Development of Novel Anti-Respiratory Syncytial Virus Therapies

by

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Laboratory Medicine and Pathobiology
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Abstract
Respiratory syncytial virus (RSV) is a leading cause of mortality in infants and young children. Despite the RSV disease burden, no vaccine is available and treatment remains non-specific. New drug candidates are needed to combat RSV. Towards this goal, we investigated two broad strategies to control RSV infection. First, we examined the utility of delivering an anti-4-1BB agonist antibody to enhance CD8 T cell costimulation in a mouse model of RSV infection. This strategy enhanced CD8 T cell numbers, however failed to reduce RSV titers in the lung. We found that this was likely due to reduced RSV specific CD8 T cells in the lung. Overall, this strategy did not attenuate RSV associated disease outcomes and was associated with increased morbidity. Second, we screened over 2000 FDA approved compounds to identify approved drugs with novel anti-RSV activity. Cardiac glycosides, inhibitors of the membrane bound Na+/K+-ATPase, were identified to have anti-RSV activity. Cardiac glycosides diminished RSV infection in HEp-2 cells and in primary human airway epithelial cells grown at an air liquid interface. Digoxin, an FDA approved cardiac glycoside, was further able to inhibit RSV infection of community isolates of RSV in primary nasal epithelial cells. Our results suggest that the antiviral effects of cardiac glycosides are primarily dependent on changes in the intracellular Na+ and K+ composition. Consistent with this mechanism, we subsequently demonstrated that
the ionophoric antibiotics salinomycin, valinomycin, and monensin inhibited RSV in HEp-2 cells and primary nasal epithelial cells. Our data indicate that the $K^+/Na^+$ sensitive steps in the RSV lifecycle occur within the initial 4 hours of virus infection, but do not include virus binding/entry. We employed an RSV mini-replicon assay allowing the independent assessment of RSV-specific RNA synthesis. Our findings demonstrated that digoxin does not alter the RSV RNA polymerase. Future studies will focus on the possibility that digoxin and other ion-modulating drugs may impact viral uncoating (i.e. release of viral nucleic acid from RNP complex).
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List of Abbreviations

RSV    Respiratory syncytial virus
N      Nucleoprotein
P      Phosphoprotein
M2-1   Transcription processivity factor protein
L      Large polymerase subunit protein
RNP    Ribonucleoprotein
M      Matrix protein
G      Attachment glycoprotein
F      Fusion protein
SH     Small hydrophobic protein
TNF    Tumor necrosis factor
RNA    Ribonucleic acid
NS1/NS2 Nonstructural protein 1/2
IFN    Interferon
h.p.i.  Hours post infection
SP     Surfactant protein
PRR    Pattern recognition receptor
TLR    Toll-like receptor
NK     Natural Killer cell
DC     Dendritic cell
BAL    Bronchoalveolar lavage
HIV    Human immunodeficiency virus
VZV    Varicella-zoster virus
HR     Heptad repeat
HSV    Herpes simplex virus
HCMV   Human cytomegalovirus
MVC    Maraviroc
HPV    Human papilloma virus
HBV    Hepatitis B virus
HCV    Hepatitis C virus
K+     Potassium
Na+    Sodium
Ca2+   Calcium
Li+    Lithium
Ab     Antibody
i.p.   Intraperitoneally
i.n.   Intranasal
ALI    Air-liquid interface
NMDG   N-methyl-D-glucamine
GFP    Green fluorescent protein
EMEM   Eagle’s minimum essential medium
PFU    Plaque forming units
MOI    Multiplicity of infection
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>TOA/TOR</td>
<td>Time of addition/Time of removal</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% inhibitory concentration</td>
</tr>
<tr>
<td>CC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>50% cytotoxicity concentration</td>
</tr>
<tr>
<td>SI</td>
<td>Selectivity index</td>
</tr>
<tr>
<td>PNEC</td>
<td>Primary nasal epithelial cell</td>
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<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
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1. Review of the Literature

1.1 Respiratory Syncytial Virus

Classification

RSV was first isolated in 1956 from a chimpanzee as an agent causing coryza and initially named chimpanzee coryza agent\(^1\). Soon after the virus was isolated from children with respiratory illness, it was renamed RSV because in tissue culture, infection of cells resulted in cell fusion or syncytial formation\(^2\). RSV belongs to Genus \textit{Orthopneumovirus}, Family \textit{Pneumoviridae}, and Order \textit{Mononegavirales}\(^3\). RSV is found as a single serotype with two antigenic subgroups (A and B) based on variation in the surface G protein that results in different antibody cross-reactivity patterns\(^4,5\). More recently, these subgroups have been further classified into phylogenetically related strains or genotypes based on genetic variation within the G protein\(^6-8\).

Virion

The virion consists of the viral RNA encased by the nucleoprotein (N), which forms the helical nucleocapsid. The nucleocapsid along with the phosphoprotein (P), the transcription processivity factor (M2-1), and the large polymerase subunit (L) are bound together to form the ribonucleoprotein (RNP) complex. This complex, surrounded by the matrix (M) protein, make up the inner part of the virion. The M protein is thought to form a two-dimensional lattice layer on the inner face of the envelope and together with the M2-1 protein tethers the RNP to the lipid envelope derived from the host cell plasma membrane during budding\(^9,10\). The envelope contains three virally encoded
transmembrane surface glycoproteins: the major attachment glycoprotein (G) involved in binding the virus to the cell surface, the fusion glycoprotein (F) responsible for mediating fusion of the viral and cell membrane, and the small hydrophobic (SH) protein which forms pore like structures with cation-selective channel-like activity (Figure 1.1A). The viral glycoproteins are present as homo-oligomers that are short (11 to 20 nm), closely spaced surface projections or spikes. Viral particles appear to be heterogeneous in size and shape and can appear as irregular spherical particles of 100 to 350 nm in diameter or long filamentous forms that are 60 to 200 nm in diameter and up to 10 µm in length when visualized by electron microscopy\textsuperscript{11,12} (Figure 1.1B).

RNA

The RSV genome (Figure 1.1C) is a single-stranded, non-segmented, negative sense RNA of approximately 15,000 nucleotides (subgroup A strain A2, GenBank accession number M74568, is the reference strain). The genome lacks a 5’ cap and a 3’ polyadenylated tail. The RSV genome is organized into 10 genes in the order 3’-NS1-NS2-N-P-M-SH-G-F-M2-L-5’ that are transcribed sequentially into the corresponding mRNA. The mRNAs have methylated 5’ caps and 3’ polyA tails. Each mRNA encodes a single protein except for M2 that encodes the M2-1 and M2-2 proteins\textsuperscript{13}. The 3’ end of the genome consists of an extragenic leader region and the 5’ end consists of an extragenic trailer region. Highly conserved nine-nucleotide gene-start signals are present at the beginning of each gene and moderately conserved 12-13 nucleotide gene-end signals are present at the end of each gene\textsuperscript{14,15}. Intergenic regions that lack any conserved motifs, are poorly conserved, and appear to be unimportant spacers that separate the first nine genes. The last two genes, M2 and L, overlap by 68 nucleotides\textsuperscript{16}. 
Figure 1.1: Structure and genome organization of RSV.

(A) Schematic representation of the RSV virion. The RSV envelope consists of the F, G, and SH proteins. The M protein underlies the lipid bilayer. The proteins of the RNP complex make up the inner viral lumen. Image adapted from Bawage et al., (2013)\textsuperscript{17}. (B) Cryo-electron tomographic reconstructions of the two RSV virion morphologies; spherical and filamentous. Scale bars, 100 nm. Images adapted from Kiss et al., (2014)\textsuperscript{10}. (C) Genomic map of RSV RNA. The map illustrates the 3’ to 5’ negative sense RNA genome of RSV. The overlapping open reading frame of the M2 messenger RNAs are depicted above the M2 genes. Numbers beneath the map indicate nucleotide (nt) lengths \textit{(in italics)} and the amino acid (aa) lengths. Colour code: genes of the nucleopcapsid or that are involved in RNA synthesis are in blue, surface glycoproteins in green, matrix protein in purple, and the two nonstructural proteins in red.
Proteins

As RSV has a relatively small genome, many of its proteins perform multiple functions that allow the virus to usurp host cells for its own replication. For instance, in addition to its role in attachment, the G protein aids in evading host immunity by binding to TNF-α or other homologs modulating the host antiviral response\textsuperscript{18-20}. TNF-α/β are proinflammatory cytokines implicated in the antiviral response to RSV\textsuperscript{21}. The RSV N protein primarily binds the RNA genome to create an RNase-resistant nucleocapsid, which serves as the template for RNA synthesis by the viral polymerase. However, because the RSV genome lacks stabilizing features, such as capping and polyadenylation, the tight encapsidation by the N protein also protects the genome from degradation and recognition by host cell pattern recognition receptors\textsuperscript{22}. The RSV P protein is a multifunctional adapter protein that facilitates interactions between components of the nucleocapsid/polymerase complex during transcription and replication. However it also plays a role in uncoating of the viral core\textsuperscript{23}. The L protein contains the polymerase catalytic domains and is therefore essential for transcription and replication. Studies with RSV minireplicons demonstrate that the minimum requirements for RNA replication are the N, P, and L proteins\textsuperscript{24}. Although N, P, and L alone have transcriptase activity, M2-1 is an essential transcription processivity factor. In the absence of M2-1 the viral polymerase terminates prematurely within several hundred nucleotides of the 3’ end of the genome\textsuperscript{25}. M2-1 is also involved in the trafficking of the M protein to the site of replication and facilitates the interaction with nucleocapsids\textsuperscript{26}. The RSV M protein mediates the association of the nucleocapsid with the nascent viral envelope, and also helps silence viral RNA synthesis prior to packaging into the viral particle\textsuperscript{27}. The RSV
genome encodes three non-structural proteins (NS1, NS2 and M2-2), which are produced in infected cells but are not incorporated in virus particles. The NS1 and NS2 proteins are unique within the family *Pneumoviridae* to the genus *Orthopneumovirus*. NS1 and NS2 strongly interfere with the induction and signaling of type I IFN and type III IFN in human epithelial cells, macrophages, and dendritic cells. This suppresses a major component of host innate defense thereby activating prosurvival pathways, and subsequently prolonging the life of the cell and increasing virion production\(^28\).

Interestingly, NS1 (and to a lesser extent, NS2) may inhibit viral transcription and replication\(^29\) in part to avoid the accumulation of viral dsRNA that would otherwise activate innate immunity. Lastly, M2-2 plays a role in shifting RNA synthesis from transcription to RNA replication\(^30\) and may play a role in increasing the efficiency of packaging\(^31,32\).

1.2 Replicative Cycle of RSV

RSV preferentially infects the apical surface of ciliated airway epithelial cells\(^33,34\). Although specific details are somewhat unclear, it is currently thought that productive entry of RSV into cells relies on two different binding events mediated by the RSV surface proteins G and F. Infection begins with attachment of RSV to the cell surface through the G protein\(^35,36\). Interactions with cellular glycosaminoglycans, especially heparin sulfate and chondroitin sulfate B, stabilize the virion on the cell surface\(^37,38\) and allow for the F protein to mediate subsequent fusion. Several potential receptor molecules have been tentatively identified for RSV, including intracellular adhesion molecule (ICAM-1)\(^39\), RhoA\(^40\), CX3CR1\(^18,41,42\), and annexin II\(^43\). More recently, efficient RSV
infection both in vitro and during experimental infection of mice identified nucleolin as a functional receptor for RSV through interaction with the F protein.\textsuperscript{44}

After the binding and uptake of RSV by host cells, a second cleavage in F by a furin-like protease provides the cue for viral entry to the cytoplasm by an acid-independent membrane fusion event.\textsuperscript{45} Currently, the cellular pathway of entry remains unclear. Previous studies employing a lipid-dequenching assay suggested that RSV, as most other paramyxoviruses, fuses its membrane directly with the plasma membrane of target cells\textsuperscript{46}. That RSV entry is pH-independent is consistent with this view\textsuperscript{47}. On the other hand, evidence has suggested that RSV uses clathrin-mediated endocytosis to infect cells based on interactions with clathrin light chain proteins and association with cholesterol micro-domains and membrane Rho-GTPases\textsuperscript{48,49}. In a recent publication, Krzyzaniak and colleagues suggested macropinocytosis as the initial uptake step of RSV, based on the dependence of RSV infection on Rab5 and other macropinocytosis-associated proteins\textsuperscript{50}. Thus, RSV appears to utilize one or more of these pathways to gain access to the host cell cytoplasm.

Following fusion, uncoating of the viral core is necessary for the delivery of the genome to the site of replication. In general, uncoating is dependent on cellular cues including receptors, low pH, osmolytes, proteases, or physical forces\textsuperscript{51}. Currently, little is known about the RSV uncoating process. Phosphorylation of S54 on the RSV P protein has been shown to be required for disassociation of the RNPs from the M protein, making them active for transcription, however the mechanism remains unclear\textsuperscript{23}.

After uncoating, genome transcription and replication occur concurrently in the cytoplasm. Upon entry into the cytoplasm, the virally encoded RNA dependent RNA
polymerase sequentially transcribes the RSV genome into messenger RNAs (mRNAs). The virus then utilizes host ribosomes for protein synthesis52. RSV transcripts and proteins can be detected as early as 4-6 hours post infection (h.p.i.) and reach peak abundance by 15-20 h.p.i53. Viral proteins accumulate in the cytoplasm in discrete aggregates, termed cytoplasmic inclusions54. These viral cytoplasmic inclusions contain RSV M, N, P, L, M2-1, M2-2, and the viral RNA26,54. Upon accumulation of the M2-2 protein, the balance of RNA synthesis is shifted from transcription to RNA replication31. RNP complexes form in the inclusions54 and then traffic to the apical membrane, where they meet with F, G, and SH that arrive from the Golgi through the secretory pathway34,55. The viral M protein and host components such as actin have been implicated in trafficking the RNP complexes from inclusion bodies to the cell surface for budding56-58.

At the inner cell surface, RSV proteins and viral RNA assemble into virus filaments59. Assembly appears to occur in regions containing virus-modified lipid rafts involving all three viral surface proteins and the M protein60-62. The minimum viral protein requirements for the formation of virus-like particles capable of delivering genomic material to target cells are the F, M, N, and P proteins32. The viral M protein is thought to direct the assembly and budding of the virion from the plasma membrane10,26,27,57. Interestingly, The M protein has been found as both a monomer63 and a dimer9. The dimer appears to be essential for the formation of higher-order oligomers that trigger viral filament assembly by interacting with F9,64. At the budding step, M is crucial for viral filament maturation and elongation57.
The final step in viral assembly and budding involves a membrane scission event severing the assembled viral particle from the host cell membrane and forming a free particle. Viruses often accomplish this through the use of host machinery involved in multivesicular body formation. Vesicle formation depends on endosomal sorting complex required for transport (ESCRT) proteins and a common ATPase, Vsp4, to perform membrane scission\textsuperscript{65}. Interestingly, membrane scission of RSV particles appears to occur in a Vps4-independent manner, suggesting RSV uses a novel mechanism to accomplish membrane scission\textsuperscript{66}. Some evidence has suggested that RSV may hijack cellular apical recycling endosomes for budding\textsuperscript{34,66}. The release of progeny virions begins by 10 to 12 h.p.i, reaches a peak after 24 hours, and continues until the cells deteriorate by 30 to 48 h.p.i. A schematic of the RSV replication cycle is depicted in Figure 1.2.

