instillations. On days 3, 6, and 10 following infection, 5 mice from each group were weighed, anesthetized with an overdose of sodium pentobarbital and exsanguinated. BALs and whole lungs were collected from the mice on those days post-infection.

Following infection, upon external examination the mice exhibited no visible cold-like symptoms or signs of distress (i.e. fur ruffling, huddling, lack of activity). However variations in weight gain were detected between the treatment groups **(Figure 20)**. Animals in the control group (mock infected with vehicle) appeared to steadily gain weight as the days progressed, always weighing heavier compared to animals in the other

groups and did not appear to have an initial drop in weight observed with all other treatments. Mice infected with the highest dose of virus (1×10^6 PFU) always weighed the least compared to the other treatment groups (either mock-infected, UV-treated hRSV and low titer hRSV groups), with an initial drop in weight on day 2 post infection. Nevertheless even this group of mice showed steady weight gain after the initial weight drop. Mice infected with the lower dose of hRSV (1×10^5 PFU) also had an initial drop in weight as measured on Day 2 post infection, but by the end of the time course animals in this group were approaching weight gains similar to control animals suggesting that the low titer of virus was not bringing long term physiological changes to the mice.

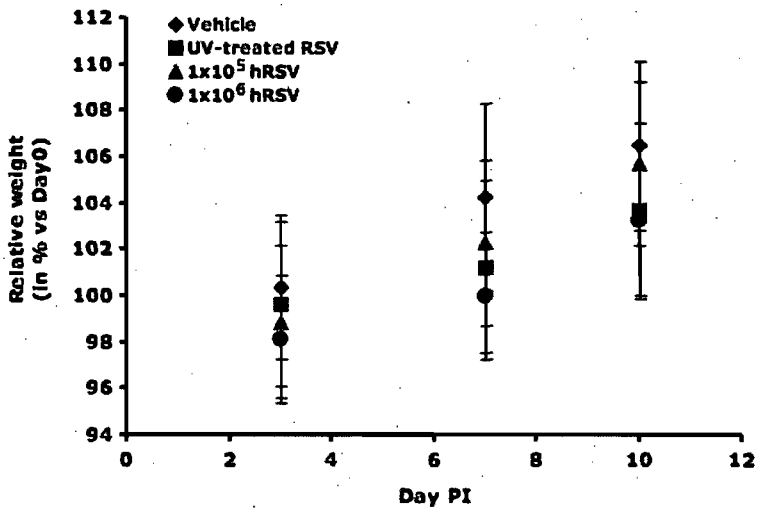


Figure 20. Mouse weight difference in percentage measured over time. BALB/c mice were weighed on ay 0, 3, 7 and 10 post-infection. Percent weight differences of the mean of each group were calculated compared to the starting weight at day 0 post-infection (± SEM).

Mice were sacrificed on days 3, 6 and 10 post-infection for BAL and whole lung collection. Total and differential cells were determined from BAL **(Figure 21, 22 and 23)**. On day 3 post treatment, animals from all groups demonstrated a consistent elevation in total number of cells in BAL regardless of whether they were infected with the virus or not suggesting that there is a rapid influx of cells to the lungs following intranasal administration **(Figure 21)**. The total number of BAL cells in both control groups, vehicle only and UV-irradiated hRSV, decreased gradually by day 10 indicating that the immune system was no longer activated. Mice infected with either the low or high titer

virus did not demonstrate this drop in cell numbers over time and instead appeared to maintain or have an increase in total cells in BAL, suggesting that these animals were to efficiently infected with the virus. The drop in the total number of cells observed on day 6 might in all treatment groups reflect the regress of cells, which were called into the lung in response to a non-specific assault of nasal instillation. The maintenance of the elevated cell numbers by day 10 in the RSV infected animals **(see figure 21)** suggest that these animals are expressing an immune response to the virus, specifically from the lymphocytes **(Figure 22 and 23).**

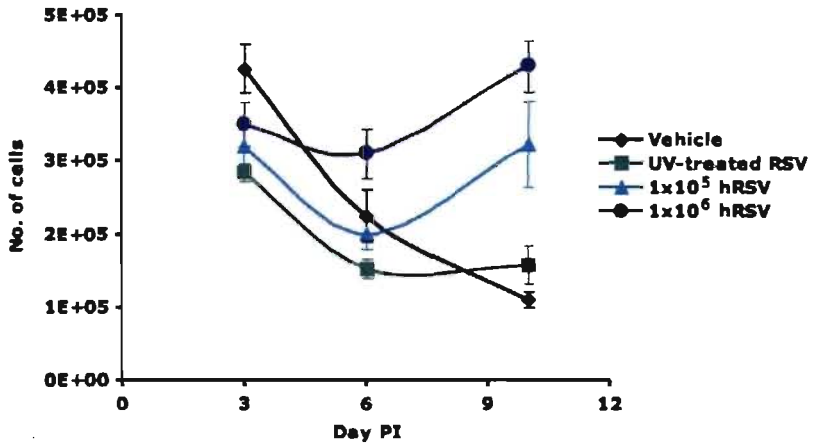


Figure 21. Total cells in BAL of hRSV infected BALB/c mice over time. Mice were sacrificed on either day 3, 6 or 10 post-infection. BALs were then performed by injecting and retrieving 5 times 0.8 mL of cold PBS into the lungs. Total cell count was determined for each sample and expressed as mean ± SEM per group (n=5 mice per group).

Differential cells counts (percent and absolute cell numbers) were also performed on the BAL **(Figure 22 and 23)**. Mice in either vehicle treated group or UV-treated RSV groups demonstrated similar profiles in cell sub-populations present in the BAL over time. Macrophages made up the largest percentage of infiltrating cells in animals of these groups at all time points. In contrast, animals infected with viable hRSV demonstrated an initial high level of macrophages on Day 3, which decreased over the time course. Instead the levels of lymphocytes in these animals increased by Day 6 and Day 10.

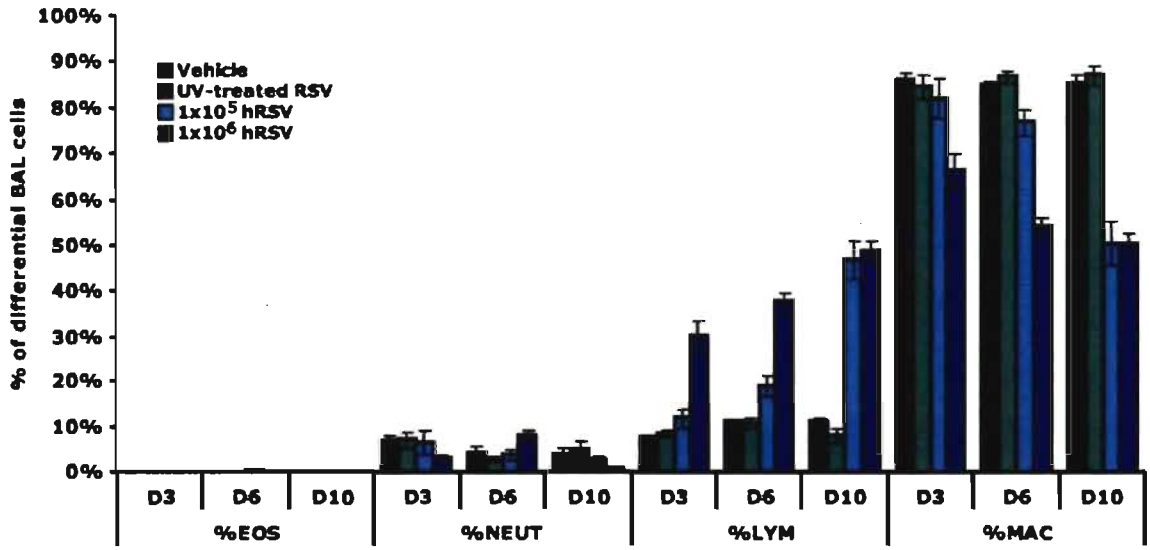


Figure 22. Percent differential cells in BAL of hRSV infected mice over time. Mice were sacrificed on either day 3, 6 or 10 post-infection. BALs were then performed by injecting and retrieving 5 times 0.8 mL of cold PBS into the lungs. Total cell counts were determined for each sample and then differential cell counts were prepared on slides using cytospins. Slides were stained with the Hema 3 stain set. Percent differential cell count was determined for each sample expressed as mean ± SEM per group (n=5).

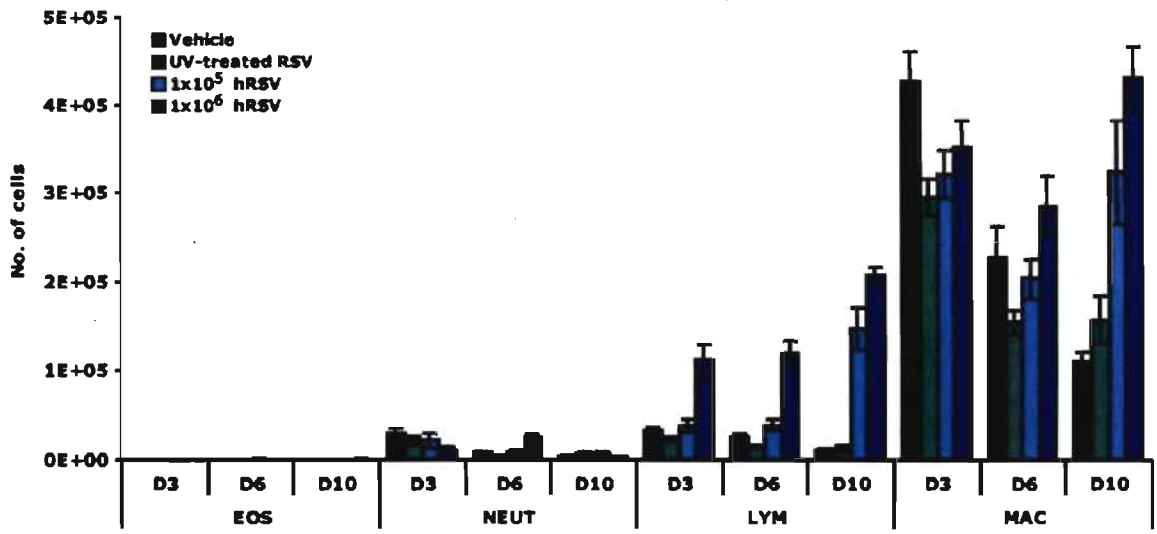
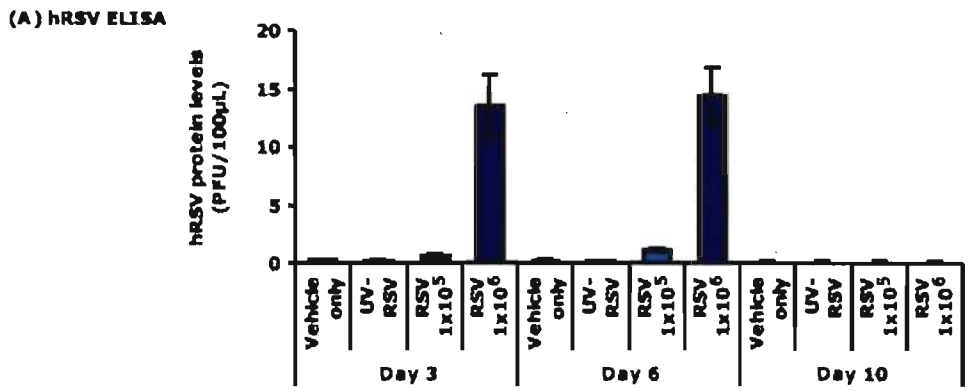


Figure 23. Absolute number of differential cells in BAL of hRSV infected mice over time. Mice were sacrificed on either day 3, 6 or 10 post-infection. BALs were then performed by injecting and retrieving 5 times 0.8 mL of cold PBS into the lungs. Absolute cell counts was determined for each sample expressed as mean ± SEM per group (n=5).