1.3 RSV Pathogenesis and Pathology

RSV disease can manifest in different ways\textsuperscript{67}. Disease can range from mild upper airway symptoms including nasal congestion and rhinorrhea to otitis media or more severe disease\textsuperscript{68} with lower respiratory tract involvement including life-threatening bronchiolitis and pneumonia\textsuperscript{69}. RSV associated mortality is rare in developed countries, largely due to the improved supportive care, however globally, RSV is the most common viral cause of infant death\textsuperscript{52,70,71}. In addition to acute morbidity and mortality, epidemiological studies demonstrate that RSV infection is associated with the development of recurrent wheeze and asthma in later life\textsuperscript{72-75}. Although it remains controversial whether RSV actually causes asthma, some studies suggest RSV prophylaxis reduces the subsequent diagnosis of asthma\textsuperscript{76}. 
Figure 1.2: Schematic representation of the RSV life cycle.
(1) After attachment to cell surface receptors, RSV is thought to enter the cell in one of two ways. (2a) The first is a direct membrane fusion event between the plasma membrane and the viral membrane. (2b) The second, RSV enters through macropinocytosis where cleavage of the F protein by furin-like proteases causes a membrane fusion event in late macropinosomes. (3) After membrane fusion, a relatively unknown uncoating mechanism occurs, resulting in the release of the RNP into the cytoplasm. (4) Primary transcription and genome replication occur concurrently. The genome is replicated to produce the antigenome, which serves as a template to synthesize additional genomic RNA. The additional genomic RNA is then used as a template for secondary transcription or is incorporated into progeny virions. (5) After translation, M proteins and RNPs are transported intracellularly to the plasma membrane and the viral glycoproteins F, G, and SH are transported from the endoplasmic reticulum to the Golgi apparatus and then the plasma membrane. (6) Finally, new virions are assembled and are subsequently released from the plasma membrane by a budding process. Figure adapted from Schildgen et al., 2011\textsuperscript{77} and De Clerq & Li, 2016\textsuperscript{78}.
Conversely, there is evidence that infants presenting with severe RSV disease may have been at higher risk for asthma even prior to their RSV disease and hence the severe RSV infection is simply a marker of asthma predisposition79,80.

RSV is a highly infectious virus, and humans are its only natural host. RSV is predominantly spread through droplets or fomites that make contact with the nasal mucosa81. More recently there is evidence that RSV may spread through the aerosol route82. The incubation period from time of infection to onset of illness is about 3 to 5 days83-85. Typically, RSV infection initiates in the nasopharynx and if the infection is not cleared by the immune system, progression to the lower airways may occur via mechanical aspiration of infectious material. Infection of the lower respiratory tract results in a worsening of disease symptoms, including onset of tachypnoea, diffuse rhonchi, and wheezing69. Signs of lower respiratory tract infection usually appear 1 to 3 days following the onset of rhinorrhea. At this point in infection the disease can resolve within 1–2 weeks. However, further spread of infection into distal airways results in exacerbation of pulmonary disease. Clinically this presents as acute bronchiolitis, with or without evidence of pneumonia69.

RSV disease arises from both direct viral damage as well as the host immune response, however the relative contributions of each remain controversial and likely vary between individuals (Figure 1.3). There is a positive correlation between RSV viral load and disease severity83,84,86,87, however, the extent to which this reflects increased viral damage versus an increased corresponding immune response remains unclear88,89. RSV infection of human airway epithelial cells is limited to the superficial layer of ciliated cells and does not induce syncytia in vivo or invade the underlying layer of cells33,90,91.
The combination of both RSV related viral factors and the host immune response contribute to the severity of RSV associated disease. The cytopathic effects of RSV may explain many of the pathological findings in RSV infected airways. However the immune response to RSV has been suggested to result in increased inflammation that may also explain many of the pathological findings. Coupled with various external factors, environmental or genetic, and the long lasting immune dysfunction associated with RSV, chronic airway disease may also result from RSV infection.
This is based on histopathological findings from airways of children infected with RSV\textsuperscript{92-94}. Additionally, RSV does not appear to be highly cytopathic as there is little damage to the tissue aside from inhibition of ciliary function\textsuperscript{33,91}. Despite the low cytopathology associated with RSV infection, there is evidence to suggest that the viral load drives disease severity in humans\textsuperscript{95,96} as in most cases, individually infected cells eventually undergo apoptosis. Furthermore, different RSV clinical isolates can induce variable amounts of disease in BALB/c mice\textsuperscript{97} suggesting that virulence factors in the virus itself are important. In post mortem studies of infants with severe RSV disease, few inflammatory cells have been found in the lung parenchyma, yet virus is found throughout the lungs, emphasizing the importance of viral load in disease severity\textsuperscript{94}.

Conversely, studies in humans and in animal models demonstrate that the host immune response also plays a major role in the severity of disease. Multiple studies have demonstrated that airway epithelial cells produce inflammatory cytokines and chemokines, such as IL-1\textalpha, IL-6, and RANTES, in response to RSV infection\textsuperscript{98-102}. Activated macrophages and neutrophils, as well as epithelial cells, secrete IL-1\textalpha, which serves to stimulate inflammation early after the onset of infection. The cytokine IL-6 and chemokine RANTES are secreted by several cell types and are later acting mediators of inflammation. Additionally, IL-8 and activated granulocytes have been found in the airways of infants and children with severe RSV disease, with neutrophils being by far the most abundant immune cell\textsuperscript{94,103-105}. Moreover, genetic variations in the host’s inflammatory immune response, such as cytokine gene polymorphisms or surfactant protein polymorphisms, could in part explain the wide range of disease severity seen in RSV infection\textsuperscript{106-109}. Furthermore, the host capacity to produce pro-inflammatory
responses has also been shown to correlate with human RSV disease severity. While this host response aims to be protective, when exaggerated it can contribute to lung damage and dysfunction.

1.4 Host Immune Response to RSV

The Innate Immune Response and Inflammation

The lung is equipped with an innate immune response that provides the first line of host defense in RSV infection. This system is made up of a number of cells and each plays an important role. The airway epithelium forms a mechanical barrier between the environment and the host however these cells are the primary targets for RSV. The epithelium produces mucous that traps pathogens including RSV. Epithelial cells are also lined with cilia that function to move mucous (and trapped pathogens) out of the lung. The mucous and airway surface liquid are enriched with various antimicrobial factors that can also inhibit RSV. Surfactant proteins are one example. Mice deficient in SP-A, SP-C, or SP-D exhibited increased RSV titers and disease. Additionally, there is some evidence that severe RSV disease in infancy is associated with genetic polymorphisms in SP-A, SP-B, and SP-D. These proteins not only bind and neutralize RSV, but also promote phagocytosis by monocytes and macrophages.

Respiratory epithelial cells are often the first cells to interact with RSV and are equipped to sense RSV and trigger an inflammatory response to aid in viral defense. Pattern recognition receptors (PRRs) are essential for the recognition of RSV. In response to RSV infection, these signaling molecules activate a proinflammatory innate immune cell response that is both protective and immunopathogenic. There are two families of PRRs involved in the recognition of RSV: Toll-like receptors (TLRs) and RIG-I.
(retinoic acid inducible gene)-like receptors (RLRs)\textsuperscript{121-123}. A number of TLRs have been suggested to play a significant role in the host response to RSV\textsuperscript{124}. Stimulation of TLR4 by the RSV F protein, independent of viral replication, leads to the activation of NFκB-mediated innate immune responses and inflammation\textsuperscript{125,126}. Additionally, RSV has been shown to persist longer in the lungs of infected mice deficient in TLR2, TLR4, or TLR6, suggesting these TLRs play a protective role\textsuperscript{125,127}. Although, inactivation of TLR3 or TLR7 does not significantly impair RSV clearance in the lungs of infected mice, production of proinflammatory cytokines and goblet cell hyperplasia are enhanced suggesting these TLRs play a role in preventing severe disease\textsuperscript{128-130}. In addition to TLRs, RLRs play an important role in the activation of RSV induced innate immune responses by stimulating the production of a range of inflammatory cytokines and chemokines\textsuperscript{131-133} including the type-I and type-III interferons (IFNs) which play a crucial role in the antiviral response\textsuperscript{123,134}.

Epithelial cells produce a broad array of proinflammatory cytokines in response to RSV that have been implicated in the initiation of the immune response to RSV. These cytokines include IL-1, IL-6, IL-8/CXCL8, IL-10, TNF-\(\alpha\), RANTES/CCL5, MCP-1/CCL2, MIP-1\(\alpha\)/CCL3, MIP-1\(\beta\)/CCL4, MIP-2/CXCL2, IP-10/CXCL10, and eotaxin-1/CCL11\textsuperscript{100,135-141}. This initial response contributes significantly to inflammation of the lung, and promotes the migration of immune cells, such as eosinophils, neutrophils, macrophages, and monocytes into the lungs, which are in turn activated to produce additional pro-inflammatory molecules\textsuperscript{140,142} (Figure 1.4).
Figure 1.4: Innate and adaptive immune response to RSV infection.

Upon RSV infection of the airways, lung epithelial cells begin to secrete inflammatory signals, such as cytokines and chemokines. Proinflammatory cytokines and chemokines then activate neutrophils, monocytes, and eosinophils and recruit them to the lung. These cells further release proinflammatory cytokines. Alveolar macrophages and NK cells also play an important role in decreasing the viral load in the lung. Between the early innate response and the adaptive response are DCs. DCs that are infected by RSV are impaired in their ability to both migrate to lymph nodes and their ability to activate T cells (denoted with small dotted lines). This triggers an inefficient T cell response for virus clearance and promotes a Th2 biased response. This decreases the effectiveness to kill infected cells due to the down regulation of proteins such as Granzyme B and Perforin. CD8 T cells are also impaired by the release of inhibitory molecules by the respiratory epithelium. After RSV infection, B cells generate non-protective antibodies that rapidly decay. Figure adapted from Gomez et al., 2014\textsuperscript{143}. 

\begin{figure}[h]
    \centering
    \includegraphics[width=\textwidth]{figures/immune_response.png}
    \caption{Innate and adaptive immune response to RSV infection.}
    \end{figure}
Inflammatory molecules produced by epithelial cells in response to RSV also promote the recruitment of innate immune cells, such as macrophages, dendritic cells, and natural killer (NK) cells. Studies in mice have indicated that macrophages provide an immediate response of proinflammatory cytokines following RSV infection\(^\text{144}\), and are the primary producers of type I IFN\(^\text{145}\). Although macrophages are dispensable for the development of the adaptive immune response to RSV, alveolar macrophages appear to be important both in restricting RSV infection and in clearing debris later in infection that would otherwise promote further damage and inflammation\(^\text{146}\) (Figure 1.4). Dendritic cells (DCs) are the major antigen-presenting cells and play an essential role in the induction and regulation of adaptive immune responses\(^\text{147,148}\). The lung contains three major subsets of DCs, conventional DCs (CD11b\(^+\)), CD103\(^+\) DCs, and plasmacytoid DCs (pDCs). All three subsets act to detect RSV infection and promote B cell responses, generate cytotoxic CD8 T cell responses, and aid in the differentiation of CD4 T cells\(^\text{149}\). pDCs are also believed to be important in limiting RSV associated immunopathology as well as promoting viral clearance in mouse models of RSV\(^\text{150,151}\). Upon RSV infection lung resident DCs rapidly migrate to the lung-draining lymph nodes and play a role in the activation of both naïve CD8 and CD4 T cells\(^\text{152}\). Simultaneous to this, there is a substantial influx of all three types of DCs into the lung. Despite only a small fraction of human DCs exposed to RSV becoming productively infected, a number of studies have suggested that RSV infection may modulate the function and T cell stimulatory capacity of both infected and uninfected DCs\(^\text{153-155}\). Additionally, RSV infection of human DCs results in minor increases in the expression of lymph node homing receptor CCR7 effectively preventing their migration, while also decreasing their capacity to activate
CD4 T cells, thereby preventing the induction of a robust T cell response\textsuperscript{153,154} (Figure 1.4). NK cells play a critical role in killing infected cells and produce IFN-\(\gamma\) early in infection. This in turn plays a critical role in priming DC function and T cell responses\textsuperscript{156}. NK cells likely play an important role during RSV infection, as infants presenting with severe RSV bronchiolitis have been reported to have poor infiltration of NK cells into the airway\textsuperscript{94}. On the other hand, animal models of RSV infection have suggested that NK cells play a role in disease pathogenesis and may contribute to lung injury during the early stages of RSV infection\textsuperscript{157}.

**The Adaptive Immune Response**

The adaptive immune response plays a crucial role in the acute infection and in mediating protection against reinfection from viruses. While long-lived immunity against RSV is not achieved\textsuperscript{158,159}, previous work has indicated that high serum titers of neutralizing antibodies correlate with increased protection\textsuperscript{160-164}. In addition to neutralizing antibodies, the cytotoxic T cell response is critical for mediating viral clearance during primary RSV infection\textsuperscript{165,166}.

Antibodies play an essential role in antiviral immunity by either directly neutralizing or aiding in the opsonization of free viral particles\textsuperscript{167}. Infection of both young infants and adults results in the induction of an RSV specific antibody response, however long lasting immunity fails to develop due to the rapid decay of antibody titers\textsuperscript{168-170} (Figure 1.4). The two major surface proteins of RSV, F and G, are the most important targets of RSV-specific neutralizing antibodies\textsuperscript{171}. The effective prophylaxis of high-risk infants with the humanized monoclonal antibody, palivizumab, directed to the RSV F protein demonstrates that neutralizing antibodies confer protection against RSV.
infection. Indeed the passive transfer of maternal IgG antibodies protects neonates, however levels of maternal antibodies rapidly decline and neutralizing RSV-specific antibody responses can only be detected in 50-70% of children less than six months of age. Lower than average titers of RSV-specific nasal IgA and serum IgG antibodies are associated with increased rates of RSV infection.

Induction of an appropriate T-cell response is required to clear a primary RSV infection. This is especially evident in adoptive transfer experiments where the transfer of RSV-primed T cells (CD4 or CD8) from infected mice effectively clear persistent RSV infections in immunodeficient mice.

The priming of different CD4 T cell subsets appears to determine the quality and magnitude of the CD8 T cell response and subsequent disease pathogenesis during infection with RSV. In mice, the RSV F protein primes both CD8 and CD4 T cells toward a Th1-type biased response, however the G protein primes CD4 T cells that are polarized toward a Th2-type cytokine response (Figure 1.4). The relevance of Th2 responses in the development of RSV-induced immunopathology in young infants is unclear. Some studies have reported a correlation between increased Th2 cytokines in bronchoalveolar lavage and the severity of RSV disease, while others failed to detect this correlation. There are a number of factors that could account for these discrepancies, including differences in age, the source of samples, and the time after infection the samples were collected. It is possible that both Th1 and Th2 cells contribute to various manifestations of RSV-induced disease. In animals, there is a clear correlation between Th2 biased responses and increased mucus production and airway hyperreactivity following RSV infection. A strong Th2 response in the host promotes
the recruitment of proinflammatory cells and the production of numerous cytokines, all of which dampens the CD8 T cell response and prevents viral clearance\textsuperscript{188,189}.

CD8 T cells possess multiple pathways to kill infected cells including lysis through the perforin-dependent release of granzyme-containing granules or the induction of apoptosis of infected cells through the interaction of Fas with the death inducing molecule FasL\textsuperscript{190,191}. In a prospective study of infants with RSV bronchiolitis, virus-specific T cell responses were found during the convalescent phase, however these responses did not provide protection from reinfection the following year and secondary infection did not boost RSV-specific T cell proliferation\textsuperscript{192}. Interestingly, it was recently shown that RSV-specific CD8 T cells isolated from infected lungs were impaired in their cytolytic ability and capacity to produce IFN-\(\gamma\)\textsuperscript{193}. Moreover, feedback mechanisms, such as the upregulation of PD-1/PD-L1, are activated in RSV infected lungs and function to inhibit CD8 T cell responses\textsuperscript{194} (Figure 1.4). It is probable that these mechanisms contribute to impaired RSV-specific T cell immunity and may partially explain the lack of long-lived immunity to RSV.

In addition to their critical role in viral clearance, there is some data to suggest CD8 T cells also contribute to lung damage. Mouse studies transferring RSV-specific monoclonal CD8 T cells demonstrated that RSV clearance was associated with significant CD8 T cell mediated lung pathology\textsuperscript{195}. T cell depletion studies in mice also suggest a role for CD8 T cells in both virus clearance and lung pathology\textsuperscript{165}. Importantly, findings in humans differ from those observed in mice, as there is no clear evidence for a CD8 T cell role in lung pathology in humans with severe RSV infections\textsuperscript{196}. Infants are able to mount an RSV-specific CD8 T cell response, however activated CD8 T cells in
the blood and bronchoalveolar lavage (BAL) are almost undetectable during peak illness. Rather CD8 T cells reached maximal levels 9-12 days after the onset of primary symptoms, suggesting CD8 T cell responses better correlate with recovery from disease\textsuperscript{196,197}. Moreover, histological analysis of fatal RSV infections found very high viral titers with relatively few CD4 and CD8 T cells in the lung, suggesting T cells do not contribute to lung damage\textsuperscript{94}. Finally, the role of CD8 T cells in reducing RSV viral titers is especially evident in children with defective T cell responses who show an increase in RSV-mediated disease severity and prolonged virus shedding in the absence of T cells\textsuperscript{198,199}.

1.5 Burden of RSV Infection

RSV is the most important viral cause of lower respiratory tract illness in infants and young children worldwide\textsuperscript{200}. RSV infections occur in yearly epidemic outbreaks during the winter months (November to April) in northern temperate locations or during the cool seasons, either dry or wet, in subtropical regions\textsuperscript{201}. In the first year of life, RSV infects 60-70\% of children, and nearly all are infected by the age of two\textsuperscript{159}. Re-infection is common in the first few years of life. In one prospective study, for infants infected by age 1 year, the reinfection rate was 74-83\% in their second year, and 46-65\% in their third year of life\textsuperscript{158}. Hospitalization for severe RSV disease occurs in approximately 2-3\% of all children and is most frequent between 6 weeks and 6 months of life, with a peak incidence at 2-3 months of life\textsuperscript{200}.

Globally, RSV is estimated to cause 33.8 million cases annually of acute respiratory illness in children less than 5 years of age. These infections resulted in approximately 3.4 million hospitalizations, and up to 199 000 deaths in 2005 alone\textsuperscript{202}. 

20
RSV remains the most frequent viral cause of death in infants worldwide and second only to malaria amongst all causes of infant death\textsuperscript{71}. As 99\% of these deaths occurred in developing countries, reducing the burden of RSV infections has become a priority of the World Health Organization’s new BRaVe (Battle Against Respiratory Viruses) initiative\textsuperscript{203}. The incidence and severity of disease in the developing world is not well documented, however life-threatening disease is far more common than in the developed world\textsuperscript{204,205}.

In affluent countries, such as the United States, approximately 2.1 million children under the age of 5 require medical attention each year due to RSV. This is estimated to result annually in 18\% of all emergency room visits, and 20\% of all hospitalizations\textsuperscript{200}. Rates in Canada and European countries are similar\textsuperscript{206-209}. The burden of RSV disease extends to the outpatient setting where it is associated with considerable acute and long-term morbidity\textsuperscript{200}. In the United States, population based surveillance estimated that among children <5 years of age, RSV results in one of 13 visits to a primary care office each year\textsuperscript{200}. Moreover, the annual rate of RSV infection of German outpatients was 77 per 1000 children under 3 years of age\textsuperscript{208}. Children presenting in an outpatient setting tend to have moderate to severe illness and approximately three quarters exhibit labored breathing. It is also important to note that in addition to acute morbidity and mortality, RSV infection is associated with recurrent wheeze and has been linked to the development of asthma\textsuperscript{72,73,210}.

Epidemiological data indicates that the most important risk factor for severe RSV infection is young age\textsuperscript{211,212}. There are a number of other important risk factors associated with severe RSV disease early in life, including prematurity\textsuperscript{213,214}, low birth weight\textsuperscript{215},
male sex\textsuperscript{216}, underlying cardiopulmonary disease\textsuperscript{217,218}, Down syndrome\textsuperscript{219,220}, and immunosuppression or immunodeficiency disorders\textsuperscript{221}. In one study, the estimated number of RSV hospitalizations per 1000 during the first year of life was 388 for infants with chronic lung disease, 92 for those with congenital heart disease, 66 for those born at 29 to <33 weeks, and 30 for term infants with no underlying disease\textsuperscript{222}. Canada also has a unique group of infants in Northern Communities suffering the highest rates of RSV infection in the world with over 30\% of term babies requiring admission to hospital with RSV\textsuperscript{223-226}. Morbidity and mortality due to RSV are substantially increased in adults and children who are severely immunosuppressed, especially those with T cell deficiencies\textsuperscript{227}. Retrospective studies of hematopoietic stem cell transplant recipients showed that approximately 29\% developed RSV associated lower respiratory tract illness\textsuperscript{228}. Additionally, the mortality rate associated with severe RSV infection in adults with profound immunosuppression can be as high as 80–100\%\textsuperscript{227}. However, it is important to note that more than half of RSV hospitalizations occur in previously healthy, full term individuals. In these term healthy children, researchers have attempted to identify individual characteristics that may place a child at risk. Some factors that have been suggested include maternal smoking, daycare attendance and older siblings\textsuperscript{229-231}.

There is an increasing recognition that RSV is an important cause of illness in adults. RSV re-infects healthy adults at a rate of approximately 5-10\% per year, a rate that significantly increases with increased exposure to the virus, such as with health care workers\textsuperscript{232,233}. Family members of sick children are readily infected, with approximately 40\% of all family members >1 year old becoming infected. However these adults often go undiagnosed\textsuperscript{234}. Among healthy working adults, absence from work due to viral
infection resulted from 38% of RSV infections compared to 66% of influenza cases, however the mean duration of RSV illness (9.5 days) was significantly longer than that of influenza (6.8 days)\textsuperscript{235}. In the elderly population, morbidity and mortality due to RSV infection are significant. Surveillance studies of elderly long-term care facilities found that approximately 5-10% of residents developed RSV infection annually\textsuperscript{236}. RSV infection has also been associated with 10.6% of hospitalizations in the elderly with pneumonia, 11.4% with chronic obstructive pulmonary disease, 7.2% with asthma, and 5.4% with congestive heart failure\textsuperscript{232}. RSV is estimated to cause on average 17,358 deaths a year in the United States, with 78% of deaths occurring in adults over the age of 65\textsuperscript{237}. Thus, in affluent countries RSV mortality is greater in the elderly population than in infants.

\subsection*{1.6 Strategies and Design of Antiviral Drugs}

Viral infections as a group have caused millions of human casualties worldwide, yet for most viruses that cause life-threatening disease there is no vaccine or therapy available. Since the first antiviral drug, idoxuridine, was approved in 1963\textsuperscript{238,239} ninety antiviral agents have been approved for the treatment of 9 human infectious diseases. Most of these are used for the treatment of human immunodeficiency virus (HIV)\textsuperscript{78}. There are still no antiviral drugs or vaccines available for more than 200 infectious diseases caused by viruses. The development of effective antiviral drugs is critical for global human health\textsuperscript{240,241}.

Antiviral drug design strategies can be divided broadly into those that target the virus and those that target the host. Targeting the virus typically yields less toxic compounds however these agents often have a narrow spectrum of antiviral activity and
drug-resistant variants often emerge. Targeting the host frequently affords broadly active antiviral compounds with low chances of developing resistance, but a higher likelihood of toxicity. Both routes have resulted in highly effective approved compounds. This review is not aimed at being comprehensive; rather, it is aimed at offering some insight into the current strategies used for combating viral infections with example therapies presented for illustrative purposes.

**Virus-Targeting Antivirals**

Virus-targeting antivirals inhibit specific functions of viral proteins, thereby blocking discrete steps in the viral life cycle including virus attachment, virus entry, viral uncoating, viral replication and, viral budding (Figure 1.5).

*Attachment inhibitors*

Viral attachment to the host cell surface is a critical step for viral entry into cells. This is achieved through the specific interaction of viral surface glycoproteins with host cell surface receptors. Thus, blocking the interaction between viral particles and host cells is an attractive strategy to inhibit viral infection. Varicella-zoster virus (VZV), the causative agent of chickenpox, shingles, and herpes zoster, has been successfully treated with anti-VZV IgG antibodies. These neutralizing antibodies prevent VZV surface glycoproteins from anchoring to host cells. Two antibody formulations have been developed, varicella-zoster immunoglobulin (VariZIG) and varicella-zoster immune globulin (VZIG). VZIG was discontinued in 2004 and was later superseded by the more effective VariZIG. VariZIG is a detergent treated, lyophilized preparation of IgG purified from human plasma with high levels of anti-VZV antibodies. VariZIG offers passive immunization for immunocompromised patients in addition to post exposure
Figure 1.5: Virus-targeting antiviral strategies.

Five virus-targeting strategies that interfere with major stages of the viral life cycle of most RNA viruses are highlighted in red. Solid black arrows indicate direct biological pathways involving viral replication. Major viral stages of RNA virus infection are illustrated, including viral attachment, endocytosis, virus entry/fusion, viral transcription and replication, and virus budding/release. Other pathways associated with cellular compartments (Golgi apparatus, endoplasmic reticulum, ribosome) are illustrated. Notably RNA viruses replicate in the cytoplasm and usurp host machinery to complete their replication cycle. Shapes and sizes of proteins and cellular components are not to scale. Figure adapted from De Clerq & Li, 2016.\(^{78}\)
prophylaxis. Due to the nature of the antiviral mechanism, VariZIG must be administered within 96 hours of VZV exposure\textsuperscript{242,245}.

**Entry inhibitors**

After attaching to host cells, a virus must release its genome into the cytoplasm through a membrane fusion event between the viral envelope and the cellular plasma membrane. This fusion process can occur during endocytosis or direct membrane fusion at the surface of the cell\textsuperscript{246}. Because viral entry is one of the key early steps in the viral life cycle, entry inhibitors have been successfully developed for antiviral therapies. Perhaps the best characterized is that of HIV\textsuperscript{247}. HIV enters susceptible cells through membrane fusion mediated by the viral Env protein. After attachment, a series of binding events lead to conformational changes in Env exposing and activating a transmembrane moiety, gp41. The extracellular portion of gp41 contains two heptad repeat domains (HR1 and HR2) separated by a loop region and a hydrophobic fusion peptide. The folding of HR2 into the hydrophobic grooves of HR1 is required to complete the fusion process. The peptide inhibitor, enfuvirtide (also known as T20 or Fuzeon (Roche)), is in clinical use to block the fusion of HIV with the extracellular membrane of host cells\textsuperscript{248}. Enfuvirtide is homologous to the helix in HR2 and therefore prevent the interaction between HR1 and HR2\textsuperscript{249,250}. As a result enfuvirtide potently inhibits HIV replication in humans with minimal systemic toxicity\textsuperscript{251}.

**Uncoating inhibitors**

After fusion, the viral capsid core must be disassembled or “uncoated” to allow for the subsequent transcription and replication of the viral genome\textsuperscript{252}. For many viruses this step is dependent on an acidic environment. In the case of influenza virus, the
causative agent of devastating respiratory tract infections responsible for the death of hundreds of thousands each year\textsuperscript{237}, passage through acidic endosomes is an essential step for uncoating and infection\textsuperscript{253}. Influx of protons into the virion lumen, through the viral ion channel M2, separates the viral RNP core from the matrix (M1) protein, thereby releasing the viral genome for efficient infection\textsuperscript{254}. Blocking the M2 proton channel with amantadine or rimantadine effectively restricts proton translocation into the interior of viral particles, thus preventing the uncoating within endosomes\textsuperscript{255,256}. Unfortunately due to widespread resistance, amantadine is no longer used in the treatment of influenza infections\textsuperscript{257}.

\textit{Viral replication inhibitors}

Viral replication occurs by different mechanisms depending on whether the viral genome is DNA or RNA. However, almost all viruses encode polymerases for replication and transcription. Thus, viral polymerases are attractive targets for antiviral development. There are two major types of polymerase inhibitors: (i) nucleoside/nucleotide analogs and (ii) allosteric inhibitors. The majority of approved polymerase inhibitors are nucleoside/nucleotide analogs. Nucleoside analogs undergo several phosphorylation steps by both viral kinases and host kinases to form a triphosphate derivative. The triphosphate derivative mimics the natural nucleoside triphosphates of the viral polymerase and incorporates into the growing viral nucleic acid chain causing termination of replication. Compounds inhibiting the replication of herpes simplex virus (HSV), VZV, and human cytomegalovirus (HCMV) DNA polymerases include acyclovir\textsuperscript{258}, penciclovir\textsuperscript{259} and ganciclovir\textsuperscript{260-262} respectively. A well-known RNA polymerase inhibitor is favipiravir, which has been primarily pursued for the treatment of influenza infections, including
highly pathogenic H5N1 viruses. Interestingly, favipiravir has also been shown to inhibit a broad range of negative-sense RNA viruses.