Besides determining the total and differential cell counts in BAL, we also measured BAL for levels of hRSV (using hRSV ELISA and real-time PCR), as well as cytokines, mMIG (mouse monokine induced by IFN-gamma) and TNF-alpha, as a marker of immune cell activation. Both MIG and TNF-α have been previously linked to persistent airway inflammation, due to continuous chemotaxis of mononuclear cells [298] [299] [8] and also to RSV disease severity [300], respectively in BALB/c mice. In mice treated with the highest titer of hRSV levels of viral infection (hRSV proteins) were observed on Day 3 and Day 6 post infection, with clearance by Day 10. Mice infected with lower titer of virus had modest levels of hRSV proteins in BAL on Day 3 and Day 6 post infection and also cleared by Day 10 **(Figure 24)**. Real-time RT-PCR results indicated that hRSV P mRNA could be identified on both day 3 and 6 with both RSV infected groups (high and low titer), however with a stronger dose-dependent signal at the peak of infection on day 6 **(Figure 24 B)**. With regard to cytokines, MIG levels were highest on Day 6 post infection in both high and low titer infected animals with clearance of MIG by Day 10 post infection. In contrast, TNF-α was only detectable on day 3 post-infection for all animal groups and was highest in the group infected with the highest titer of RSV. A consistent pattern in hRSV protein, viral P mRNA and mMIG levels appears throughout, in which a peak in viral replication is observed 6 days following infection and becoming undetectable by day 10.



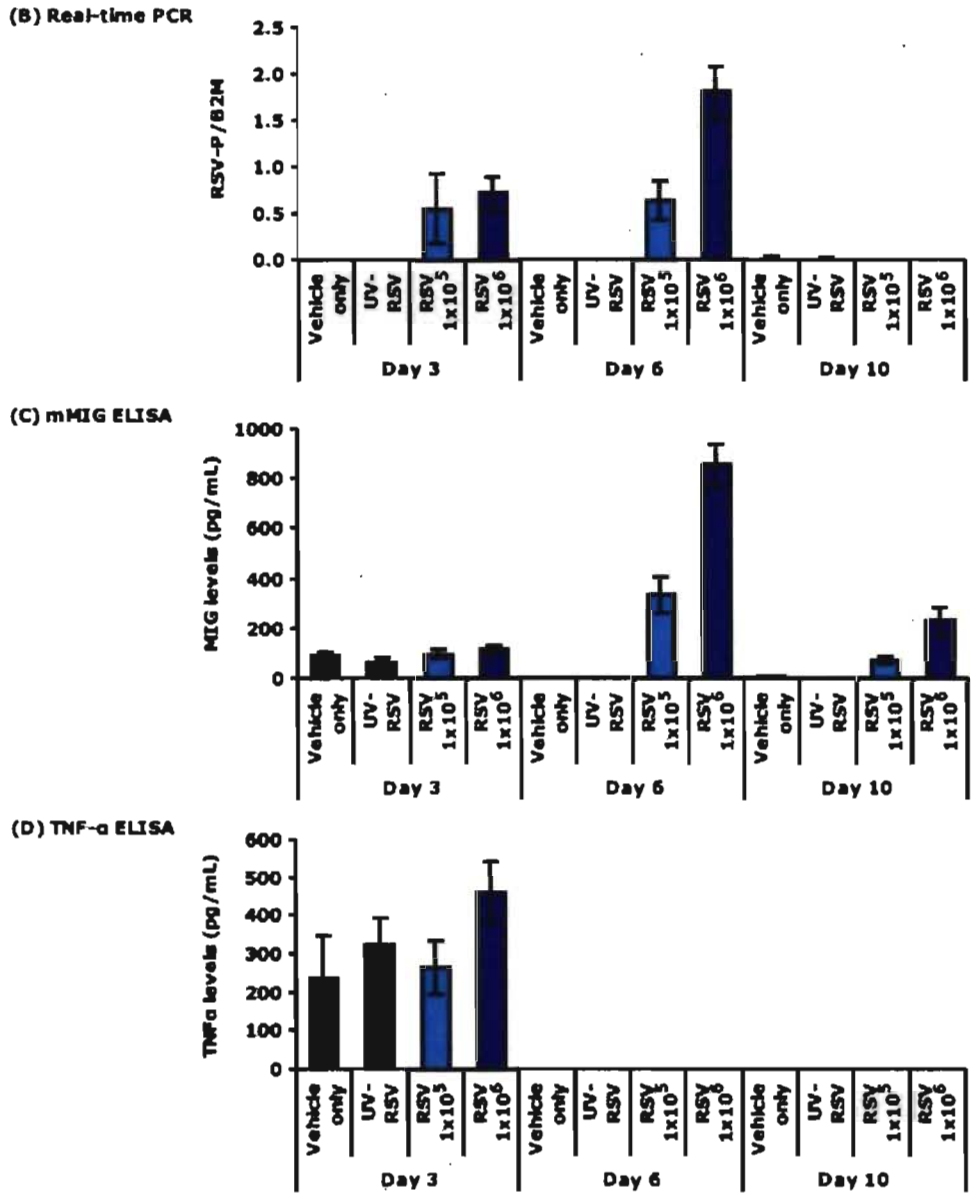


Figure 24. Determining hRSV infection in BAL and lung homogenates of hRSV infected BALB/c mice over time. BALB/c mice were infected by nasal installation with vehicle, UV-irradiated hRSV at 1×10^6 PFU, active hRSV at 1×10^5 PFU or 1×10^6 PFU in 50 μL in one nostril. Animals were sacrificed at time points post infection and BAL fluids were collected and analyzed by **(A)** hRSV protein ELISA, **(C)** mouse MIG ELISA, and **(D)** TNF-alpha ELISA assays. Whole lungs were collected after BALs were performed and real time PCR was performed **(B)** on RNA isolated from the lung homogenates. cDNA was made with Random Hexamers by reverse transcription and then quantitated by real-time PCR using viral RSV-P primers. β2M was the housekeeping gene used as reference. Samples expressed as mean ± SEM per group (n=5 mice per group).

5.3.2 Optimizing the *in vivo* mouse hRSV model

Due to the abundant number of mice used and the lengthy amount of time for each set of experiments a single optimal dose and single harvest day would be highly favorable to conduct efficacy testing of our selected siRNAs. We had previously determined that both low and high titer of hRSV were sufficient to infect the mice and elicit an immune response, however the lower titer of 1×10^5 PFU was barely detectable by hRSV protein ELISA while infections with 1×10^6 PFU would require an great amount of virus. We therefore tested a median dose of 5×10^5 PFU per mouse and included an additional harvest on Day 1 post-infection to further optimize this model of hRSV infection in the BALB/c mice.

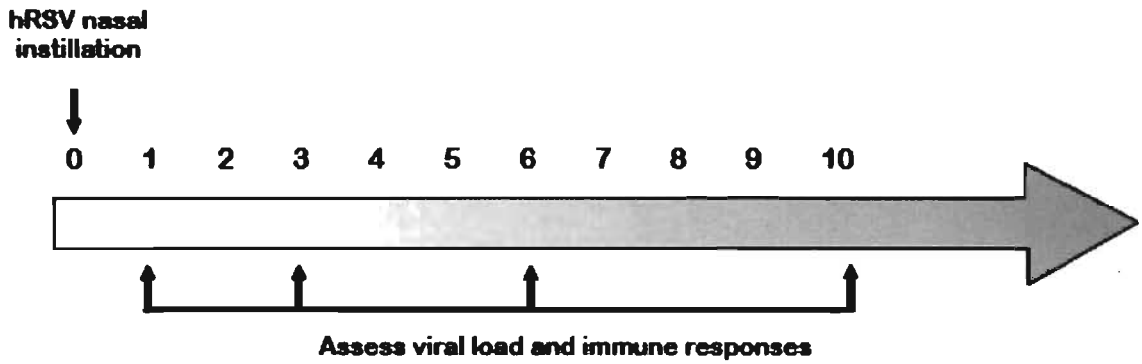


Figure 25. Timeline of hRSV infection. BALB/c mice were anesthetized with a mixture of ketamine-xylazine then infected with either 50 µL of vehicle or active hRSV at $5x10^5$ PFU by nasal instillations. On days 1, 3, 6, and 10 following infection, 5 mice from each group were weighed, sacrificed and BALs and whole lungs were collected.

On Day 1 post infection, BAL from animals in both treatment groups demonstrated increased numbers of total cells **(Figure 26)**. In animals infected with hRSV (5×10^5 P.F.U), again there was an increase in total cells in BAL on Day 6 and Day 10 post infection while in control animals total cell numbers appeared to plateau. For both treatment groups, between 40-50% of the cells present at Day 1 were neutrophils **(Figure 27)** and these levels fell by Day 3 suggesting that this early influx of cells into the lungs may be a non-specific response to nasal instillation rather than a specific immune

response to virus, although in the case of RSV infection, neutrophils have been shown to be the early responders [301, 302].

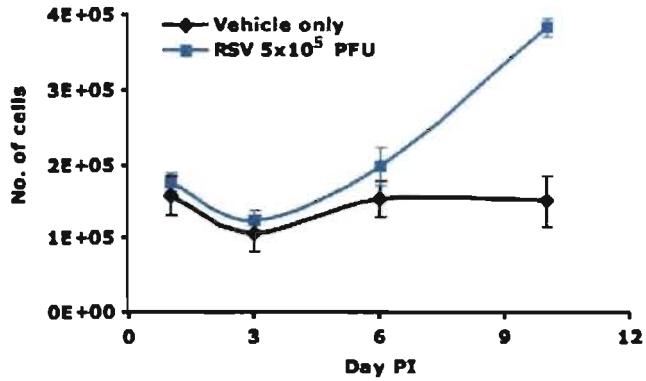


Figure 26. Total cells in BAL of hRSV infected BALB/c mice over time. Mice were sacrificed on either day 1, 3, 6 or 10 post-infection. BALs were performed and total cell counts were determined for each sample and then averaged per group (n=5 mice per group) ± SEM.

Similar to what was previously observed with high and low titer infections, the medial inoculum of 5×10^5 PFU per mouse of virus led to an increase in lymphocytes over the time course of infection **(Figure 27 and 28)** as well as an increase in the absolute number of macrophages **(Figure 28)**.

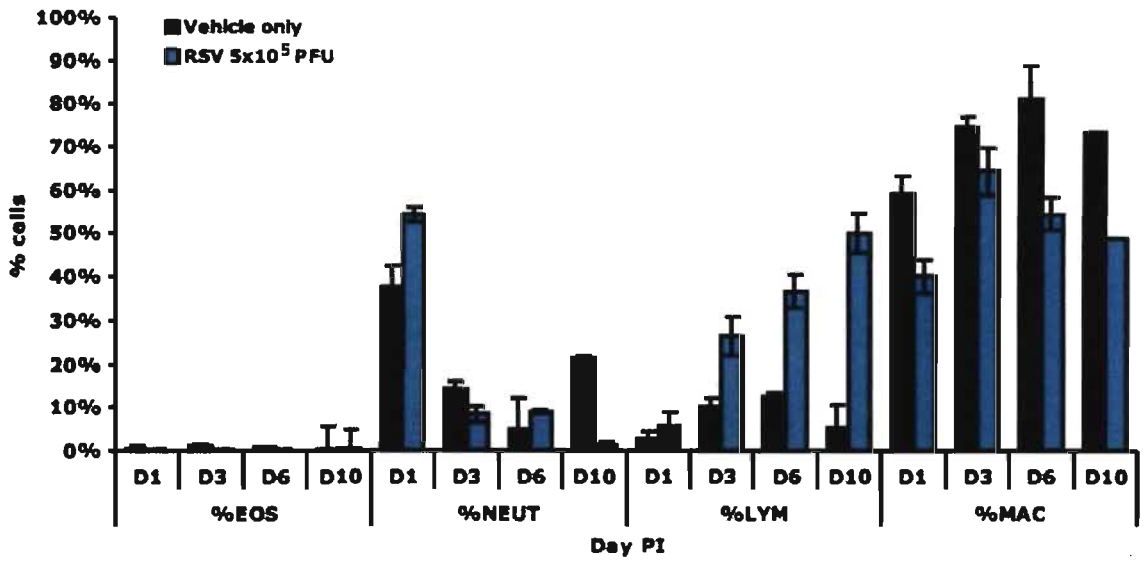


Figure 27. Percent differential cells in BAL of hRSV infected mice over time. Mice were sacrifice on either day 1, 3, 6 or 10 post-infection. BALs were then performed and total cell counts determined. Cell concentrations were then adjusted to 1×10^6 cells/mL and cytospins were prepared on glass slides. Slides were stained with the Hema 3 stain set. Percent differential cell count was determined for each sample expressed as mean ± SEM per group (n=5).

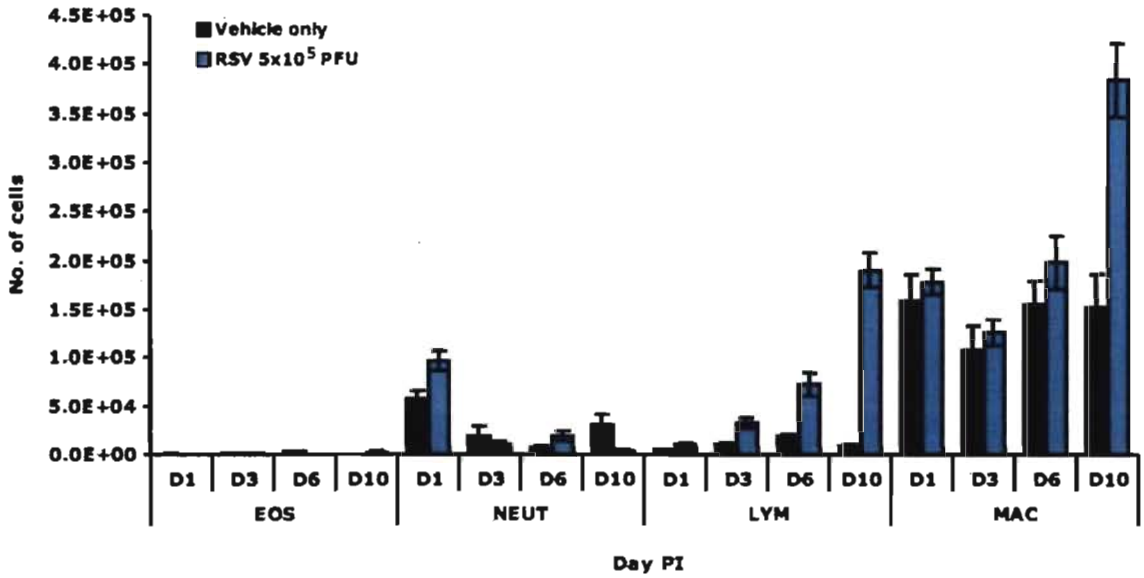


Figure 28. Absolute number of differential cells in BAL of hRSV infected mice over time. Mice were sacrificed on either day 1, 3, 6 or 10 post-infection. BALs were performed, differential performed and absolute cell count was determined for each sample, expressed as mean ± SEM per group (n=5).

Again to further characterize the infection model in response to 5×10^5 PFU we measured levels of hRSV protein, mRNA and murine cytokines MIG and TNF-α **(Figure 29)** in the BAL. hRSV proteins and mRNA were detected as early as the next day following infection, increased on Day 3, peaked on day 6 **(Figure 29 A and B)** and fell by Day 10, similar to the pattern observed with low and high titer infections **(Figure 24)**. With regard to cytokines, only the hRSV-infected group of mice exhibited levels of MIG and only on day 6 post-infection, whereas TNF-α was detectable immediately after nasal instillation on Day 1 in both treatment groups **(Figure 24 C and D)**. Taken together, these results suggested that a Day 6 post-infection time point would be an optimal harvest day to assess viral infection.

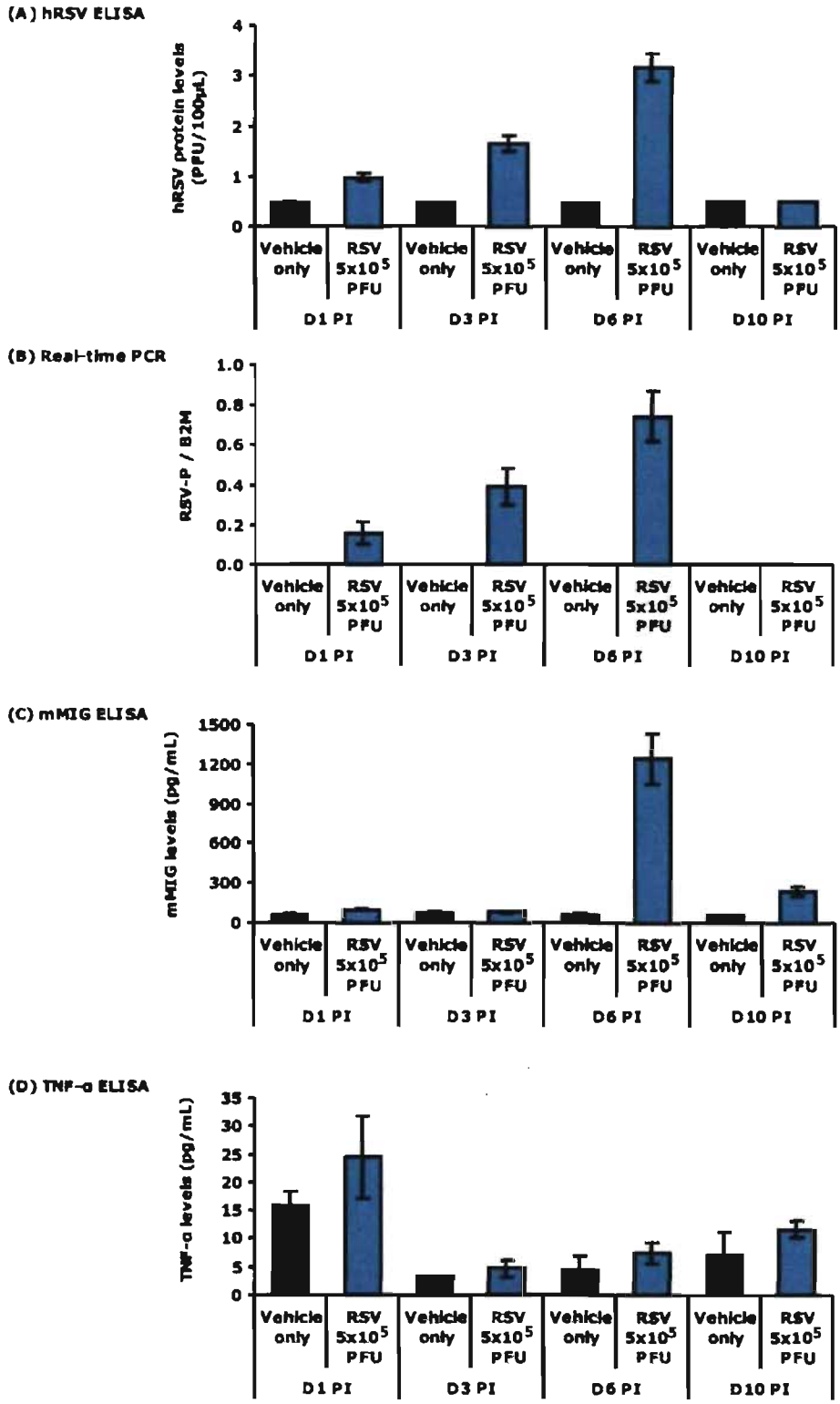


Figure 29. **Determining hRSV infection in BAL and lung homogenates of hRSV infected BALB/c mice over time.** BALB/c mice were infected by nasal installation with vehicle or active hRSV at 5×10^5 PFU in 50 µL in one nostril. Animals were sacrificed at indicated times post infection and BAL fluids were collected and analyzed by **(A)** hRSV protein ELISA, **(C)** mouse MIG ELISA, and **(D)** TNF-alpha ELISA

assays. Whole lungs were collected after BALs were collected and real-time PCR performed on **(B)** RNA isolated from the lung homogenates. cDNA was made with Random Hexamers by reverse transcription and then quantitated by real-time PCR using viral RSV-P primers. β2M was the housekeeping gene used as reference. Samples expressed as mean ± SEM per group (n=5 mice per group).

5.3.3 Validation of murine hRSV model via treatment with ribavirin

As our previous results indicated an optimal viral dose of our hRSV to be 5×10^5 PFU per mouse for our model, we felt it was important to further validate this optimized animal model by attempting to reduce the infection with the current treatment available on the market, ribavirin. To this end, 5×10^5 PFU of hRSV was nasally instilled into mice followed by subcutaneous injections of ribavirin daily (low and high doses) until peak of infection where they were sacrificed to assess viral load and immunological responses **(Figure 30)**.

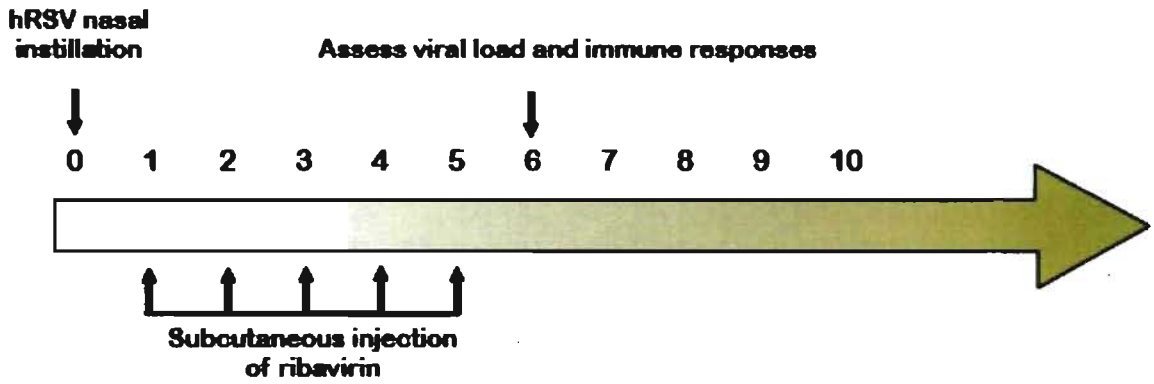


Figure 30. Timeline of hRSV infection. BALB/c mice were anesthetized with a mixture of ketamine-xylazine then infected with 50 μL of vehicle or active hRSV at 5×10^5 PFU by nasal instillations. In addition to being infected, mice were then either given a low (50 mg/kg/day) or high (500 mg/kg/day) dose of ribavirin subcutaneously daily. Day 6 following infection, 5 mice from each group sacrificed, and BALs and whole lungs were collected for further analyses.