Viral budding/release inhibitors

After a complex, multistep process involving the transport and organization of viral proteins and nucleic acid on the host plasma membrane, enveloped viruses must then complete the replication cycle by forming vesicles that bud from the plasma membrane in a membrane fission step. At present, antiviral treatment of human influenza infections are based on the use of oseltamivir and zanamivir that act to impede the release of infectious virus from the host membrane. The influenza virus glycoprotein, hemagglutinin, recognizes sialic acid on the surface of cells. Although sialic acid is the receptor for virus entry, newly formed virions remain attached to the cell membrane during budding due to the interaction between hemagglutinin and sialic acid. The viral neuraminidase abrogates this by cleaving sialic acid residues, thereby releasing viral particles. Oseltamivir and zanamivir inhibit this hydrolytic activity. Oral oseltamivir and inhaled zanamivir significantly reduce mortality and the duration of influenza symptoms and complications.

Host-Targeting Antivirals

Viruses are obligate intracellular pathogens, and, as such, they rely on host factors for replication. Targeting host factors dramatically increases the number of therapeutic targets and offers a greater barrier to the emergence of resistance. Moreover, targeting host molecules has the potential for broad-spectrum inhibition when targeting pathways shared by variants of a given virus or different types of viruses. However, this strategy is still somewhat controversial since inhibiting host factors may result in toxicity or adverse
effects in the host. The development of host-targeting antiviral strategies will likely depend on which host factor is targeted and how the viruses and the cell depend on its function. Despite concerns of toxicity, several host-targeting antivirals are currently approved for use in humans.

A highly successful example of an antiviral agent targeting a host protein is in the area of HIV research. HIV enters host cells though the binding of its envelope glycoprotein to the CD4 receptor and/or co-receptors, C-C chemokine receptor type 5 (CCR5) and C-X-C chemokine receptor type 4 (CXCR4), on the surface of CD4 T cells. HIV has two tropic variants; R5-tropic viruses use CCR5 as their co-receptor and X4-tropic viruses use CXCR4 as their co-receptor for entry. Given CCR5’s importance in HIV transmission and infection, drugs that block the interactions between HIV and CCR5 have been highly sought after. Maraviroc (MVC) is the only CCR5 antagonist currently approved for the treatment of patients infected with R5-tropic HIV. MVC is a small molecule antagonist that reversibly binds to the chemokine receptor CCR5 on the surface of CD4 T cells and macrophages. For patients with R5-tropic HIV infections, MVC is a safe and highly effective treatment option. Unfortunately, MVC does not inhibit X4-tropic viruses and because these viruses occur at the late stages of disease progression, it is possible that CCR5 antagonists could select for CXCR4 tropic viruses thereby accelerating disease progression. Nevertheless, MVC provides a strong proof-of-principle that small molecule antagonists of viral receptors or co-receptors on the surface of host cells is a viable treatment option worth exploring for other viral diseases.
Another area of interest in host-targeting antivirals is the use of agents that trigger apoptotic-signaling cascades. Induction of apoptosis not only helps to limit viral replication and spread to adjacent cells, it also simultaneously promotes the host innate immune system and inflammatory responses\textsuperscript{281,282}. Podofilox (Condylox) is an antimitotic compound that is safe and effective in the treatment of external genital warts caused by human papilloma virus (HPV)\textsuperscript{283,284}. Podofilox is a cytotoxic drug that blocks the formation of the mitotic spindle during metaphase of the cell cycle. This invariably leads to the interruption of cell division, followed by the activation of apoptosis and thus blocks HPV infection\textsuperscript{284,285}. Topical treatment with 0.5\% podofilox solution results in wart clearance rates from 45\% to 77\%, with recurrence rates between 4\% and 33\%\textsuperscript{286}.

**Immune Modulating Antivirals**

Although the immune system provides a necessary response to infection, it may not always successfully eliminate the threat posed by the pathogen or its actions may be damaging to the host. This is because some viruses circumvent the immune system (chronic viral hepatitis\textsuperscript{287}, HIV\textsuperscript{288}), while others, cause cytokine dysregulation, upregulate proinflammatory cytokines, and lead to a fatal outcome (influenza virus\textsuperscript{289}, RSV\textsuperscript{290}). The use of immune modulators to either boost the immune response or return the immune response to homeostasis is a promising strategy to combat a broad range of viral infections.

Current clinical practice already includes an approved form of immune modulation as an antiviral approach. Three interferons have been licensed for the treatment of hepatitis B virus (HBV) and hepatitis C virus (HCV) infections: interferon alfacon 1, pegylated interferon alfa 2a (PegIFNα-2a), and PegIFNα-2b. Interferon alpha
(IFNα), predominantly secreted by hematopoietic cells (e.g., plasmacytoid dendritic cells), is a well defined type I interferon that stimulates the immune system for antiviral defense\textsuperscript{291-293}. PegIFNα-2a and PegIFNα-2b interfere with viral replication in two important ways. First, they stimulate immune cells, such as CD8 T cells and natural killer T cells, to enhance the noncytolytic clearance of viruses by cytokines or cytolysis of infected cells\textsuperscript{294}. Second, they stimulate the expression of innate antiviral genes and proteins, such as APOBEC3A/B and MxA, to block viral replication\textsuperscript{294}. Unfortunately, due to the immune stimulatory nature of IFNα based drugs, a wide range of adverse side effects have been reported limiting their use clinically\textsuperscript{295}.

1.7 Management of RSV Infection

Unfortunately there are currently no specific therapies for RSV and thus management remains non-specific and supportive in nature (Table 1.1).

Current Therapies

Supportive Care

The majority of infants and children with mild RSV infection can be managed safely as outpatients. Indications for hospitalization include: severe respiratory distress, hypoxia, and dehydration\textsuperscript{296}. Inpatient treatment of RSV infection can require considerable supportive care, including removal of secretions, administration of humidified oxygen and intravenous fluids, and in severe cases, mechanical ventilation. The American Academy of Pediatrics currently recommends the use of supplemental oxygen when the oxyhemoglobin saturation is below 90\%\textsuperscript{296,297}. Supplemental oxygen is currently the most accessible therapeutic modality for RSV in resource-limited settings\textsuperscript{298}. 
**Bronchodilators**

Drugs used to treat airway smooth muscle constriction, such as nebulized albuterol, salbutamol, and epinephrine, have been shown in some studies to produce modest short-term improvements in clinical scores. However, most randomized control trials have not demonstrated a consistent benefit in infants and young children with acute RSV bronchiolitis\(^1\). Given the greater strength of evidence suggesting no benefit of bronchodilators and a lack of method to predict which children would benefit from bronchodilators, these drugs are no longer recommended for the routine treatment of infants and children with RSV bronchiolitis\(^2\). However, typically bronchodilators are often trialed, as few options exist for management. The use of aerosolized hypertonic saline, presumably to aid in removal of airway secretions, has shown to be safe and effective at improving symptoms of mild to moderate bronchiolitis, however its potential beneficial effects appear to be greater in hospitalized patients compared with patients presenting to the Emergency Department\(^3\). For this reason, it is recommended that nebulized hypertonic saline only be given to infants that have been hospitalized due to RSV bronchiolitis\(^4\).

**Anti-inflammatories**

Corticosteroids have been used for RSV bronchiolitis since the 1960s. However, several large placebo-controlled studies have demonstrated that both nebulized and systemic glucocorticoids alone, or in combination with bronchodilators, do not provide significant improvement in oxygenation, respiratory rate, or length of hospitalization\(^5\). Additionally, a large controlled study indicated that high dose inhaled glucocorticoids provided no significant improvement in wheezing after RSV infection\(^6\).
<table>
<thead>
<tr>
<th>Current Therapies</th>
<th>Drug</th>
<th>Target</th>
<th>Development phase</th>
<th>Target population</th>
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<tr>
<td><strong>Supportive care</strong></td>
<td>Bronchodilators</td>
<td>Airway smooth muscle</td>
<td>Approved</td>
<td>All infants</td>
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<td></td>
<td>Hypertonic saline</td>
<td>Airway epithelium</td>
<td>Approved</td>
<td>Hospitalized infants</td>
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<td></td>
<td>Anti-inflammatories</td>
<td></td>
<td>Approved – not often used</td>
<td>Hospitalized infants</td>
</tr>
<tr>
<td><strong>Antibodies</strong></td>
<td>RSV IVIG</td>
<td>Polyclonal</td>
<td>Approved – no longer used</td>
<td>Prophylaxis of high-risk infants</td>
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<td></td>
<td>Palivizumab</td>
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<tr>
<td><strong>Replication inhibitors</strong></td>
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<td>Unknown</td>
<td>Approved</td>
<td>Hospitalized, severe disease</td>
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<tr>
<td><strong>Emerging Therapies</strong></td>
<td>Drug candidate</td>
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<tr>
<td><strong>Entry inhibitors</strong></td>
<td>GS-5806</td>
<td>F protein</td>
<td>Phase II/ongoing</td>
<td>Adults/children</td>
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<td></td>
<td>MDT-673</td>
<td>F protein</td>
<td>Phase I</td>
<td>Adults</td>
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<td>TMC353121</td>
<td>F protein</td>
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<td>BTA-C585</td>
<td>F protein</td>
<td>Phase I</td>
<td>Adults</td>
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<tr>
<td><strong>Replication inhibitors</strong></td>
<td>ALS-8176</td>
<td>Nucleoside analog</td>
<td>Phase I</td>
<td>Adults/children</td>
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<tr>
<td></td>
<td>RSV-604</td>
<td>N protein</td>
<td>Phase I/Phase IIa</td>
<td>Adults</td>
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Other anti-inflammatory approaches, such as leukotriene receptor antagonists (montelukast), have been assessed for their utility in ameliorating acute RSV bronchiolitis and have yielded disappointing results. Thus, currently available anti-inflammatory agents are not effective and should not be used in the management of RSV bronchiolitis in children.

**Antiviral Interventions**

After RSV was first isolated in humans, attempts were made to create a vaccine by formalin inactivating the virus. This methodology was not successful and rather resulted in a phenomenon known as vaccine enhanced disease. As described in an initial report in 1969, vaccine recipients experienced more severe disease and in some cases even death when subsequently infected with RSV, whereas unvaccinated subjects fared fairly well. This result slowed RSV vaccine development for many years. However, there has been a wealth of information obtained on vaccine enhanced disease which is now thought to be related to a number of factors including lack of affinity maturation, altered antigens by formalin, and a key role for CD4 T cells. A better understanding of RSV vaccine enhanced disease has also led to renewed efforts to generate a protective RSV vaccine and indeed, there are now candidates undergoing clinical testing however despite extensive efforts, there is no licensed vaccine available for RSV.

The current mainstay for prevention of RSV in high-risk infants is the seasonal prophylactic use of polyclonal RSV intravenous immunoglobulin (RespiGam) or human anti-F monoclonal antibodies (palivizumab, motavizumab). Due to concerns of adverse effects, motavizumab has not been approved for clinic use. Currently, the American Academy of Pediatrics recommends the seasonal prophylactic use of palivizumab in the
first year of life for premature infants (<29 weeks gestation) and those of <32 weeks gestation with chronic lung disease or heart disease. Due to the high cost associated with palivizumab, healthy infants are not recommended to receive prophylactic palivizumab\textsuperscript{296}. Under these recommendations seasonal prophylactic use of palivizumab has been widely successful in reducing RSV associated hospitalization in high-risk infants\textsuperscript{313}. Both palivizumab and motavizumab have been evaluated as a therapy for hospitalized infants with established RSV infection. One study found a consistent reduction in viral load by quantitative culture, however this may have reflected \textit{ex vivo} neutralization by the therapeutic antibody\textsuperscript{314}. A subsequent larger double-blind, randomized study found no antiviral effect, as measured by quantitative PCR\textsuperscript{315,316}. Thus, monoclonal antibodies are restricted for prophylaxis of high-risk infants and not for treatment of acute disease.

Ribavirin (Virazole), a nucleoside analog, is a broad-spectrum antiviral compound with potent activity against RSV in culture and experimental animals\textsuperscript{317-319}. Although the exact mechanism of action remains unknown, evidence suggests a few potential mechanisms including: inhibition of the cellular enzyme inosine 5-monophosphate dehydrogenase, suppression of innate and cell-mediated immune responses, chain termination during viral RNA synthesis, inhibition of viral mRNA capping, and accumulation of mutations in viral genomes\textsuperscript{320}. Ribavirin is the only licensed drug for use in the treatment of RSV infection in children. Several small proof-of-concept studies have shown a reduction in RSV load and disease severity in infants treated with aerosolized ribavirin\textsuperscript{321-324}. Despite this, ribavirin efficacy is questionable, as many of the supportive studies had methodological issues and a number of additional studies
demonstrate no benefit\textsuperscript{325}. Additionally, ribavirin is difficult to administer via aerosol and has the potential for mutagenicity, teratogenicity, and carcinogenicity\textsuperscript{325}. Due to its unclear efficacy and concerns for toxicity, its use has been restricted to very high-risk infants with severe disease\textsuperscript{296,326,327}. Given the lack of specific RSV therapies, novel approaches to management of RSV infection are desperately needed.

**Emerging Therapies**

In recent years a number of promising anti-RSV strategies have been investigated in preclinical studies. New antivirals currently being evaluated for the treatment of RSV in humans are summarized in Table 1.1.

*Entry Inhibitors*

Entry of RSV in host cells is mediated by the class I viral F protein in response to binding of a host receptor. A series of conformational changes in the F protein bring the host and viral membranes together, allowing fusion and entry of the viral RNP complex\textsuperscript{328}. The F protein is critical for entry and is therefore the subject of intense investigation for antivirals.

The orally bioavailable GS-5806 (Presatovir), is an allosteric inhibitor of the F-protein that blocks viral entry. It has been found to be active against a wide range of RSV clinical isolates \textit{in vitro}\textsuperscript{329}. Additionally, in a phase IIa trial, GS-5806 treatment reduced viral load in nasal washes and severity of symptoms in healthy volunteers experimentally infected with RSV\textsuperscript{330}. However, it is important to note that the adult subjects in this study showed treatment-emergent GS-5806 resistant RSV variants\textsuperscript{331}. Similar resistance patterns were seen in a mouse model of RSV infection\textsuperscript{332}. These observations have raised concerns that these mutations could lead to highly resistant circulating strains of RSV if
this drug is used clinically. However, GS-5806 is advancing in clinical development, with active recruitment for placebo-controlled phase 2 studies investigating the safety and efficacy in naturally infected patients who have been hospitalized with acute respiratory symptoms.