The clinically relevant dose of ribavirin calculated for the murine model was determined to be 50 mg/kg/day. In order to exaggerate any inhibitory effect of treatment, an extreme dose of 500 mg/kg/day was also included in this study. Results of total cell numbers in BAL of treated mice demonstrate no effect of the 50-mg/k/day dose of ribavirin to reduce

cellular infiltrate whereas 500-mg/kg/day ribavirin dose demonstrated a modest inhibition of 8.5% **(Figure 31)**.

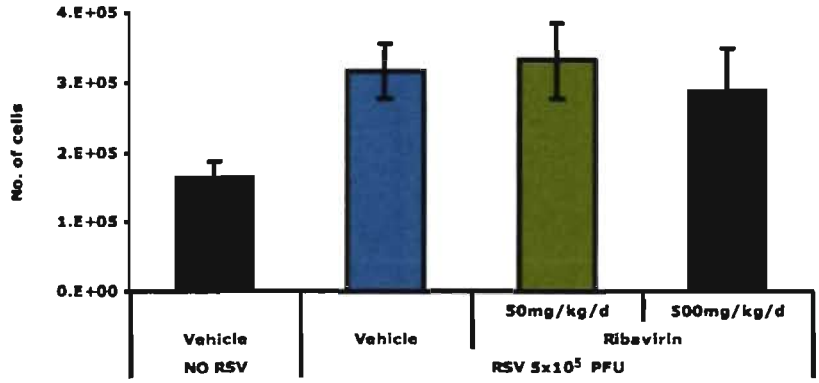


Figure 31. Total cells in BAL of hRSV infected BALB/c mice over time. Mice were sacrificed on day 6 post-infection. BALs were performed and total cell counts determined. Data expressed as mean ± SEM per group (n= 5 mice per group).

Ribavirin treatment did significantly inhibit the increase in lymphocytes by almost half the amount with just 50 mg/kg/day (see % lymph, bar 3 in **Figure 32** and **33**), and a similar effect with the high dose of ribavirin (refer to bar 4 in % lymph).

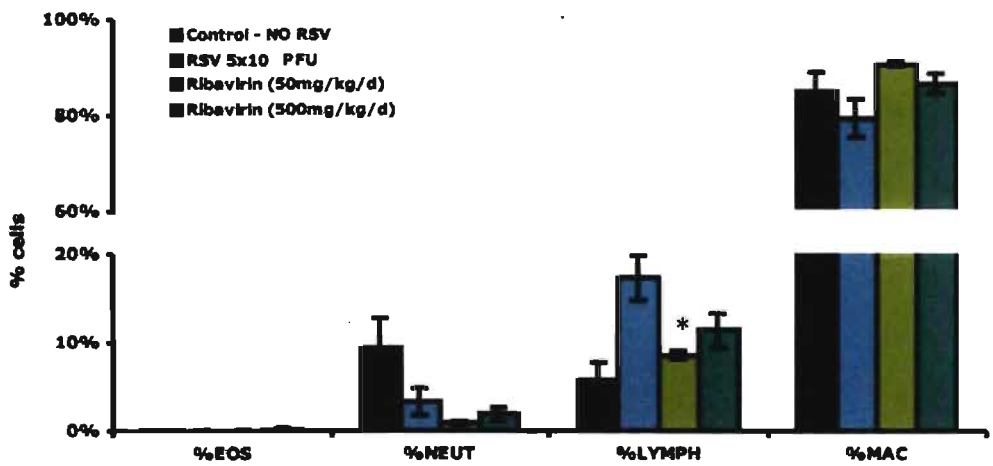


Figure 32. Percent differential cells in BAL of hRSV infected mice over time. Mice were sacrificed on day 6 post-infection. BALs were performed and total cell counts were determined for each sample. Cell concentrations were then adjusted to 1×10^6 cells/mL and cytospins were prepared on glass slides. Slides were stained with the Hema 3 stain set. Percent differential cell count was determined for each sample expressed as mean ± SEM per group (n=5). Asterisks indicate significant inhibition ($P < 0.05$).

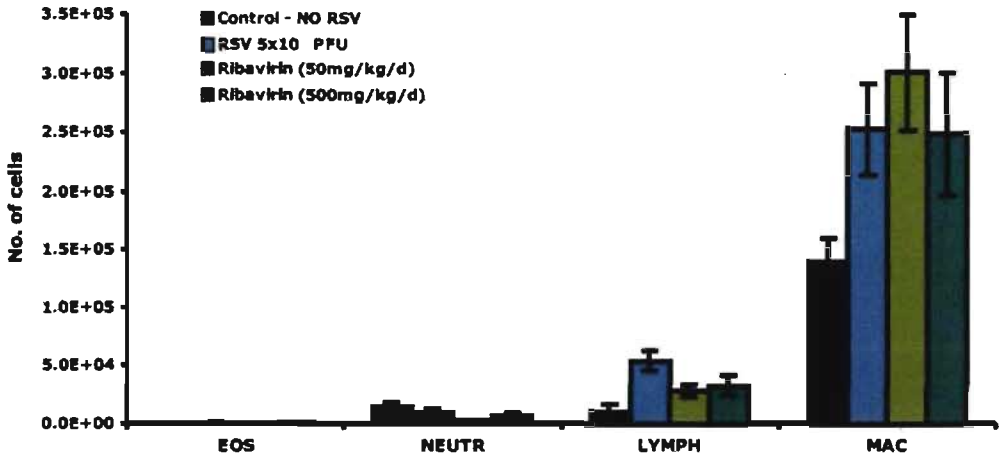


Figure 33. Absolute number of differential cells in BAL of hRSV infected mice over time. Mice were sacrificed on day 6 post-infection. BALs were performed and absolute cell counts determined. Data expressed as mean ± SEM per group (n= 5 mice per group).

Treatment of mice with ribavirin was also able to inhibit both viral protein and mRNA levels, with the greatest efficacy observed with the high dose ribavirin, which significantly inhibited protein levels by 53% **(Figure 34 A)** and viral P mRNA levels by 33%, **(Figure 34 B)**. As animals were sacrificed on Day 6 post infection we opted to measure mouse MIG levels only as an indication of immune cell activation. Ribavirin (high dose) treated significantly abrogated mouse MIG induction by 97% **(Figure 34 C)**. Although these levels of inhibition are impressive, 500 mg/kg/day exceeds the recommended dose by 10 times, while the modest effect of the 50 mg/kg/day dose would be more representative of a clinical response to this therapy. The use of ribavirin as a method of validating the hRSV mouse model was effective. The inhibition observed with the high dose also validated the infection dose of 5×10^5 PFU per mouse, the day 6 post-infection harvesting time-point and the readout methods.

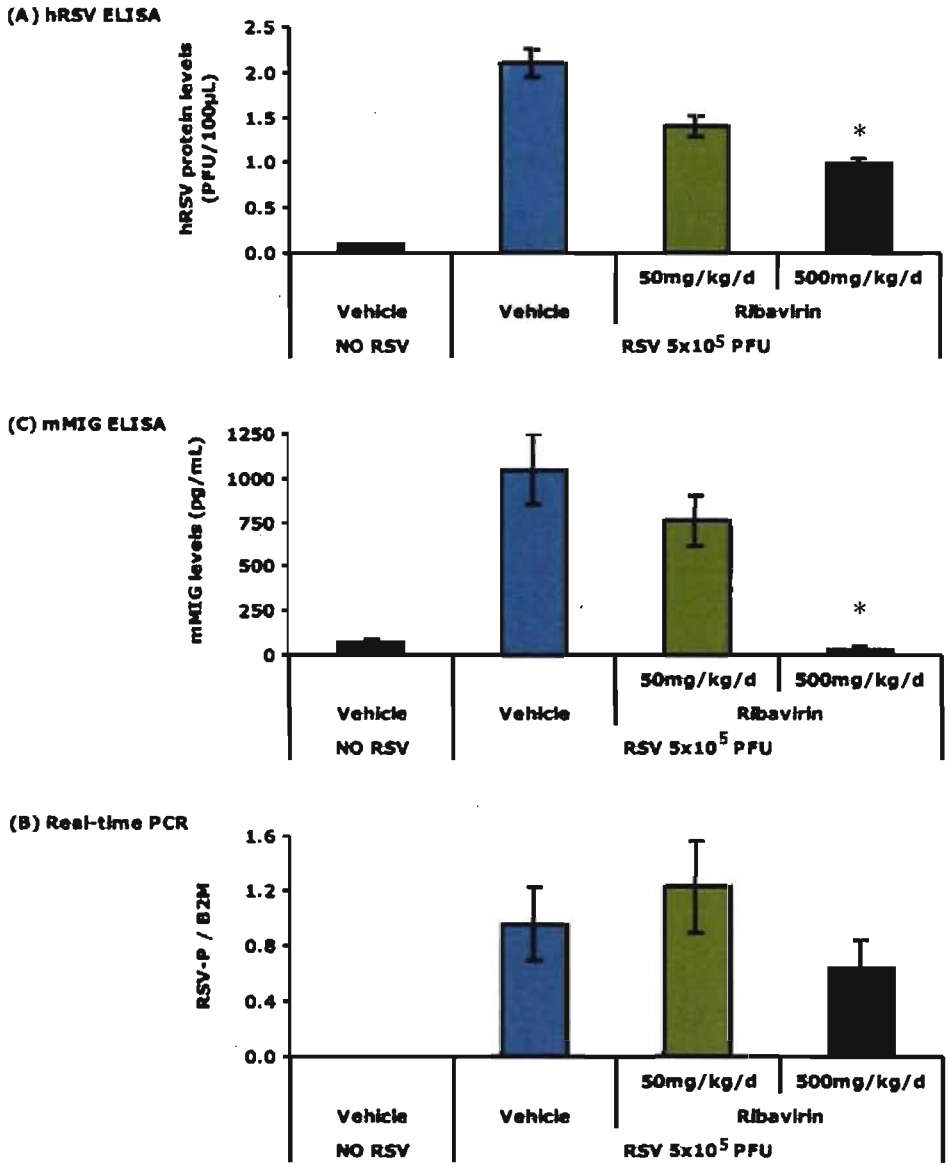


Figure 34. Determining hRSV infection in BAL and lung homogenates of hRSV infected BALB/c mice over time. BALB/c mice were infected by nasal instillation with vehicle or active hRSV at $5x10^5$ PFU in 50 µL in one nostril, and injected daily with ribavirin subcutaneously at 50mg/kg/day or 500mg/kg/day. BAL fluids were collected and analyzed by **(A)** hRSV protein ELISA and **(C)** mouse MIG ELISA assays. Whole lungs were collected after BALs were performed. **(B)** RNA was isolated from the lung homogenates. cDNA was made with Random Hexamers by reverse transcription and then quantitated by real-time PCR using viral RSV-P primers. β2M was the housekeeping gene used as reference. Samples expressed as mean SEM per group (n=5 mice per group). Asterisks indicate significant inhibition ($P < 0.05$).

5.4 DETERMINATION OF EFFICACY OF siRNA IN AN ANIMAL MODEL OF hRSV INFECTION:

5.4.1 Determination of *in vivo* efficacy of unmodified siRSV-P2, preliminary experiments

The optimized infection model thus far required for the nasal instillation of hRSV at 5×10^5 PFU per mouse with harvesting of BAL and lungs 6 days post-infection. With the model set up and validated, we set out to assess the efficacy of the best siRNA sequences selected from *in vitro* studies.

Two doses of unmodified siRSV-P2 (also denoted as siRSV-P2 O/O) was administered intranasally in 50 µL volume (naked in PBS) followed by an infection with 5×10^5 PFU into the same nostril. BAL and whole lungs were collected 6 days later for assessment of viral load and immunological reactions **(Figure 35)**. The following data shows a representative experiment of the efficacy screening of siRSV-P2 O/O.

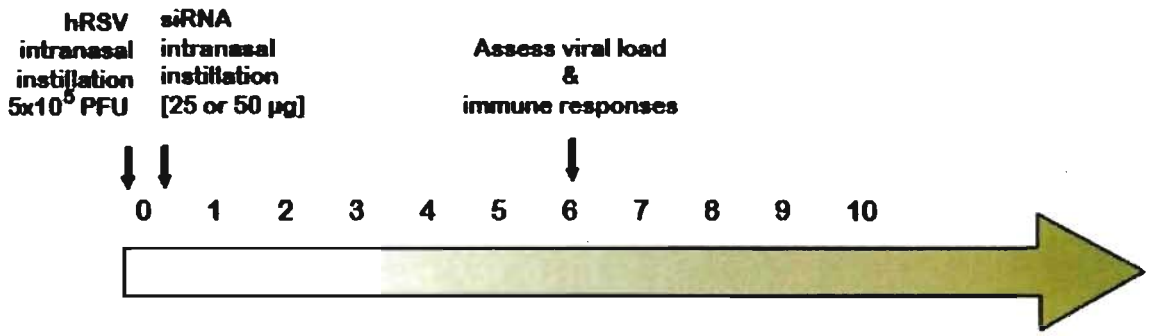


Figure 35. Timeline of hRSV infection. BALB/c mice were anesthetized with a mixture of ketamine-xylazine then infected with 50 µL of vehicle or active hRSV at 5×10^5 PFU by nasal installations. Following infection, 50 µL of naked 25 µg or 50 µg siRSV-P2 O/O was administered intranasally. 6 days post-infection, BALs and whole lungs were collected from 5 mice per treatment group.

Upon analysis of the cellular infiltrate into the lungs on Day 6 post-infection, in this experiment the level of total cells in the hRSV-infected group was similar to levels observed in the vehicle control group **(Figure 36)** mainly linked to the surge of neutrophils and decrease in macrophages in the hRSV-infected group (data not shown)

consistent with results previously seen. Treatment of animals with either dose of siRSV-P2 resulted in a non-significant stimulation instead of inhibition of total cells when compared to either the hRSV only control group or their respective mismatches.

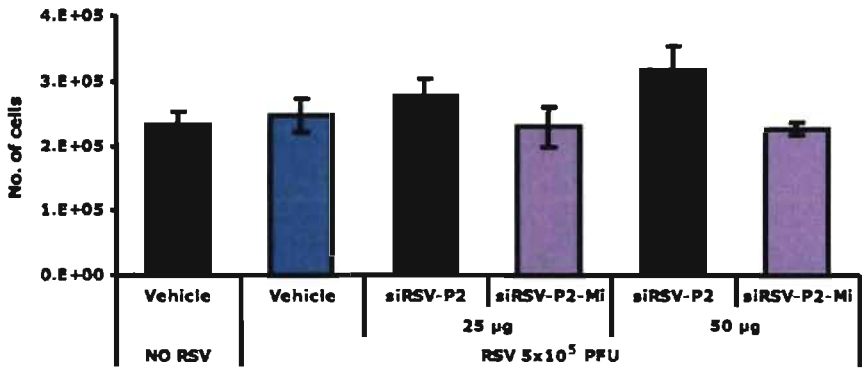


Figure 36. Total cells in BAL of hRSV infected BALB/c mice over time. Mice were sacrificed on day 6 post-infection. BALs were performed by injecting and retrieving 5 times 0.8mL of cold PBS into the lungs. Total cell count was determined for each sample and expressed as mean ± SEM per group (n=5).

Although no decrease in total cell numbers was observed in response to siRSV-P2 treatment, there was a modest inhibition of percent of lymphocytes in BAL when hRSV-infected mice were treated with siRSV-P2 O/O **(Figure 37)**. At both the 25 µg and 50 µg doses, the increase in lymphocytes was impeded by 15% and 12%, respectively, compared to the control hRSV only group (non-significant). Only a 6% inhibition of the absolute number of cells was detectable with the lower dose of siRSV-P2 **(Figure 38)**.

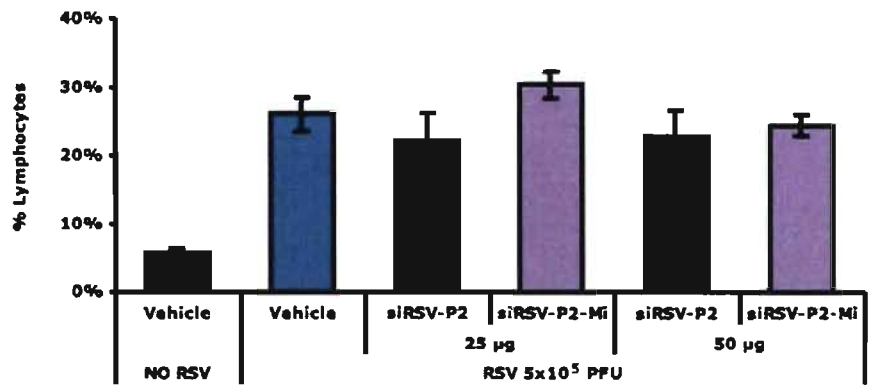


Figure 37. Percent lymphocytes in BAL of hRSV infected mice over time. Mice were sacrificed on day 6 post-infection. BALs were then performed by injecting and retrieving 5 times 0.8 mL of cold PBS into the lungs. Total cell count was determined for each sample. Cell concentrations were then adjusted to $1x10^6$ cells/mL and cytospins for differential cell counts were prepared on glass slides. Slides were stained with the Hema 3 stain set. Percent differential cell count performed and the percent lymphocytes for each sample was expressed as mean ± SEM per group (n=5).

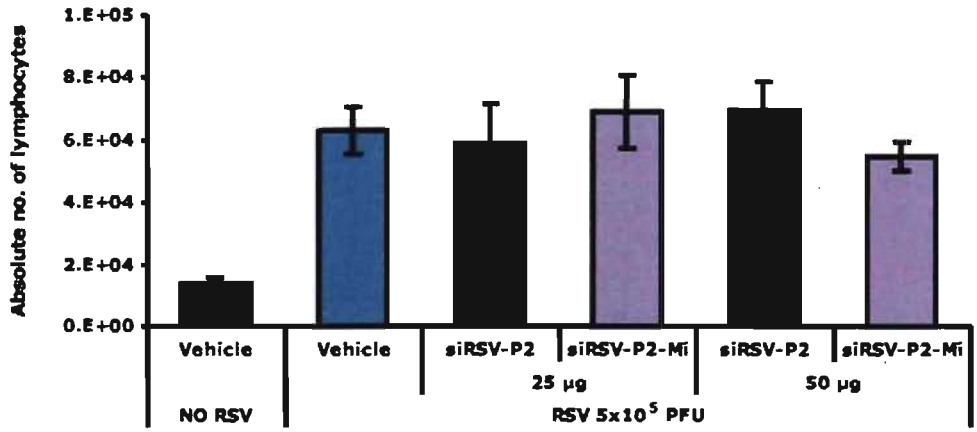


Figure 38. Absolute number of lymphocytes in BAL of hRSV infected mice over time. Mice were sacrificed on day 6 post-infection. BALs were then performed by injecting and retrieving 5 times 0.8 mL of cold PBS into the lungs. Absolute number of lymphocytes was determined for each sample, expressed as mean ± SEM per group (n=5).

Looking at levels of RSV proteins in the BAL of these animals it was observed that unmodified siRSV-P2 at 25 µg and 50 µg per mouse inhibited the levels of hRSV proteins by 17% and 24% respectively (**Figure 39 A**, bar 3 and 5). The effect of mismatch siRNA sequences were often inconsistent and appeared to have inhibitory effects therefore for *in vivo* evaluations of all data, all inhibitions were compared to the hRSV-infected only control group (i.e. bar 2 in all three readouts of **Figure 39**). With regard to inhibition of mRNA, levels were not inhibited in response to treatment with the low dose of siRSV-P2 however an inhibition of 33% was observed in response to the higher dose treatment (**Figure 39 B**, bar 5). Inhibition of mouse MIG levels was observed with low dose siRSV-P2 to be 17% and 36% with the high dose of siRSV-P2 (50 µg) (**Figure 39 C**, bar 3 and 5 respectively). The results from all three readouts are suggestive of a dose-dependent inhibitory response to hRSV by the siRNA.