VP-14637, now reformulated for improved aerosol delivery and renamed MDT-673, is a novel triphenol-based inhibitor of RSV fusion. It binds directly to the heptad repeat domains of the F protein trimer and demonstrates significant anti-RSV activity against lab strains (EC$_{50}$ 1.4 nM) and clinical isolates (EC$_{50}$ 1.72 nM)$^{329,333}$. In vivo data from cotton rat models demonstrates significantly lower pulmonary RSV titers and reduced histopathology when compared to placebo. However, a phase IIa trial in healthy volunteers challenged with RSV was halted in 2012 for unknown reasons$^{331}$.

TMC353121, another RSV fusion inhibitor, is an optimized derivative of JNJ-2408068 with reduced tissue retention$^{334,335}$. Inhibit of fusion is thought to occur either through interaction with both heptad repeat regions of the F protein and disruption of the post-fusion formation$^{336}$, or antagonization of fusion by preventing the pre-fusion confirmation$^{337}$. Although TMC353121 has not progressed to Phase I clinical studies yet, extensive preclinical studies demonstrate a reduction in viral load in mice$^{338}$, rats$^{339}$, and non-human primates$^{340}$.

BTA-C585 is an additional fusion inhibitor thought to bind to the RSV F protein in a similar manner as MDT-673, and has shown EC$_{50}$ values below 100 nM against lab and clinical strains of both RSV-A and B. In the RSV cotton rat model it was shown to significantly reduce the viral mRNA detected in the lung 5 days post infection when
dosed orally\textsuperscript{331}. Two studies in healthy volunteers (NCT02558413, NCT0266867) have been completed, however results have not been released publically.

\textit{Viral Replication Inhibitors}

The RSV viral polymerase is another target of significant interest in the development of RSV antivirals. It is the main protein involved in replicating the viral genome and transcribing viral mRNAs. Viral polymerases are the leading therapeutic target for treating viral infections because inhibitors typically work both prophylactically and have a large window of activity after infection.

ALS-8176 is a first-in-class cytidine nucleoside analog. Similar to other nucleoside analogs, this drug is present as a pro-drug and must be metabolized by the host to generate the active form. Upon oral administration of ALS-8176, plasma enzymes metabolize it into ALS-8112, which is actively taken up by many cells including those of the respiratory tract. Once internalized by the cell, it is further metabolized by cellular enzymes to produce the active metabolite ALS-8112-5’-triphosphate which selectively inhibits the RSV polymerase by a classic chain termination mechanism\textsuperscript{341}. Phase I clinical trials of ALS-8176 are ongoing, however the results from a human challenge study found reductions in viral load of treated volunteers within 12 hours after the start of the treatment and the mean time to undetectable RSV RNA ranged from 1.3 to 2.3 days compared to 7.2 days for placebo treated individuals. In addition, the clinical severity of RSV disease was significantly lower in ALS-8176 treated volunteers. This provides proof-of-principle that a nucleoside inhibitor is a viable antiviral strategy for the treatment of RSV infections\textsuperscript{342}.
In addition to the viral polymerase, the RSV N protein plays an indispensible role during replication of the viral genome. RSV-604 is a small molecule inhibitor of both viral RNA synthesis and the infectivity of released virus that inhibits RSV N protein interactions with the viral polymerase\textsuperscript{343}. In a Phase I clinical study, RSV604 was found to be safe and well tolerated in healthy adult volunteers\textsuperscript{344}. However, a proof of concept trial in hematopoietic stem cell transplant patients, demonstrated no significant difference in viral load as compared to placebo controls. This was attributed to low RSV-604 plasma levels in these patients\textsuperscript{344}.

**Need for Additional RSV Therapies**

Compared to those of HIV and HCV, industry explorations of antiviral therapies for RSV infections have been limited. The majority of RSV antivirals are directed against the F protein or the viral polymerase, and the bulk of compounds identified have yet to pass through phase II trials demonstrating efficacy in human subjects. In addition, most compounds tested thus far have been associated with the selection of resistant RSV strains. Successful antiviral therapies currently approved against other RNA viruses, including HIV and HCV, have demonstrated that combinational therapy using different mechanistic classes of antiviral drugs is the best option for avoiding resistance. Thus, the development of a second generation of RSV inhibitors belonging to broad mechanistic classes would be useful to combat RSV.

**1.8 Opportunities for Novel Anti-RSV Therapies**

Studies in the last decade have confirmed the significant impact of RSV infection in the pediatric population worldwide and highlighted the need for developing novel therapeutic and preventive strategies beyond traditional high-risk groups. There is
extensive ongoing research aimed at the development of better RSV therapeutics, and although in recent years a number of promising new antiviral agents have shown efficacy, none of them have been approved for clinical use yet. The majority of the new anti-RSV therapies have targeted the fusion protein, however there are a number of opportunities for the development of antiviral agents with different mechanisms of action.

**Viral Targets**

The SH protein is one of three envelop proteins expressed on the surface of the RSV virion. It appears to play an important role in RSV pathogenesis, however its specific role in the RSV life cycle remains unclear. The SH protein is a viroporin, a family of small hydrophobic proteins able to induce membrane permeability to ions or small molecules. The influenza virus viroporin has been targeted with some success and therefore targeting the RSV SH protein may open up novel antiviral therapies. A recent preclinical study identified pyronin B as an inhibitor of the RSV SH protein. Detailed biophysical characterization demonstrated that the molecule is an allosteric inhibitor of SH, similar to that of rimantadine. Cell-based assays indicated potent activity of pyronin B against lab strains of RSV (EC50 <100 nM), however cellular toxicity of the molecule was not addressed. Nevertheless, this indicates that the RSV SH protein represents an opportunity to develop novel RSV antivirals.

The RSV M2-1 protein is an essential transcription elongation factor that binds viral RNA in a competitive manner and prevents the premature termination of transcription by the viral polymerase. It is clear that M2-1 binds preferentially to viral gene ends and poly-A sequences, however the mechanism by which it improves transcription efficiency remains unknown. Additionally, M2-1 has been suggested
to play a key role in mediating association of the RNP complex with the matrix protein during assembly and is present between the matrix layer and the RNP in filamentous viral particles\(^{350}\). The recent crystal structure of M2-1 has identified surfaces critical for its function and could provide the basis for novel anti-RSV compounds. Interestingly, it was recently reported that cyclopamine is a potent inhibitor of RSV infection both \textit{in vitro} and \textit{in vivo}\(^{351}\). Cyclopamine is an antagonist of smoothened protein (Smo)\(^{352}\), however its anti-RSV activity was associated with direct inhibition of M2-1 mediated transcription independently of the Smo receptor. Evidence of this came from cyclopamine resistant RSV strains that had a single mutation in M2-1 in a region previously thought to play a role in RNA and P binding\(^{348,351}\).

**Host Targets**

Targeting a host factor that is essential in the viral replication cycle has been proposed as an alternative strategy to develop therapies for RSV infection. Prevention of virus attachment to cells by targeting RSV receptors represents an attractive strategy. Nucleolin has been identified as a cell surface receptor for the RSV F protein\(^{44}\) and thus blocking nucleolin may reduce RSV infection. Nucleolin blocking drugs are currently being tested in humans for efficacy in the setting of malignancy, as nucleolin is highly expressed on cancer cells. By leveraging the work from these trials, it should be possible to repurpose these drugs and test their ability to improve outcomes for RSV infection.

The host immune response associated with RSV is thought to be inadequately activated or even suppressed in infants with severe RSV disease\(^{353}\). For instance, CD8 T cells from the lungs of mice experimentally infected with RSV are impaired in their cytolytic ability and their capacity to produce IFN-\(\gamma\)\(^{354}\). Additionally, in the RSV infected
airway, programmed death ligands (PD-1/PD-L1) are upregulated on antigen presenting cells inhibiting CD8 T cells. T cells are believed to play an essential role in clearance of RSV from infected airways. Mice depleted of T cells and children with defective T cell responses show increased RSV-mediated disease severity and increased viral titers with prolonged virus shedding. Therefore, strategies that enhance CD8 T cell responses to RSV may be beneficial. This approach could include the use of therapeutic agonists that stimulate CD8 T cell activation early during the course of infection. Such agents would have the added benefit of being broadly effective against different strains of RSV. Certainly the use of immune modulating agents, such as IFNα, in combination with direct acting antivirals has been widely effective at controlling viral infections.

As previously mentioned, RSV encodes an SH protein that functions as a viroporin to manipulate host ion homeostasis. This indirectly suggests that host ions, such as potassium (K⁺), sodium (Na⁺), or calcium (Ca²⁺), play an essential role in the viral replication cycle. Cellular ions as a group are universal and versatile signaling molecules involved in almost every aspect of cellular processes. In mammalian cells, large gradients of both monovalent and divalent cations are maintained across the plasma membrane that serve many functions within the cell. For example, there is a >10,000-fold gradient of Ca²⁺ maintained across the plasma membrane of mammalian cells. Calcium adaptor proteins, such as calmodulin, can detect small changes in this gradient and function to activate calcium-signaling pathways. The ability of viruses to manipulate or exploit these intracellular ion systems enables them to control host processes creating a tailored cellular environment to optimize steps of their replication cycle under normally hostile host conditions. Recently, a cell-based assay screening for natural compounds with anti-
RSV activity identified cyclopiazonic acid, an intracellular calcium ATPase inhibitor\textsuperscript{357}. The results of this study indicated that increased intracellular Ca\textsuperscript{2+} is detrimental to RSV early in its replication cycle. Thus, targeting host ion homeostasis with pharmacological interventions offers an exciting opportunity to combat RSV infection.

1.9 Proposed Research

The overriding purpose of this research is to identify novel RSV therapeutics for clinical use and improve outcomes for individuals infected with RSV. In the general population, RSV is singlehandedly the most important cause of lower respiratory tract infections in infants and young children worldwide. Globally, of all causes, RSV is the most frequent viral cause of death in infancy and second only to malaria amongst all causes of infant death\textsuperscript{71}. All children are infected by the age of 2 and there is no lasting protective immunity afforded by infection and so re-infection throughout life is common. In adults, RSV causes equivalent or more disease than influenza virus, a well-recognized threat, with approximately 2\% of all adults over 65 requiring medical attention each year for RSV infection\textsuperscript{358-360}. Despite the disease burden presented by RSV, no vaccine is available and treatment remains non-specific.

In the absence of effective and economical treatments, new drug candidates and treatment therapies are desperately needed to combat RSV. The work herein aims to investigate two broad strategies to control RSV infection. First, building on the recognized critical role of CD8 T cells in antiviral defense, I describe the utility of delivering an antibody to enhance CD8 T cell costimulation and subsequently boost CD8 T cells against RSV. Second, after analyzing the results of a high throughput screen, we identified that cardiac glycosides inhibit RSV replication. My work tests the hypothesis
that cardiac glycoside concentrations required to inhibit RSV are within the range of what is safely achievable in human lungs based on previous pharmacokinetic studies. As part of this work, I determine if the antiviral effects of cardiac glycosides are directly related to their ability to alter intracellular ion concentrations and test the modality of targeting intracellular ions to control RSV replication. Mechanistic work further defines where in the replication cycle intracellular ions exert their effect.

This work will be completed in several extensively investigated model systems. Initial validation work will be done in cell lines and human airway epithelial cells. In vitro RSV replicates most efficiently in immortalized cell lines of human epithelial origin. The most commonly used cell line to grow RSV is the human HEp-2 cell line. I also study primary human airway epithelial cells grown at an air-liquid interface. These cells generate a pseudostratified, airway epithelium that displays similar morphological and phenotypic characteristics of the in vivo human airway epithelium making it more representative than submerged culture for modeling in vivo lung epithelial cell responses. Finally, my work also involves the inbred laboratory mouse model of RSV infection. Although mice are only semi-permissive for human RSV replication, requiring a very high intranasal inoculum, BALB/c mice are one of the more susceptible strains and have been used to elucidate the roles T cells play in RSV clearance, immunopathology, and immune evasion. In this model, some signs of lower respiratory tract infection are detectable and peak pulmonary replication occurs on day 4 post infection with virus clearing from the lungs by day 7 post infection.

My work explores novel strategies for modifying RSV replication and has the potential to inform our understanding of RSV infection and its management.
Chapter II

2. Agonistic 4-1BB antibody fails to reduce disease burden during acute respiratory syncytial virus (RSV) infection


2.1 Abstract

Respiratory Syncytial Virus (RSV) remains a leading cause of infant morbidity and mortality worldwide. Despite this, there are limited therapeutic options. CD8 T cells have an integral role in controlling viral infections; strategies to enhance these responses may be clinically relevant. The T cell costimulatory receptor, 4-1BB, is known to play a role in expansion of antiviral CD8 T cells. In this study, we examined the effect of agonistic 4-1BB antibody at the time of RSV infection in mice. We show that this antibody did not improve outcomes in the setting of RSV infection but rather, led to increased weight loss and a reduction in RSV specific CD8 T cells in the lung. This work suggests caution in the use of agonistic 4-1BB antibody in the setting of viral infections.

2.2 Introduction

Respiratory syncytial virus (RSV) is a member of the Pneumoviridae family of viruses and is a leading cause of bronchiolitis and hospitalization in infants and young children worldwide\textsuperscript{366,367}. Globally it is estimated there are 64 million cases of infection and 160,000 deaths attributable annually to RSV\textsuperscript{368}. Disease morbidity and mortality is increased in premature infants, the elderly and immunocompromised\textsuperscript{67}. All children are infected at least once by the age of two and remain susceptible to reinfection throughout
life, as sterilizing immunity does not occur\textsuperscript{158}. Despite the disease burden presented by RSV, no commercial vaccine is available and treatment remains non-specific.

It is well accepted that CD8 T cells are critical for the control and clearance of viruses\textsuperscript{369}. In the context of RSV, there is evidence that CD8 T cell immune responses reduce pathology. Studies in both humans and animals have demonstrated that the absence of CD8 T cells results in poor outcomes with RSV infection\textsuperscript{199,370,371}. Moreover, in animal models, immunization with CD8 epitopes generates protective CD8 T cell based immunity\textsuperscript{372-374}. Strategies that boost CD8 T cell responses may therefore provide a therapeutic strategy for RSV.