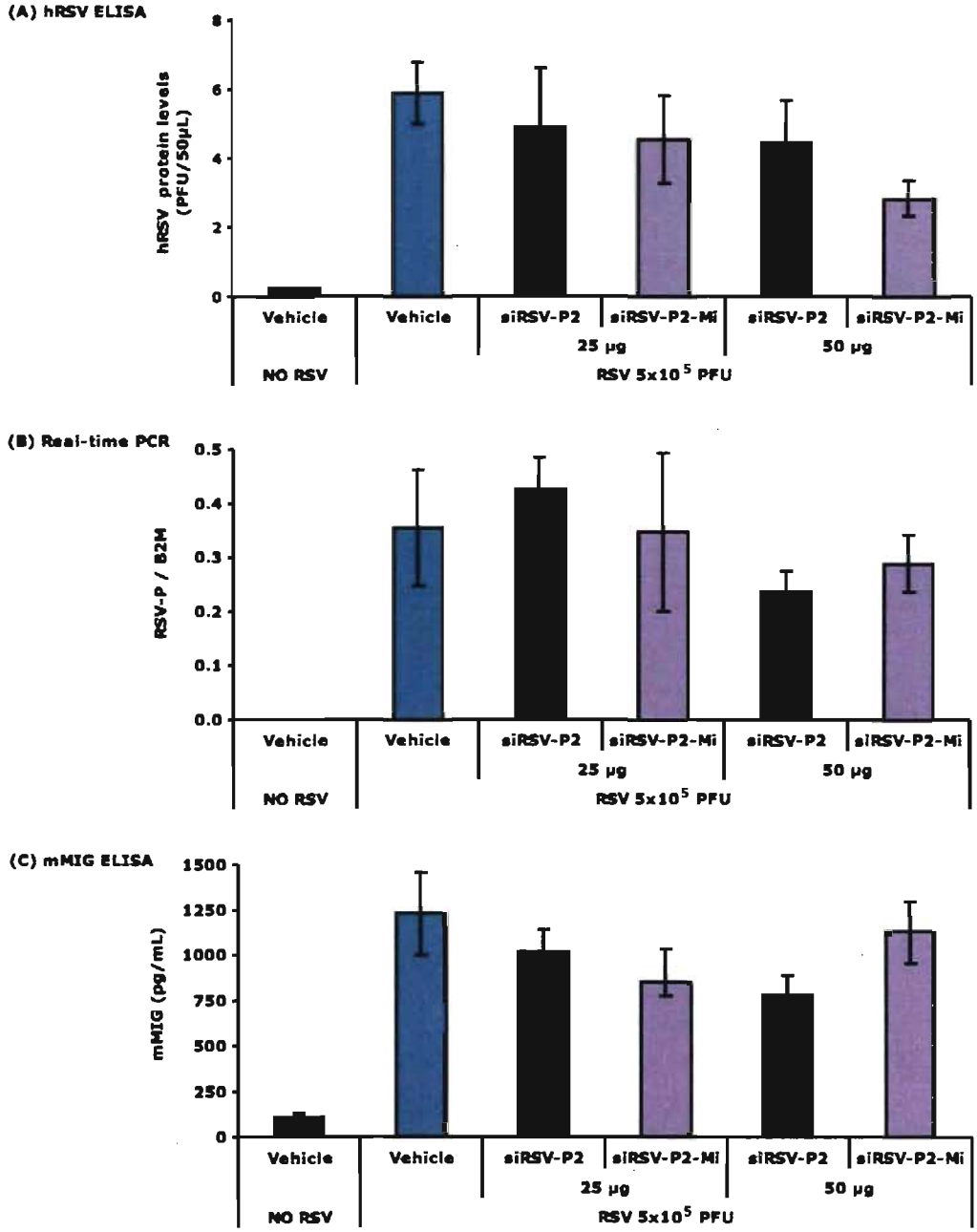


Figure 39. Determining hRSV infection in BAL and lung homogenates of hRSV infected BALB/c mice over time. BALB/c mice were infected by nasal installation with vehicle or active hRSV at $5x10^5$ PFU in 50 µL in one nostril, followed by 50 µL of naked 25 µg or 50 µg siRSV-P2 O/O administered intranasally. Lungs were washed 5 times with 0.8 mL of cold PBS and BAL fluids were collected and analyzed by **(A)** hRSV protein ELISA and **(C)** mouse MIG ELISA assays. Whole lungs were collected after BALs were performed. **(B)** RNA was isolated from the lung homogenates. cDNA was made with Random Hexamers by reverse transcription and then quantitated by real-time PCR using viral RSV-P primers. β2M was the housekeeping gene used as reference. Samples expressed as mean ± SEM per group (n=5 mice per group).

In view of the fact that these data are from a single experiment representative of the efficacy of unmodified siRSV-P2 at two doses, no concrete conclusions can be drawn from such results. In the following section, all experiments using unmodified siRSV-P2 were pooled and compared to pooled data of treatments with F-ANA modified siRNAs.

5.5 COMPARISON OF *IN VIVO* EFFICACY OF UNMODIFIED VS. F-ANA MODIFIED siRNA.

5.5.1 Comparison of *in vivo* efficacy of unmodified siRSV-P2 O/O vs. F-ANA modified siRSV-P2 O/F4, pooled analysis

In vitro screening of siRNAs showed promising results with siRSV-P2 O/O and O/F4 and next we wished to assess the efficacy of the unmodified and F-ANA modified siRNA in our animal model of hRSV infection. Unmodified and F-ANA modified siRSV-P2 were intranasally administered at two doses followed by infection with hRSV at 5×10^5 PFU per mouse. Viral load and immune responses were analyzed 6 days post-infection **(Figure 40)**. As a control treatment, ribavirin was delivered subcutaneously to a subgroup of infected mice on a daily basis as described below **(Figure 50)**.

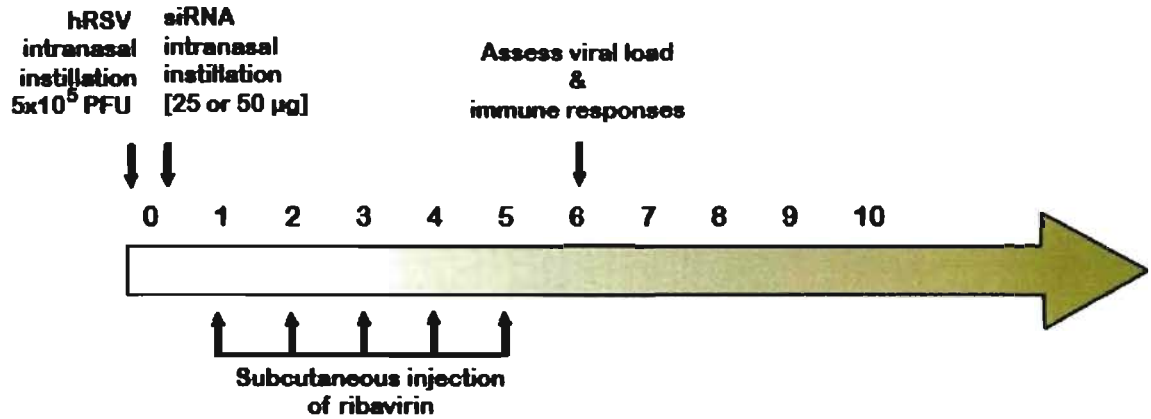


Figure 40. Timeline of hRSV infection. BALB/c mice were anesthetized with a mixture of ketamine-xylazine then infected with 50 µL of vehicle or active hRSV at $5x10^5$ PFU by nasal installations. In addition to being infected, mice were then either given a low (50mg/kg/day) dose of ribavirin subcutaneously daily. Day 6 following infection, 5 mice from each group were weighed, anesthetized with an overdose of sodium pentobarbital BALs and whole lungs were collected from the mice on those days post-infection.

Results from these studies showed the failure of ribavirin and siRSV-P2 (either unmodified or F-ANA modified) to affect the total cell numbers present in BAL **(Figure 41)**. These results coincided with the slight stimulation of total cell counts observed previously with unmodified siRNAs at both high and low doses. Upon examination of the differential cell counts however, inhibitions varied from cell type to cell type **(Figure 42)**. hRSV infection stimulated an infiltration of percent lymphocytes and both siRSV-P2 O/O and siRSV-P2 O/F4 successfully inhibited this lymphocyte influx at both the 25 μg and 50 μg doses (**Figure 42 A**, on the left). Unmodified siRSV-P2 demonstrated moderately better inhibitory effects than F-ANA modified with 18% and 20% at the low and high dose respectively while F-ANA modified siRSV-P2 inhibited 15% at high dose only. In contrast, mice treated with ribavirin had over twice the levels of inhibition, inhibiting percent lymphocyte levels by 47% (**Figure 42 A**, on the left, bar 5). With regard to neutrophils, ribavirin treatment inhibited by 76% (**Figure 42 C**, on the left, bar 5) and treatment with low doses of siRSV-P2 also decreased the percent neutrophils in the lungs by 38% for O/O and 41% for O/F4 (**Figure 42 C**, on the left, bar 1 and 3). In contrast to these cell types, macrophages (percent and absolute numbers) were increased in mice with all treatments **(Figure 42 B)**. Similarly, both unmodified and F-ANA modified siRSV-P2s did not inhibit percent and absolute levels of eosinophils, whereas ribavirin was effective by 47% and 16%, respectively **(Figure 42 D)**.

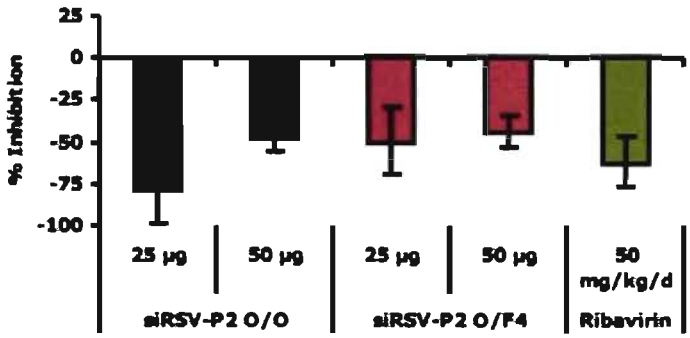


Figure 41. % Inhibition of total cells in BAL of hRSV infected BALB/c mice over time. Mice were sacrificed on day 6 post-infection. BALs were then performed by injecting and retrieving 5 times 0.8 mL of cold PBS into the lungs. Total cell count was determined for each sample and expressed as mean (n=15-33 mice per siRNA treatment group, n=17 per ribavirin group) ± SEM.

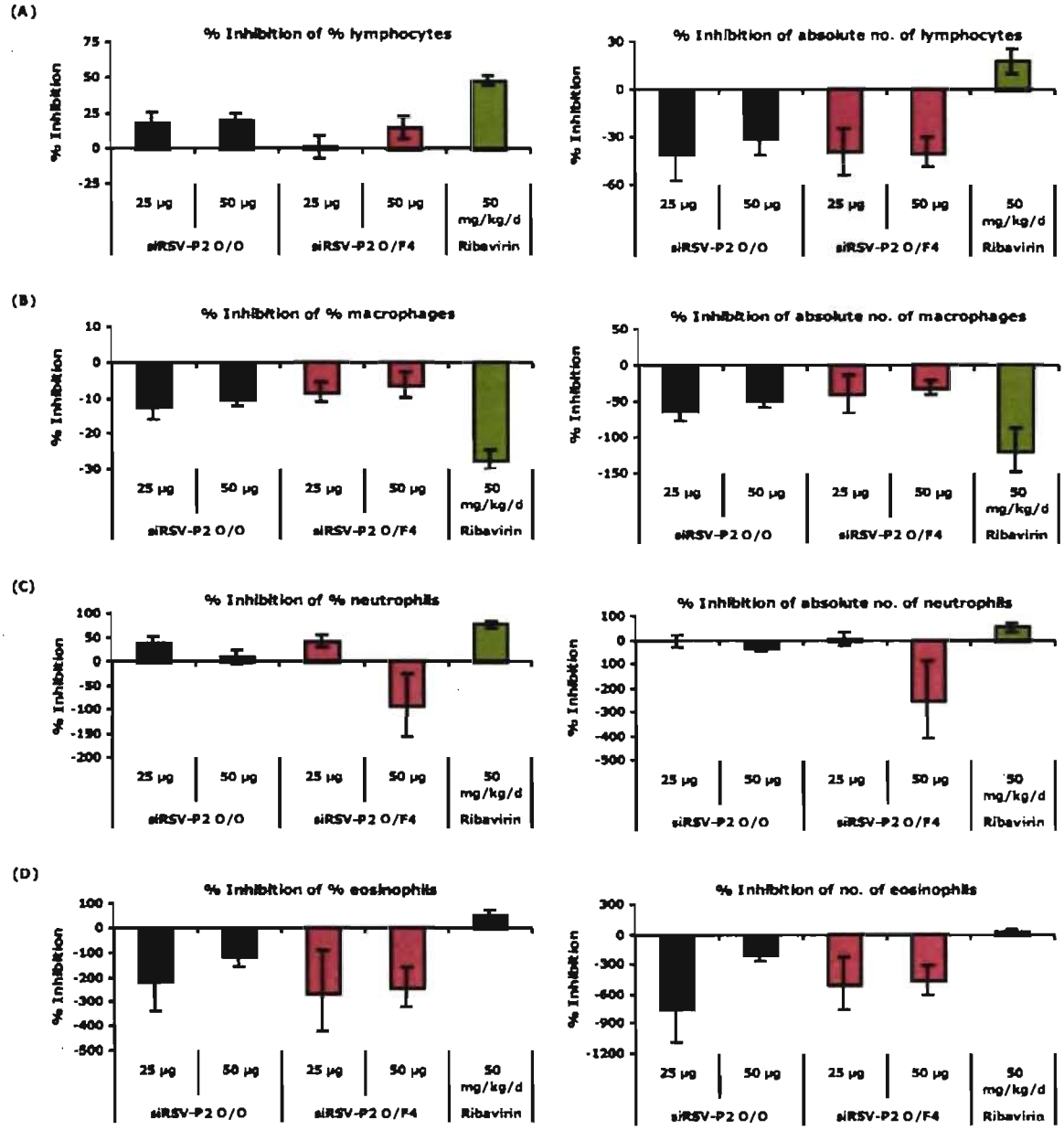


Figure 42. % Inhibition of cells released in BAL of hRSV infected BALB/c mice (measured by total and differential cell counts). Mice were sacrificed on day 6 post-infection. BALs were then performed by injecting and retrieving 5 times 0.8 mL of cold PBS into the lungs. Total cell count was determined for each sample. Cell concentrations were then adjusted to $1x10^6$ cells/mL and cytospins for differential cell counts were prepared on glass slides. Slides were stained with the Hema 3 stain set. Percent and absolute cell counts were determined for each sample, expressed as mean (n=15-33 mice per siRNA treatment group, n=17 per ribavirin group) ± SEM.

The efficacy of the unmodified and F-ANA modified siRSV-P2 upon RSV proteins, mRNA levels and cytokines in BAL were also investigated. Inhibition of hRSV viral proteins occur in a dose dependent manner in mice treated with both unmodified and F-

ANA modified siRSV-P2 **(Figure 43)**. At the low and high dose, hRSV viral proteins were inhibited at 31% and 44%, respectively, by unmodified siRSV-P2. The efficacy of F-ANA modified siRSV-P2 inhibited viral proteins at similar levels to that of unmodified siRSV-P2 at both the high and low doses, with 25% and 48%. Protein levels inhibited by ribavirin at 50 mg/kg/day everyday were comparable to the inhibition levels observed with either siRSV-P2 O/O and O/F4, with 50 μg given at a single time point immediately prior to infection.

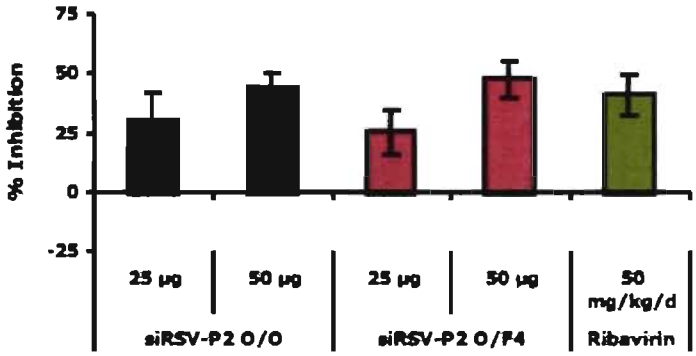


Figure 43. % Inhibition of hRSV proteins in BAL of hRSV infected BALB/c mice (measured by hRSV ELISA). Animals were sacrificed on day 6 post infection and BAL fluids were collected and analyzed by hRSV protein ELISA. Inhibition of hRSV proteins expressed as % mean ± SEM per group mean (n=15-33 mice per siRNA treatment group, n=17 per ribavirin group).

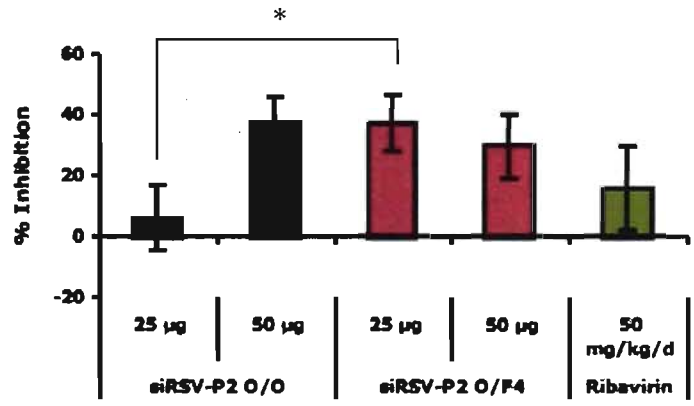


Figure 44. % Inhibition of hRSV P mRNA levels detected in lung homogenates of hRSV infected BALB/c mice (measured by real-time RT-PCR). Animals were sacrificed on day 6 post infection and whole lungs were collected after BAL. Real-time PCR was performed and analyzed by RNA isolated from the lung homogenates. cDNA was made with Random Hexamers by reverse transcription and then quantitated by real-time PCR using viral RSV-P primers. B2M was the housekeeping gene used as reference. Inhibition of hRSV P mRNA expressed as mean ± SEM per group (n=15-33 mice per siRNA treatment group, n=17 per ribavirin group). Asterisks indicate significant inhibition ($P < 0.05$).

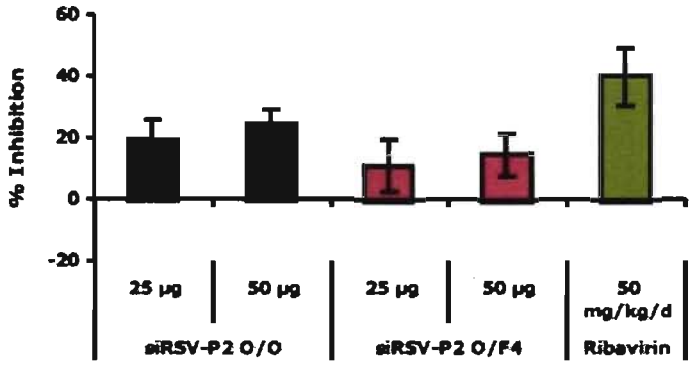


Figure 45. % Inhibition of mouse MIG levels in BAL of hRSV infected BALB/c mice (measured by mMIG ELISA). Animals were sacrificed at indicated times post infection and BAL fluids were collected and analyzed by mouse MIG ELISA, Inhibition of mouse MIG expressed as mean ± SEM per group (n=15-33 mice per siRNA treatment group, n=17 per ribavirin group).

At the mRNA level, a 37% target knockdown of hRSV P mRNA was observed at the low dose with F-ANA modified siRSV-P2, which was significantly higher than the efficacy of the unmodified siRSV-P2 (6%) at this same dose (**Figure 44**, bar 3 and 1, respectively). This enhancement in efficacy of siRSV-P2 O/F4 at the low dose is 83% greater than that of the unmodified siRSV-P2. At the high dose of 50 µg per mouse, both unmodified and F-ANA modified siRSV-P2 inhibited at similar levels, of 38% and 29%, suggesting that a plateau in dosage was reached. Unlike with the other readouts, interestingly, ribavirin was less effective at inhibiting viral mRNA, with only a 16% target knockdown (**Figure 45**, bar 5). With regard to mouse MIG levels, inhibition achieved by siRSV-P2 O/O and O/F4 were of approximately similar levels at both low and high doses, averaging 17%, however treatment with ribavirin was greater with an inhibition of 40% **(Figure 45)**. These results in conjunction demonstrated that both unmodified and F-ANA modified siRSV-P2 exhibited inhibition of viral mRNA and proteins, both direct viral readouts, regardless of the lesser inhibition levels observed with the indirect mouse MIG readout.

CHAPTER VI

GENERAL DISCUSSION

Human respiratory syncytial virus is the leading cause of severe lower respiratory tract disease in young children and an important cause of morbidity and mortality in the elderly and the immunosuppressed. Despite extensive research, development of an effective vaccine against the virus has been difficult due to the partial immune response towards hRSV. Currently, the only antiviral therapy available is ribavirin, however its use is controversial both in regards to efficacy and cost-effectiveness [6]. Recent studies with small interfering RNAs have demonstrated inhibition of hRSV replication by targeting the viral P [8] and NS1 proteins [9] in a murine model. Despite the high inhibition efficacy levels achieved by their siRNAs of RSV replication in mice, in order to become a potential optimized drug, critical changes must be addressed including prolonging their half-lives, enhancing their stability, and increasing their affinity with RNA, safety and bio-distribution without compromising biological activity. We therefore investigated the efficacy of a chemically modified siRNA with F-ANA at inhibiting hRSV replication both in culture and also in a mouse model.

After the initial set up of an *in vitro* hRSV infection in the A549 cell line, four siRNAs were screened for their efficacies. Two siRNAs, siRSV-P1 and siRSV-P2 were targeting the viral P gene and which the other two were targeting the viral NS1 gene. By targeting the genes essential in the process of hRSV replication, we expected to inhibit not only replication but also viral load. *In vitro* results suggested that although both siRSV-P2 and siRSV-NS1 inhibited almost completely viral plaque growth, only siRSV-P2 demonstrated consistent inhibition of mRNA, of viral proteins, and also cytokine levels in a dose-dependent manner. Up to an 89% knockdown of hRSV P mRNA and a 68% inhibition of viral proteins was observed with siRSV-P2 at a dose of 0.4 μg when compared to their respective control mismatches. Cytokine levels, such as MIP-1α and IL-8, were also inhibited up to 73% and 40% with siRSV-P2, respectively. SiRSV-NS1 on the other hand, demonstrated at times inhibition of the virus, however the results did not show consistency throughout the various assays. The siRSV-NS1 had no effect on the viral proteins, yet siRSV-NS1 had inhibition levels of viral titer of approximately 90%, similar to that of siRSV-P2. Zhang *et al* [9] previously had shown that this siRSV-NS1 sequence greatly attenuated RSV replication in their cellular system, when transfected

with a DNA-vector. Our results differed from that of Zhang *et al* [9] possibly because of the difference in transfection methods, where instead of a DNA-vector, a lipid complex (i.e. Lipofectamine2000) was used in order to enhance stability. Using this type of carrier has been proven effective by Bitko *et al* at inhibiting RSV replication however it may not have been the optimal mode of delivery to the cells for the siRSV-NS1 in particular. It can also be hypothesized that the inhibition seen at the cellular level may simply be due to non-specific off-target effects as the siRSV-NS1 failed to decrease the protein level but did attenuate the viral titer. These off-target effects could have been caused by partial complementarity to the RSV mRNA, or by the binding to the target mRNA causing it to frequently induce a moderate mRNA reduction [303]. As some of the tested siRNAs showed promising inhibitory effects while others did not, the differences can be influenced by a gamut of factors. SiRNA guide (antisense) strands must properly bind to the RISC complex in order to search for complementary target mRNA sequences [191]. This linkage to the RISC complex sometimes may not form, which in consequence, will also be difficult to form the 3° structure, thus not allowing for the siRNA to fully carry out its silence pathway to the end. Improper hybridization of the siRNA to target mRNA may also lead to variations in efficacy [209]. As for the pro-inflammatory cellular response observed, generally siRNAs of 21-23 nt can be expected to avoid inducing the interferon response, however even smaller siRNAs have been shown to activate this pathway, resulting in global translational blockade and even cell death [304].