The modulation of T cell costimulatory pathways has been used in various models as a way to enhance or suppress T cell activity. With respect to CD8 T cells, CD137 (or 4-1BB) signaling is known to enhance antiviral responses\textsuperscript{375-378}. The receptor 4-1BB is a member of the TNFR family and is expressed on activated T and NK cells\textsuperscript{375,379,380}. Its ligand, 4-1BBL, is primarily found on activated B cells, macrophages and dendritic cells\textsuperscript{381}. T cell stimulation via the T cell receptor combined with additional message provided by 4-1BB signaling improves T cell activation and proliferation. Notably, stimulation of 4-1BB in various models improves CD8 T cell responses to several viruses including influenza A, herpes simplex virus 1, vesicular stomatitis virus and poxvirus\textsuperscript{382-385}. The use of agonist 4-1BB specific antibody (Ab) has been used to stimulate 4-1BB signaling \textit{in vivo}\textsuperscript{386-389}. We therefore tested the hypothesis that agonist 4-1BB Ab would improve outcomes in RSV infection by enhancing cytotoxic CD8 T cell responses.

\section*{2.3 Methods}

\textbf{Mouse infection and treatment with 4-1BB agonistic antibody}
RSV animal studies were approved by the Hospital for Sick Children Animal Care Committee in accordance with the regulation of the CCAC. We obtained 6-8 week-old female BALB/c mice from Charles River Laboratories (Wilmington, Massachusetts, United States). Animals were housed in a specific pathogen–free environment and fed food and water *ad libitum*. For intranasal instillation, lightly sedated mice (isoflurane) inhaled 50 µl of instillate, which we applied to the nares with a P-200 pipette while their mouths were held closed. On day 0, mice received 5x10^6 PFU (50 µl) of RSV A2 via intranasal instillation as previously described44. Within 20 minutes of inoculation with RSV, mice received PBS or 100 µg of monoclonal (clone 3H3) agonistic 4-1BB Ab (gift from Dr. Tania Watts and Dr. Robert Mittler) intraperitoneally (i.p.) or intranasally (i.n.). Control mice were given 100 µg of rat IgG2a isotype control antibody i.p. or i.n. All mice were monitored daily for changes in weight. In this model, mice lose approximately 10-15% of their original body weight by day 4, corresponding to peak viral titres, and begin to gain weight by day 6. On day 4, the peak of viral replication, the lungs were excised, homogenized, and a standard plaque assay was performed to obtain plaque-forming units as previously described44. On day 6, the spleen and the lungs were excised for fluorescence activated cell sorting.

**Airway responsiveness measurements**

Airway responsiveness was assessed 6 days after RSV infection in response to increasing doses of nebulized methacholine (Sigma-Aldrich, Oakville, ON, Canada) using a previously described protocol391. In brief, mice were anesthetized with inhaled isoflurane (3% with 1 L/min of O₂), paralyzed with pancuronium bromide (1 mg intraperitoneal), tracheotomized using a blunted 18-G needle, and mechanically ventilated using a small
animal computer-controlled piston ventilator (flexiVent, SCIREQ, Montreal, QC, Canada). The response to nebulized saline and increasing doses (0-40 mg/ml) of methacholine was measured and the data fit to the constant phase model. Model parameters of airway resistance and dynamic compliance were calculated as described previously.392

**Fluorescence activated cell sorting (FACS)**

Organs were removed from mice and re-suspended in HBSS after passage through a 70 µm filter with the aid of a 5 mL syringe plunger. Prior to resuspension, the lungs were perfused via the right ventricle with 5–10 mL of PBS to remove cells in the pulmonary vasculature. Spleen and lung lymphocyte suspensions were hemolysed by a 2-min incubation with ACK lysis buffer. In addition, lung lymphocytes from individual mice were enriched by isolation over an 80/40% percoll gradient. After RBC lysis, the cell suspensions were used for tetramer analysis. Splenocytes and lung cells were analyzed using a Gallios Flow Cytometer (Beckman Colter) and FlowJo (TreeStar Inc., Ashland, OR, USA) software. Where indicated, cells were labeled with antibodies to CD8a, CD3, CD4, CD19, CD14, CD11c, CD11b, CD45, and CD25 (eBioscience). RSV M2 specific CD8 T cells (M2 H-2K(d)/SYIGSINNI) were enumerated using MHC tetramers (obtained from the NIH tetramer facility).

**2.4 Results**

We examined the *in vivo* effect of 4-1BB costimulation on the primary CD8 T cell response to RSV. The 4-1BB agonist Ab was not associated with a reduction in lung viral titres 4 days post infection (Figure 2.1A) or a change in histological appearance of the lung (Figure 2.2C).
Figure 2.1: RSV associated disease outcomes.
BALB/c mice were infected i.n. with $5 \times 10^6$ PFU of RSV on day 0. Mock infected mice were included as a control and received PBS on day 0. Within 20 min of inoculation, mice received PBS or 100 µg of monoclonal (clone 3H3) agonistic 4-1BB Ab i.p. or i.n. (A) On day 4, the peak of viral replication, lung homogenates were analyzed by standard plaque assay for viral load. (B) Weight loss of the infected mice was monitored daily. Results are representative of 4 experiments with N = 3–4 mice per group. (C, D) Airway hyperresponsiveness (AHR) was assessed in response to increasing doses of nebulized methacholine (Sigma–Aldrich, Oakville, ON, Canada) on day 6 following RSV infection. Airway resistance (C) and dynamic compliance (D) were measured. Results are shown as mean ± SEM and p values were derived from a 1-way ANOVA analysis with a Tukey’s post hoc test for group comparisons. ns $P > 0.05$, * $P \leq 0.05$
Figure 2.2: RSV associated disease outcomes after administration of isotype IgG control Ab.

BALB/c mice were infected i.n. with $5 \times 10^6$ PFU of RSV on day 0. Within 20 min of inoculation, mice received PBS or 100 µg of rat IgG2a isotype control antibody i.p. or i.n. (A) On day 4, lung homogenates were analyzed by standard plaque assay for viral load. (B) Weight loss of the infected mice was monitored daily. Results are representative of 1 experiment with N = 3–6 mice per group. (C) On day 6 mice were euthanized, and lungs removed for fixation, paraffin embedding, sectioning and H&E staining (TCP, Toronto). Representative sections are shown. Lung histology reveals no differences between mice infected with RSV and treated with agonist 4-1BB Ab or isotype IgG2a control Ab.
This was also found with administration of isotype IgG control Ab (Figure 2.2).

However, mice treated with agonist 4-1BB Ab lost more weight than control mice post RSV (Figure 2.1B). This increased weight loss was not found in mice treated with isotype IgG control Ab (Figure 2.2B). This finding suggested increased morbidity with agonist 4-1BB Ab treatment. Inflammation post RSV infection is associated with changes in lung mechanics peaking on day 6\(^{393}\). Both 4-1BB agonist Ab treated and non-treated RSV infected mice exhibited increased resistance (Figure 2.1C) and decreased dynamic compliance (Figure 2.1D) as compared to control uninfected mice. Although there was no statistically significant difference in lung compliance between the non-treated group and agonist 4-1BB Ab treated group, a trend was seen with the agonist 4-1BB Ab group having higher resistance and lower compliance in response to inhaled methacholine. This suggested that agonist 4-1BB Ab does not attenuate RSV associated disease outcomes but rather may be associated with increased morbidity.

Agonist 4-1BB Ab should enhance antiviral CD8 T cell generation, which in turn should improve viral clearance. 4-1BB expression on T cells is inducible and transient\(^{375}\), thus, it was possible that the lack of benefit with agonist 4-1BB Ab was related to a lack of effect on T cell numbers. Alternatively, agonist 4-1BB Ab may have led to an overly aggressive T cell response associated with increased morbidity. To test this, spleens and lungs were removed on day 6 after RSV challenge and CD8 T cell numbers enumerated by FACS analysis. The data confirmed that i.p. agonist 4-1BB Ab increased the percentage and numbers of CD8 T cells in the spleen (Figure 2.3A, B) when compared to RSV infection alone.
Figure 2.3: Administration of 4-1BB agonist Ab with primary RSV infection increases total CD8 T cells in the spleen but reduces RSV M2 specific CD8 T cells in the lung.

Samples were analyzed using a Gallios Flow Cytometer (Beckman Colter) and FlowJo (TreeStar Inc., Ashland, OR, USA) software. Where indicated, cells were labeled with antibodies to CD8a and CD3 (eBioscience). RSV M2 specific CD8 T cells (M2 H2K(d)/SYIGSINNI) were enumerated using MHC tetramers (obtained from the NIH tetramer facility). Bar graphs show percent (A, C, E, G) and number (B, D, F, H) of lymphocytes positive for indicated marker in spleen or lung. Cells from uninfected mice in open bars, RSV infected mice shown in black bars, RSV infection with i.p. 4-1BB agonist Ab in dark gray bars and RSV infection with i.n. 4-1BB agonist Ab in light gray bars. All samples are from mice at day 6 post RSV infection. N = 3–15 for each panel. p values were derived from a 1-way ANOVA analysis with a Tukey's post hoc test for group comparisons. ns P > 0.05, * P ≤ 0.05, ** P ≤ 0.01, **** P ≤ 0.0001
Although, in the spleen there was no difference in the percentage of CD8 T cells that were RSV specific between mice that received i.p. agonist 4-1BB Ab and those that did not (Figure 2.3C), the numbers of RSV specific CD8 T cells were higher in the spleen (Figure 2.3D). This supports the hypothesis that agonist 4-1BB Ab enhances CD8 T cell responses, however this enhancement was not exclusive to RSV specific T cells. The CD4 percentages decreased correspondingly in the spleen though the absolute numbers did not change significantly (Figure 2.4).

In the lung, the percent and number of total CD8 T cells were not altered by i.p. agonist 4-1BB Ab (Figure 2.3E, F). Most surprising, we found that the percent and number of RSV specific CD8 T cells were reduced in the lung (Figure 2.3G, H) in mice treated with i.p. 4-1BB Ab as compared to RSV infection alone.

We questioned if these findings were related to the route of costimulation and so repeated experiments delivering agonist 4-1BB Ab intranasally following RSV infection. The route of agonist 4-1BB Ab administration did not significantly affect the viral titers (Figure 2.1A) or weight loss (Figure 2.1B).

Intranasal agonist 4-1BB Ab resulted in similar enhancement of CD8 T cells in the spleen suggesting that the route of costimulation did not alter this systemic effect (Figure 2.3A, B). However, RSV specific CD8 T cells in the spleen were slightly reduced with intranasal versus i.p. agonist 4-1BB Ab. Further, in the lung, intranasal agonist 4-1BB Ab was superior to i.p agonist 4-1BB Ab at boosting CD8 T cells numbers (Figure 2.3F). Despite this, the percentage of CD8 T cells that were RSV specific was reduced in the lung (Figure 2.3G) in mice treated with intranasal agonist 4-1BB Ab as compared to RSV infection alone.
Figure 2.4: Administration of 4-1BB agonist Ab with primary RSV infection decreases percentage of CD4 T cells in spleen though absolute numbers do not change.

Samples were analyzed using a Gallios Flow Cytometer (Beckman Colter) and FlowJo (TreeStar Inc., Ashland, OR, USA) software. Where indicated, cells were labeled with antibodies to CD4 or CD3 (eBioscience). Bar graphs show percent and number of lymphocytes positive for indicated marker in spleen or lung. Cells from RSV infected mice shown in black bars, RSV infection with i.p. 4-1BB agonist Ab in dark gray bars and RSV infection with i.n. 4-1BB agonist Ab in light gray bars. All samples are from mice at day 6 post RSV infection. N = 3-15 for each panel. p values were derived from a 1-way ANOVA analysis with a Tukey’s post hoc test for group comparisons.
The number of RSV specific CD8 T cells trended lower but given the overall increase in CD8 T cell numbers there was not a statistical difference seen (Figure 2.3H). These data suggest that route of delivery of agonist 4-1BB Ab does lead to different effects; i.p. delivery provides slightly improved antigen specific splenic CD8 T cells whereas intranasal delivery results in slightly improved lung CD8 populations. However, the important finding remained that regardless of route of delivery, agonist 4-1BB Ab did not improve outcomes and resulted in reduced RSV specific CD8 T cells in the lung. Differences associated with agonist 4-1BB Ab administration were not seen with administration of isotype IgG control Ab (Figure 2.5). Thus the effects reported are related to the agonist 4-1BB Ab and not a non-specific effect of IgG.

We questioned whether the reduction in antigen specific T cells in the lung was related to upregulation of regulatory T cell populations. In the lung, there was a trend towards higher percentage CD4/CD25 + T cells with agonist 4-1BB Ab (Figure 2.6A); this pattern was also seen with absolute numbers and a statistically significant difference was seen between mice that received intranasal agonist 4-1BB Ab versus no treatment (Figure 2.6B). These findings were not seen in the spleen (data not shown).

We also examined dendritic cell (DC) populations and saw a reduction in inflammatory (Figure 2.6C, D; CD3-, CD19-, CD14-, CD11c+, CD11b+) and plasmacytoid DCs (Figure 2.6E, F; CD3-, CD19-, CD14-, CD11c dim, CD11b-, CD45+) with agonist 4-1BB Ab administration in both i.n. and i.p. groups. No significant change was seen in conventional DCs (data not shown; CD3-, CD19-, CD14-, CD11c+, CD11b, CD103+).
Figure 2.5: Administration of isotype IgG2a control Ab does not alter outcomes with primary RSV infection.

Samples were analyzed using a Gallios Flow Cytometer (Beckman Colter) and FlowJo (TreeStar Inc., Ashland, OR, USA) software. Where indicated, cells were labeled with antibodies to CD8a and CD3 (eBioscience). RSV M2 specific CD8 T cells (M2 H-2K(d)/SYIGSINNI) were enumerated using MHC tetramers (obtained from the NIH tetramer facility). Bar graphs show percent (A, C, E, G) and number (B, D, F, H) of lymphocytes positive for indicated marker in spleen or lung. Cells from RSV infected mice shown in black bars, RSV infection with i.p. isotype IgG2a Ab in dark gray bars and RSV infection with i.n. isotype IgG2a Ab in light gray bars. All samples are from mice at day 6 post RSV infection. N = 3 for each panel. p values were derived from a 1-way ANOVA analysis with a Tukey's post hoc test for group comparisons.
Figure 2.6: Administration of 4-1BB agonist Ab with primary RSV infection alters CD25 and dendritic cell populations in the lung.

Samples were analyzed using a Gallios Flow Cytometer (Beckman Colter) and FlowJo (TreeStar Inc., Ashland, OR, USA) software. For these experiments inflammatory dendritic cells (DC) were defined as CD3-, CD19-, CD14-, CD11c+, CD11b+; plasmacytoid DCs were defined as CD3-, CD19-, CD14-, CD11c dim, CD11b-, CD45+. All antibodies were from eBioscience. Bar graphs show percent (A, C, E) and number (B, D, F) of indicated populations. All cells from the lungs of mice day 6 post RSV infection. RSV infected mice without treatment shown in black bars, RSV infection with i.p. 4-1BB agonist Ab in dark gray bars and RSV infection with i.n. 4-1BB agonist Ab in light gray bars. N = 6–8 for each panel. p values were derived from a 1-way ANOVA analysis with a Tukey’s post hoc test for group comparisons. * $P \leq 0.05$, **** $P \leq 0.0001$
2.5 Discussion

These findings suggest that administration of 4-1BB agonist Ab at the time of primary RSV infection does not influence morbidity from RSV in a favorable manner. This work was not aimed at documenting the importance of 4-1BB signaling during RSV infection. The role of the 4-1BB pathway in boosting T cell responses, and especially secondary memory T cell responses, has been documented. Rather we sought to explore if exploiting this pathway could acutely improve outcomes in the setting of RSV.

We chose the current model in order to keep the antigen (RSV) and the costimulatory signal (4-1BB) in close proximity to each other. Previously, 4-1BBL has been coupled to an influenza epitope in the form of viral vectors. In this setting, antiviral CD8 T cell production was enhanced to influenza. Physically uncoupling antigen from the 4-1BB costimulatory signal (despite temporal proximity) may have led to a less specific effect of 4-1BB signaling (i.e. expansion of non RSV or non M2 specific CD8 T cells), however, there was no benefit seen in outcomes to RSV to support an enhanced non M2 RSV response. Lee et. al. did study the use of agonist 4-1BB Ab in aged mice in order to boost CD8 responses in an RSV vaccination model. In this setting multiple administrations (4 occasions) of agonist 4-1BB Ab did improve RSV specific CD8 T cell responses 20 days later. It is likely that the effects of 4-1BB stimulation will vary depending on the timing of administration and the timing of the assessment.

These factors will influence which cells express 4-1BB or 4-1BBL, and how much time the cells have to respond to the signal.

To approximate the antigen and costimulatory signal, we delivered agonist 4-1BB Ab intranasally. To our knowledge, intranasal agonist 4-1BB Ab has not been used
previously. Both intranasal and intraperitoneal agonist 4-1BB Ab enhanced CD8 T cell generation in a similar fashion. This work suggests some minor differences between routes of administration of the costimulatory signal, however, weight loss, viral titers and percentage of lung RSV specific CD8 T cells were not appreciable altered by route of delivery of agonist 4-1BB Ab.

We did explore the impact of agonist 4-1BB Ab on CD4/CD25 +ve T cells and DCs. Both cell types can express 4-1BB and can play a role in RSV infection. The effects were minimal but did suggest some changes that will need future work with a kinetic study including examination of draining lymph nodes in order to better understand the role of these cells.

Our findings presented here suggest caution in using agonist 4-1BB Ab in vivo. Although, there is literature to support agonist 4-1BB Ab in the setting of asthma and cancer, our results suggest that agonist 4-1BB Ab may lead to increased morbidity with acute infections. Anti-4-1BB Ab treatment of otherwise naïve BALB/c or C57BL/6 mice has been previously shown to cause the development of a series of immunological abnormalities including, splenomegaly, lymphopenia, thrombocytopenia, and anemia. In vivo use of agonist 4-1BB Ab should be considered carefully with close attention paid to monitoring for adverse events with infection.

Author Contributions:
M.J.N. conceived of, performed, and planned all experiments, analyzed the data and wrote the paper. W.D. assisted with plaque assays in Figures 2.1 & 2.2. Y.C. assisted with plaque assays in Figures 2.1 & 2.2 and viral propagation. T.J.M. conceived of in
*in vivo* experiments, assisted with flow cytometry, analyzed data, wrote and edited the paper, supervised and provided support for the project.
Chapter III

3. Repurposing of the cardiac glycoside digoxin and related compounds for the treatment of RSV


3.1 Abstract

Respiratory syncytial virus (RSV) is the most important viral agent of pediatric lower respiratory tract illness worldwide. Despite the disease burden presented by RSV, no commercial vaccine is available and treatment remains non-specific. In the absence of effective and economical treatments, new drug candidates are needed to combat RSV. Towards this goal we performed an \textit{in vitro} screen to identify drugs with novel anti-RSV activity. From this screen, we found that cardiac glycosides are potent inhibitors of RSV infection. Treatment of both HEp-2 cells and primary nasal epithelial cells with digoxin or a related cardiac glycoside, digitoxin, resulted in a dose-dependent decrease in infection by RSV. This anti-RSV activity appeared to be highly dependent on changes in intracellular sodium and potassium. Digoxin was further able to inhibit clinical isolates of RSV in primary nasal epithelial cells. The cardiac glycoside mediated block to RSV infection occurred post viral entry but before a replication complex formed. These data suggest host cellular ions play a critical role in RSV infection and that further study of cardiac glycosides offers the potential to develop a treatment for RSV infections.
3.2 Introduction

RSV is the leading cause of viral lower respiratory tract infections in infants and young children worldwide. Globally, it is estimated that, among all causes, RSV is the second leading cause of death in infancy. Premature infants or children with chronic lung disease or congenital heart disease are at particularly high risk for severe RSV disease, however most RSV related hospital admissions are from healthy infants with no known risk factors. By the age of 1 year, 60-70% of children will be infected by the virus with ~2-3% of all infants requiring hospitalization. This translates to ~3.4 million yearly hospitalizations and ~200,000 deaths globally. Despite the disease burden presented by RSV, no vaccine is available and treatment remains non-specific.

The use of humanized monoclonal antibodies directed against the RSV F glycoprotein (palivizumab, motavizumab), has been successful as a prophylaxis in high risk infants, but not as a treatment in those with established RSV infection. The only therapeutic intervention for RSV infection currently available to patients is ribavirin, a nucleoside anti-metabolite prodrug. Unfortunately ribavirin is associated with inconsistent clinical benefit and severe teratogenicity limiting its use. Novel RSV specific antivirals have undergone clinical testing with promising results, however none are currently available. In the absence of effective and economical treatments, there is an urgent need for new antiviral drug candidates to combat RSV infection.

Towards this goal, using a cell-based quantitative high-throughput screening platform (Maynes & Moraes Labs, Unpublished), we tested 2560 compounds from the MicroSource SPECTRUM library to identify approved drugs with novel anti-RSV activity. Cardiac glycosides, inhibitors of the membrane bound Na⁺/K⁺-ATPase, were
identified to reduce RSV replication. Although the primary use of cardiac glycosides is for cardiac reasons, there are studies to suggest this class of drugs may also impact viral infection\textsuperscript{410-414}.

We report here that cardiac glycosides diminish RSV infection in HEp-2 cells and in primary human nasal epithelial cells. Digoxin, an FDA approved cardiac glycoside, was further able to inhibit community isolates of RSV in primary nasal epithelial cells. Initial mechanistic work highlights the essential role of intracellular potassium (K\textsuperscript{+}) and sodium (Na\textsuperscript{+}) in this process. These findings suggest that cardiac glycosides may represent a viable antiviral therapeutic class and that further understanding of the underlying mechanism of action may reveal modifiable therapeutic targets leading to broad antiviral strategies.

### 3.3 Methods

**Reagents and medium**

Digoxin, digitoxin, and ribavirin were purchased from Sigma. Virox was purchased from Virox Technologies Inc, Oakville, Canada. Eagle’s Minimum Essential Medium (EMEM) and EMEM lacking NaCl were purchased from Wisent Inc, Montreal, Canada. Basal epithelial growth medium (BEGM) for primary cell maintenance was purchased from Lonza, Walkersville, MD. Basal differentiation medium (PneumaCult) for maintenance of primary cells at an air-liquid interface (ALI) and Heparin sodium salt were purchased from StemCell Tech., Vancouver, Canada. PrestoBlue and blasticidin (Thermo Fisher Scientific, Mississauga, Canada). KCl and NaCl purchased from Ambion, Burlington, Canada. N-methyl-D-glucamine (NMDG) purchased from Sigma.
Digoxin for mouse studies, 0.9% saline, and 3% saline purchased from SickKids Research Pharmacy.

**Cells and viruses**

HEp-2 (ATCC; CCL-23) were maintained at 37°C in EMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Invitrogen, Burlington, Canada), penicillin (1 U ml⁻¹), and streptomycin (1 µg ml⁻¹). Cultures were kept in a humidified incubator containing 5% CO₂. The A2 strain of RSV was purchased from the American Type Culture Collection (ATCC; VR-1540). The recombinant strain of RSV expressing green fluorescent protein (rgRSV224; here, RSV-GFP)³⁸ was provided by Dr. M.E. Peeples (Children’s Research Institute, Columbus, OH) and Dr. P.L. Collins (National Institutes of Health, Bethesda, MD). Community isolates of RSV A and RSV B were provided by Dr. S. Richardson (The Hospital for Sick Children, Ontario, Canada) and Dr. J. Gubbay (Public Health Ontario, Ontario, Canada). RSV stocks were prepared as described³⁹.

**Viral infectivity assays**

For all inhibition experiments, HEp-2 cells were plated in black, flat, clear-bottom 96-well plates at a density of 6000 cells/well in a total volume of 200 µl per well and incubated overnight at 37°C, 5% CO₂. The next day, cells were washed twice with serum free EMEM followed by the addition of 50 µl of RSV-GFP at the indicated multiplicity of infection (MOI). Plates were incubated at 37°C, 5% CO₂ for 90 minutes under constant agitation. After this incubation unbound virus was removed and 200 µl of fresh medium containing serum ± drug treatment was added to cells. The plates were further incubated at 37°C, 5% CO₂ until 48 hours post infection (hpi), when the amount of GFP
in each well of the infected plates was determined. To assess the effect of extracellular Na\(^+\) substitution on RSV replication in the presence or absence of cardiac glycosides, RSV infected HEp-2 cells were cultured in medium containing 120 mM NaCl substituted with equimolar concentrations of N-methyl-D-glucamine (NMDG) and RSV infectivity was measured at 48 hpi. RSV-GFP infected cells were imaged on an inverted epifluorescent microscope (Nikon TE-2000) using a Hamamatsu C4742-80-12AG camera and Perkin Elmer Volocity software. The total fluorescence intensity was determined from 4 random fields per well (4X magnification) using CellProfiler software. The antiviral activity was calculated by the inhibition of GFP compared to vehicle control-treated cells.

**In vitro cytotoxicity**

To confirm that a decrease in fluorescence correlated with the inhibition of viral replication and not an increase in cell death, a viability screen was run in tandem using uninfected HEp-2 cells. Briefly, cells were seeded at a density of 6,000 cells/well in black, flat, clear-bottom 96-well plates (Costar) and allowed to attach overnight at 37°C. The next day, medium was replaced with 200 µl of fresh medium containing 5-fold serially diluted compound with a concentration ranging from 3.2 nM to 10 µM. Cells were cultured at 37°C for 2 days. Following incubation, cell viability was determined by the addition of 20 µl of PrestoBlue viability reagent to the culture medium. The mixture was incubated at 37°C for 1 hour and the fluorescent signal was quantified on a Molecular Devices SpectraMax Gemini EM fluorescent plate reader.
**Virucidal activity assay**

Approximately $10^5$ PFU of RSV-GFP and the drug treatment at the IC$_{90}$ concentration were added to serum free EMEM and mixed in a total volume of 200 ul. The virus-compound mixture was incubated for 15 min in a 37°C water bath and then serially diluted to the non-inhibitory concentration of test compound. The residual viral infectivity was determined 24 hours later by standard endpoint dilution assay using GFP as an indicator of infected cells.

**Entry inhibition assay**

HEp-2 cells in black, flat, clear-bottom 96-well plates were pretreated with indicated concentrations of ion-modulating drugs in indicated solvent for 1 hour. Cells were then infected with RSV-GFP in serum free EMEM ± ion-modulating drug at an MOI of 1 for 90 minutes. After this incubation, cells were washed twice with serum containing EMEM to remove any unbound virus and drugs. RSV-GFP was quantified at 24 hpi. Heparin (1 mg/mL), an inhibitor of RSV entry, was included as a positive control$^{44,416}$.

**Time of drug addition and removal**

HEp-2 cells were seeded at a density of 10,000 cells/well in black, flat, clear-bottom 96-well plates. Cells were infected with RSV-GFP at an MOI of 1 PFU/cell in serum free EMEM. After 90 minutes the viral inoculum was removed and cells were washed once with serum containing EMEM. Cardiac glycosides were added at selected time points, and the amount of GFP in each well of the infected plates was determined at 24 hpi as indicated prior. Drugs were added at a minimum of 10X the IC$_{50}$ to normalize for potency differences and to ensure that efficacy was not reflected in time of addition (ToA)
profiles. Time of removal (ToR) assays were done similarly, however all drugs were added at 2 hpi and replaced with serum containing EMEM at selected time points.

**RSV replicon assay**

HeLa cells containing a stably transformed subgenomic RSV replicon expressing a GFP reporter cassette was licensed from Apath, LLC and received from M. Peeples. Replicon cells were seeded at a density of 10,000 cells/well in black, flat, clear-bottom 96-well plates (Corning) and cultured overnight in growth media containing 50 µg/ml blasticidin S. The growth medium was removed and replaced with 200 µl of 5-fold serially diluted drugs prepared in EMEM supplemented with 10% (v/v) FBS, penicillin (1 U ml⁻¹), and streptomycin (1 µg ml⁻¹) and blasticidin S (50 µg/ml). After 48 hours at 37°C, the cells were imaged on an inverted epifluorescent microscope (Nikon TE-2000) using a Hamamatsu C4742-80-12AG camera and Perkin Elmer Volocity software. Replicon gene expression was quantified using CellProfiler software by measuring the total replicon GFP fluorescence signal intensity from 4 random fields per well (4X magnification).

**Primary nasal epithelial cells**

Air-liquid interface (ALI) differentiated primary human nasal epithelial cells were cultured as described. Briefly, cells were obtained from healthy volunteers (no lung disease or respiratory medication use) by lightly brushing the inferior turbinate using a sterile cytology brush (MP Corporation, Camarillo, CA). Cells were seeded on a collagen-coated flask and maintained in BEGM until 70% to 80% confluence at which point they were passaged. For experiments, cells were seeded on collagen coated Transwell inserts (6.5 mm diameter, 0.4 µm pore size, Corning) and brought to ALI by
removing the media at the apical side of the cells and changing to a basal differentiation media (PneumaCult, StemCell Tech., Vancouver). By 3 weeks, cells were differentiated with a ciliated phenotype. Transepithelial resistance was quantified with an ohmmeter (World Precision Instruments, Sarasota, FL) to evaluate epithelial barrier function prior to proceeding to experiments. Cells were infected apically with RSV at an input MOI quantified by plaque forming units or infectious units required to infect ~1% of cells. For RSV-GFP, virus replication was quantified as previously stated. For RSV A2 virus replication was quantified by qRT-PCR.

**Quantitative reverse transcription-PCR (qRT-PCR)**

Cellular RNA was extracted using an RNeasy minikit (Qiagen) according to the manufacturer’s instructions. Quantification of RSV was conducted using absolute qRT-PCR using primers specific for the RSV nucleocapsid gene. RNA levels were measured using a modified version of a previously published protocol on the ABI 7500 FAST platform (Applied Biosystems, California).