Although inhibition of RSV replication was achieved with siRSV-P2, the possibility of enhancing the potency and efficacy with F-ANA modifications was unknown. Therefore, three different F-ANA modifications were made of the siRSV-P2 in order to investigate their potential enhanced efficacies. Of the three, only siRSV-P2 O/F4 exhibited inhibition levels greater than or similar to the unmodified siRSV-P2. With just a modest modification to the siRSV-P2 strand at the 3'-end of the antisense strand, siRSV-P2 O/F4 inhibited the viral P mRNA 30% better than the unmodified siRNA at the lower doses while maintaining similar inhibitory effect of approximately 92% at the highest dose. Although this enhanced efficacy with siRSV-P2 O/F4 at the lower doses was not detected to alter levels of hRSV protein nor MIP-1α, this F-ANA version did retain equivalent

inhibition abilities at both high and low doses compared to its unmodified O/O version. These results suggested that the dose of potential F-ANA modified siRNA drugs can be reduced and still achieve similar levels of inhibition at the mRNA level. Since the presence of F-ANA within DNA or RNA increases the stability of the oligonucleotide [10, Ferrari, 2006 #255] and potentially decreases the toxicity, it was worth pursuing the comparison *in vivo*. For this, we decided to employ a murine model of hRSV infection.

Despite the shortcomings of using a murine system to study hRSV, including lack of infection of the bronchiolar epithelium, failure to propagate infection from the upper to lower airway, and relatively high inoculum required for robust infection and induction of pathology and illness [305], we were able to demonstrate efficient intra-nasal inoculation of hRSV, with 5×10^5 PFU per mouse, as determined through the detection of hRSV mRNA and proteins, as well as an increased cellular responses. The advantage of using mice is not only its relatively low cost, but also its comparable response to RSV infection, particularly in increased cytokine and chemokine production as well as the cellular patterns of lung inflammation that is comparable to that observed in humans. Immune responses in our infected BALB/c mice were similar to those previously reported by Jafri and colleagues [298], with viral replication peaking 6 days-post infection and becoming undetectable by day 10, also seen by Chavez-Bueno *et al* [297]. Although the hRSV-infected mice did not exhibit any physiological symptoms of pneumonia or loss of weight, they did demonstrate higher levels of inflammatory infiltrates compared to those mice that were sham infected. The influx of lymphocytes into lungs of hRSV-infected mice was visibly more abundant and progressively higher over time than in sham-infected mice. BAL concentrations of TNF alpha, and mouse MIG were also more elevated in hRSV-infected mice than in control mice. Taken together, as these results bore great resemblance to other BALB/c hRSV models, along with the inhibitory action of ribavirin as a control, our in vivo model of hRSV was validated and we were confident in commencing treatment with the selected siRNAs.

Based on their heightened efficacy to inhibit viral replication in vitro, the unmodified siRNA siRSV-P2 O/O and its F-ANA modified version siRSV-P2 O/F4, were both

selected to be tested for efficacy in our murine hRSV model. Although hRSV infection in humans occurs naturally through the nasal route and the virus has an affinity towards respiratory epithelial cells, which once propagated, will find itself in the bronchioles, justifying bronchoalveolar lavage analysis, however nasal instillation of siRNA may not be the optimal method in this hRSV infection murine model. Intratracheal delivery of siRNAs was initially tested in the mice (data not shown), however some deaths occurred, mainly due to their small size and the invasiveness of the procedure. From those observations, intranasal delivery was quickly established as the optimal method for administering siRNAs. Several publications have proven that nasal instillation of siRNAs was non-invasive and are specifically delivered into the lungs [8, 9, 306]. When Massaro et al [306] instilled microliter amounts of siRNA mixture into the nose of lightly anesthetized mice, results showed elevated target mRNA levels specifically in the lungs, whereas when administered intraperitoneally, elevated levels of mRNA were found in the brain cortex, cerebellum, and testes, thereby increasing the risk non-specific effects. Bitko [8] and Zhang [9] both aimed at the inhibition of hRSV with siRNAs targeting different viral genes through intranasal delivery and both showed promising results using this model. It is not so much an issue of whether the siRNAs were entering the lungs, as opposed to staying in the nasal cavity, as the mice were allowed to inhale the siRNA bolus into the lungs while lightly anesthetized. In addition, nasal lavage would be difficult to perform on such a small animal, whereas bronchoalveolar lavage is already a very standard and optimized procedure in mice, thus reinstating that intranasal delivery of siRNA was the optimal route for this hRSV murine model.

Although the control drug treatment of Ribavirin had a greater inhibitory effect in reducing the influx of immune cells, including lymphocytes, neutrophils and eosinophils and secretion of the mouse MIG cytokine, it was not as efficient as either the unmodified or F-ANA modified siRNAs to inhibit levels of RSV protein or mRNA. SiRNAs are designed to specifically inhibit and cleave target genes, therefore it can be expected that siRSV-P2 O/O and O/F4 obtained greater levels of knockdown and inhibition at the mRNA and protein levels. Of note, at lower doses, F-ANA modified siRSV-P2 successfully reduced viral P mRNA levels 5 times better than the unmodified siRSV-P2,

consistent with the results seen *in vitro*. Although both siRNAs did not inhibit the infiltration of lymphocytes in the lungs of hRSV-infected mice as efficiently as Ribavirin, it may be argued that limiting the immune regulating cells is not beneficial for an infected individual. Lymphocytes play a key role in the identification and destruction of foreign particles. Although infection by hRSV in infants and young children may cause illnesses such as bronchiolitis which is an inflammation of the small airways of the lungs, there exists the possibility that interfering with the infiltration of immune cells into target infected areas may result in more harm than allowing for the presence of inflammatory immune cells. When presented with the virus, lymphocytes are attracted to the site of infection, i.e. the lungs, and their presence is required for the termination of viral replication, recovery from primary infection and protection from re-infection [125]. Studies of RSV infection in mouse models have demonstrated that in previously naïve animals, cytotoxic T lymphocytes and antibodies both play a role in eradicating RSV from the respiratory tract [307] [125], in that T lymphocytes are an important determinant of illness and that antibodies are postulated to be an illness-sparing mechanism for the protection of the mice against hRSV infection. Therefore the allowance of lymphocyte infiltration may be in fact an advantage since it also correlated with the presence of illness. Not all immune cell influx may play a direct role in controlling the infection as the rise in neutrophils on the day following infection in both sham-infected and hRSV-infected mice demonstrates that the simple action of administering a small volume of liquid into the nasal cavity and lungs can stimulate an immediate response during the acute phase of infection.

Though daily treatment with ribavirin appeared to inhibit overall hRSV infection, a single small dose of siRNAs administered to mice once was effective enough at inhibiting viral replication by almost half. Inhibition levels of hRSV proteins achieved by both ribavirin and F-ANA modified siRSV-P2 at the highest dose are comparable, thus similar potency. Even more remarkable was the knockdown of target hRSV P mRNA in lung of hRSV-infected mice treated with the low 25 μg dose of siRSV-P2 O/F4. The effect was 61% greater than that observed with ribavirin. These results have the potential to be clinically important, as a tolerable therapeutic dose of siRNA for nasal installation could be envisioned for humans, with possibly less repeated dosing.

From the results presented in this study, it is apparent that not only did unmodified siRSV-P2 inhibit viral replication *in vitro*, but that a modest substitution of two riboses with F-ANA placed at the 3' end of the antisense strand of siRSV-P2 (siRSV-P2 O/F4) improved its efficacy by 30% at the lowest dose. Animal studies demonstrated that unmodified siRSV-P2 was effective *in vivo* at inhibiting viral replication in a dose dependent manner when simply administered intra-nasally. Although siRSV-P2 O/F4 obtained greater inhibition at the mRNA level when instilled at the lower dose, overall the F-ANA modifications to the siRNA maintained a similar levels of efficacy as the unmodified siRNA at the 50 μg dose for all readouts. Further studies may be performed to assess the potential of other placement of F-ANA along the siRNA duplexes for improved efficacy both *in vitro* and *in vivo*. Although only one chemistry of F-ANA was tested in our RSV mouse model, in other in vitro models an assortment of F-ANA modifications to siRNAs have been screened by Dowler *et al* [10] and we selected our F-ANA modification based on the success of this modification in their system. There is no guarantee that a modification that is effective in one system will be the best in another system. More F-ANA versions should be tested for more desirable levels of inhibition of hRSV infection *in vivo*. Furthermore, the beneficial effect of F-ANA over native siRNAs is often detectable at low doses or over longer periods of time because of increased stability, arguing for the need of further studies to be done.

Various dosing regimens and delivery mechanisms have been examined by others in order to improve the activity and stability of the siRNAs. In our study, various doses of up to 50 μg per mouse were examined however siRNAs were administered only at a single time point on the same day as hRSV inoculation. Different timing strategies may be employed, such as several hours and also days post-infection or even re-dosing post-infection, so as to prolong the presence of siRNAs so as to inhibit hRSV replication. Unmodified and F-ANA modified siRNAs may also be investigated as a method of prophylaxis if treatment were to occur prior to hRSV infection. The frequency of dosing may be reduced if the stability of the siRNAs were to be strengthened. As discussed in the introduction, the stability of the siRNAs can be greatly enhanced if a delivery

mechanism were to be employed, such as cholesterol-conjugation, aptamer-conjugation and liposome-formulations. Although our *in vitro* screening of siRNAs were conducted with a Lipofectamine 2000 as a lipid carrier, investigations in mice were executed with naked siRNAs instilled directly into the nose of the animals. Bitko *et al.* estimated that the naked siRNA was 70-80% as effective as the siRNA complexed with transfection reagent. This finding along with our results using naked siRNAs is of particular importance as transfection reagents themselves may have adverse effects. Our results clearly demonstrate that siRNAs delivered unguarded may offer substantial protection against hRSV.

In conclusion, our data showed that naked unmodified and F-ANA modified siRNA reduced the infection and replication by hRSV in A549 cells and in mice when administered intra-nasally. Treatment with siRSV-P2 O/F4 retains the degree of efficacy observed with siRSV-P2 O/O *in vivo*, however chemical modification of this siRNA with F-ANA did not lead to dramatic improvements of its *in vivo* efficacy, except for a trend for more efficacy at lower doses. Daily therapy with ribavirin obtained similar inhibition of RSV infection (as assessed by knockdown at the mRNA level) as F-ANA modified siRSV-P2, however the cumulative dose of ribavirin to achieve this level of efficacy was 75-fold greater than the dose of siRNA. These findings are encouraging and indicate the need for more research especially focusing on areas such as prolonging the siRNA half-life, enhancing their stability, increasing their affinity with RNA and bio-distribution without compromising biological activity, in order to convert these siRNAs into optimized prophylactic and/or therapeutic agents against hRSV infection in humans.

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