**Treatment of RSV-infected mice with Digoxin**

Pathogen free 6-8 week old female BALB/c mice (Charles River Laboratories) were lightly sedated with isoflurane and infected intranasally with 5x10^6 plaque forming units (PFU) of RSV on day 0 as described previously. One hour after inoculation with RSV, mice received a loading dose of 2 mg/kg of digoxin or saline intraperitoneally (i.p.). Following the loading dose, mice received 1 mg/kg of digoxin or saline i.p. every 12 hours until sacrifice. All mice were monitored daily for changes in weight. In this model, mice lose approximately 10-15% of their original body weight by day 4, corresponding to peak viral titres, and begin to gain weight by day 6. On day 4, the peak of viral
replication, the lungs were excised, homogenized, and a standard plaque assay was performed to obtain plaque-forming units as previously described.44

Statistics

Statistical significance was assessed with GraphPad Prism 7.0 software (GraphPad Software, La Jolla, California). Data are presented as means ± SEM (n ≥ 3). Student’s t test was used to determine statistical significance between the means of two groups. A one-way ANOVA was used to assess statistical significance between the mean of three or more groups, with Dunnett’s multiple comparisons test (single pooled variance). The IC50 and CC50 values of drugs were calculated using the Prism software and non-linear regression was used to fit curves. In all analyses, two-sided P values were used, and P < 0.05 was considered statistically significant.

3.4 Results

In vitro anti-RSV effects of cardiac glycosides

In an initial screen to identify novel RSV directed therapies, we screened 2560 compounds many of which are FDA approved. With this assay, we identified 67 compounds that significantly reduced RSV infectivity, 36% of which are known to modulate intracellular ion levels (Figure 3.1A). Cardiac glycosides potently prevented RSV infection. In a secondary confirmation assay, the effects of cardiac glycosides on RSV replication and cell viability were examined using a dose-response study. HEp-2 cells were infected with RSV-GFP (RSV modified to express green fluorescent protein reporter gene) and treated with increasing concentration of cardiac glycosides (0.0032 to 10 µM). Ribavirin was used as a control (0.16 to 100 µM). Virus replication was quantified indirectly by GFP fluorescence at 48 hpi.
Figure 3.1: Cardiac glycosides inhibit RSV without effecting cell viability.

(A) Mechanistic overview of hits from high throughput screen. Vertical axis denotes the cellular mechanism. Horizontal axis indicates the number of drugs with that particular mechanism (B, C, & D) Top, chemical structure of drug. Bottom, dose-response curves showing the effect of drug treatment on RSV-GFP (MOI = 0.5) and cell viability in HEp-2 cells. Left y-axis denotes reduction in GFP signal (representative of RSV replication) normalized to DMSO treated cells infected with RSV-GFP. Right y-axis denotes cell death normalized to DMSO treated cells (background cell free fluorescence subtracted). Values represent mean ± SEM. (n = 3 independent experiments performed in quadruplicate). Curves represent best fit for calculating the IC_{50} (solid line) and CC_{50} (dotted line) values (listed below).
Treatment with the cardiac glycosides, digoxin and digitoxin, resulted in a dose-dependent reduction in RSV replication with 50% inhibitory concentration (IC\textsubscript{50}) values of 0.026 µM and 0.020 µM respectively (Figure 3.1B & C). Ribavirin treatment resulted in a higher IC\textsubscript{50} of 8.3 µM (Figure 3.1D). To ensure the observed anti-viral effects were not due to cell toxicity, viability was determined by a resazurin-based assay (PrestoBlue, Invitrogen) in non-infected, drug treated cells. Digoxin and digitoxin had relatively high 50% cytotoxicity concentrations (CC\textsubscript{50}) of 0.839 uM and 0.585 uM (Figure 3.1B & C), with corresponding selectivity indexes (SI = CC\textsubscript{50}/IC\textsubscript{50}) of 32 and 28 respectively. The CC\textsubscript{50} of Ribavirin was >10 µM (Figure 3.1D). This strongly suggests that the observed anti-RSV effects of digoxin and digitoxin are not due to cell toxicity.

**Anti-RSV effects of cardiac glycosides are dependent on changes in intracellular Na\textsuperscript{+}/K\textsuperscript{+}**

Cardiac glycosides inhibit the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase altering intracellular ion concentrations including a reduction in intracellular K\textsuperscript{+} and an increase in intracellular Na\textsuperscript{+}\textsuperscript{420-422}. To test whether inhibition of RSV by cardiac glycosides occurs via changes in K\textsuperscript{+} concentrations, we treated RSV infected cells with DMSO or digoxin in the presence of high K\textsuperscript{+}, effectively reducing the gradient for K\textsuperscript{+} efflux. We found that addition of high extracellular KCl in the presence of digoxin significantly reduced inhibition of RSV (Figure 3.2A). These results suggest that antiviral effects of cardiac glycosides are in part mediated by K\textsuperscript{+} efflux. We next evaluated the role of Na\textsuperscript{+} influx in the inhibition of RSV, as cardiac glycosides are also known to cause Na\textsuperscript{+} influx concurrently with K\textsuperscript{+} efflux\textsuperscript{420-422}. We tested this in two ways.
Figure 3.2: Anti-RSV effects of cardiac glycosides are dependent on changes in K\(^+\) and Na\(^+\).

(A) Left, representative images of HEp-2 cells infected with RSV-GFP (MOI = 1) and incubated with DMSO or digoxin at indicated concentration in standard medium or medium supplemented with increasing concentrations of KCl and infectivity was measured at 24 hours post infection. Right, reduction in GFP signal normalized to DMSO treated cells. Values represent mean ± SEM (n = 3 independent experiments) *** P ≤ 0.001; by one-way ANOVA. (B & C) Digoxin and digitoxin were diluted in medium containing N-Methyl-D-glucamine (NMDG) iso-osmotically substituted for NaCl to maintain physiological isotonic conditions in the absence of sodium. Left y-axis denotes reduction in GFP signal normalized to DMSO treated cells. Dotted lines indicate dose response curve of compounds in EMEM (5 mM KCl and 120 mM NaCl) and solid lines indicate dose-response curve of compounds in NMDG media. For all assays, HEp-2 cells were infected with RSV-GFP (MOI = 0.5). Data is representative of mean ± SEM (n = 3 independent experiments). Curves represent best fit for calculating the IC\(_{50}\) values (listed...
below). (D) Left, representative images of HEp-2 cells infected with RSV-GFP (MOI = 1) and incubated with DMSO or digoxin at indicated concentration in standard medium or medium supplemented with increasing concentrations of NaCl and infectivity was measured at 24 hours post infection. Right, reduction in GFP signal normalized to DMSO treated cells. Values represent mean ± SEM (n = 3 independent experiments) * P ≤ 0.05, *** P ≤ 0.001, **** P ≤ 0.0001; by one-way ANOVA with a Tukey's post hoc test for group comparisons.
First, we iso-osmotically substituted extracellular Na\(^+\) with the organic monovalent cation, N-methyl-d-glucamine (NMDG), and treated RSV infected cells to varying concentrations of cardiac glycosides. Reducing the extracellular Na\(^+\) concentration reduced the anti-RSV effects of digoxin and digitoxin. When RSV-GFP infected cells were incubated in NMDG-substituted media containing digoxin or digitoxin the IC\(_{50}\) values shifted from 0.026 and 0.020 \(\mu\)M to 0.157 and 0.106 \(\mu\)M respectively (Figure 3.2B & C). These results indicate that Na\(^+\) influx plays a role in the inhibition of RSV. To further test if inhibition of RSV by cardiac glycosides is mediated by changes in Na\(^+\) influx, we treated RSV infected cells with DMSO or digoxin in the presence of increasing media concentrations of NaCl. Higher extracellular NaCl should enhance Na\(^+\) influx with glycoside treatment. We found that addition of extracellular NaCl in the presence of digoxin significantly enhanced the inhibition of RSV by digoxin in a dose-dependent manner (Figure 3.2D). This data confirms that alterations of intracellular K\(^+\) and Na\(^+\) contribute to the antiviral activity of cardiac glycosides.

**Cardiac glycosides inhibit RSV infection of primary nasal epithelial cells**

In order to enhance the clinical relevance of our *in vitro* observations, we performed studies in well-differentiated primary human nasal epithelial cell cultures (WD-PNECs). These cells are grown in an air liquid interface, have cilia and tight junctions indicating a well differentiated population of airway epithelial cells thought to better model the *in vivo* environment when compared to submerged cell cultures\(^{361,423}\). After 21 days of culture, RSV-GFP was added to the apical surface and digoxin or digitoxin (0.016, 0.08, and 0.4 \(\mu\)M) was added to the basal media.
Figure 3.3: Cardiac glycosides inhibit RSV in primary nasal epithelial cells.  
(A & B) Left, representative images of primary human nasal epithelial cells 72 hours after apical surface infection with RSV-GFP (MOI = 0.01). Indicated concentrations of drug were added to the basal medium 2 hours after exposure to RSV-GFP. Right, reduction in GFP fluorescence normalized to DMSO treated cells in RSV-GFP infected PNEC cultures treated with the indicated concentrations of digoxin or digitoxin. Human nasal epithelial cells were obtained from brushing of nasal turbinates from healthy donors (non-CF, non-asthma, nonsmokers) (n = 5 donors). Values represent mean ± SEM * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001; by one-way ANOVA with a Tukey's post hoc test for group comparisons. # indicates values slightly outside the axis limits.
There was a dose dependent reduction in RSV-GFP replication over 72 hours following RSV-GFP infection in digoxin and digitoxin treated cells with complete inhibition at 0.4 µM (Figure 3.3A & B). The cells remained viable at all drug doses as determined by microscopic appearance and maintenance of barrier integrity (i.e. apical surface remained dry).

**Digoxin inhibits clinical strains of RSV in primary nasal epithelial cells**

To further evaluate the clinical relevance of cardiac glycosides we infected WD-PNECs apically with circulating strains of RSV obtained from children presenting with bronchiolitis. The virally infected cells were treated in the basal media with 0.4 μM digoxin, 0.08 μM digoxin, or DMSO for 72 hours. RNA was isolated using the Qiagen RNeasy mini kit and viral yield was quantified using qRT-PCR. Both 0.4 and 0.08 μM digoxin significantly reduced clinical strains of RSV-A compared to DMSO treated isolates (Figure 3.4).

**Hypertonic saline accentuates anti-RSV effects of digoxin in PNECs**

Our mechanistic investigations suggest the anti-RSV effects of cardiac glycosides are enhanced by increasing Na⁺ influx (Figure 3.2D). With this in mind, we evaluated the ability of hypertonic saline to enhance the anti-RSV effects of digoxin. A recent clinical review suggested a beneficial effect of inhaled hypertonic saline (high sodium) in the setting of RSV infection³⁰¹. Although beneficial effects of hypertonic saline may be related to improved airway clearance, reduced viral replication offers an additional explanation. The apical surface of digoxin-treated, virus-infected, ALI cultures was washed with 3% saline or 0.9% saline three times a day (every 8 hours) and RSV-GFP infection was documented.
Figure 3.4: Digoxin inhibits clinical strains of RSV in primary nasal epithelial cells.

Human primary nasal epithelial cells grown obtained from brushing of nasal turbinates from a healthy donor (non-CF, non-asthma, nonsmokers) grown at an air liquid interface were infected with community isolates of RSV. At 2 hours post exposure to RSV, digoxin (400 nM or 80 nM) or DMSO was added to the basal media. RNA was harvested 72 hours post RSV and quantified by qRT-PCR. Y-axis denotes the reduction in RSV normalized to DMSO treated cultures (400 nM, n = 7; 80 nM, n = 3). \( \% \text{ Inhibition} = (1-10^{\text{Log (DMSO)}-\text{Log (Treatment)}}) \times 100 \)
Under these conditions, the addition of 0.9% saline wash did not enhance antiviral effects of 16 nM or 80 nM digoxin; 3% saline washes, however, did enhance antiviral effects at both doses (Figure 3.5).

**Digoxin failed to reduce RSV titers in a mouse model of RSV infection**

We examined the *in vivo* effect of digoxin treatment in a mouse model of RSV infection. Pathogen free 6-8 week old female BALB/c mice (Charles River Laboratories) were lightly sedated with isoflurane and infected intranasally with 5x10^6 plaque forming units (PFU) of RSV on day 0. Two hours after inoculation with RSV, mice received 0.9% saline or a loading dose of 2 mg/kg of digoxin intraperitoneally (i.p.). Following the loading dose, mice received 0.9% saline or a maintenance dose of 1 mg/kg digoxin every 12 hours until sacrifice. All mice were monitored daily for changes in weight (Figure 3.6A). In this model, mice lose approximately 10-15% of their original body weight by day 4, corresponding to peak viral titres^79^, and begin to gain weight by day 6. On day 4, the peak of viral replication, the lungs were excised, homogenized, and a standard plaque assay was performed to obtain plaque-forming units as previously described^44^. Digoxin treatment had no significant affect on peak lung viral titres 4 days post infection (Figure 3.6B). The remainder of the lung homogenate was used to isolate RNA and qPCR was performed to determine viral titers. Similarly, there was no significant change in lung viral titers as determined by qPCR from mice treated with digoxin (Figure 3.6C). The murine alpha-1 subunit of the Na^+/K^+-ATPase is resistant to inhibition by cardiac glycosides^411,424^ which may explain a lack of efficacy.
Figure 3.5: Hypertonic saline enhances the anti-RSV effects of digoxin.

Left, representative images of primary human nasal epithelial cells 72 hours after apical surface infection with RSV-GFP (MOI = 0.01). Indicated concentrations of drug were added to the basal medium 2 hours after exposure to RSV-GFP. The apical surface was either untreated, washed with 0.9% saline, or washed with 3% saline 3 times per day (every 8 hours). Right, reduction in GFP fluorescence normalized to DMSO treated cells in RSV-GFP infected PNEC cultures treated with the indicated concentrations of digoxin. Human nasal epithelial cells were obtained from brushing of nasal turbinates from healthy donors (non-CF, non-asthma, nonsmokers) ($n = 3$ donors). Values represent mean ± SEM.
Figure 3.6: Digoxin fails to reduce RSV titers in lungs of infected mice.

BALB/c mice were infected i.n. with $5 \times 10^6$ PFU of RSV on day 0. Beginning at 2 hpi mice received a loading dose of 2 mg/kg of digoxin or saline i.p. followed by a maintenance dose of 1 mg/kg digoxin or saline every 12 hours until sacrifice. (A) Weight loss of infected mice was monitored daily. (B) On day 4, the peak of viral replication, lung homogenates were analyzed by standard plaque assay (C) or qRT-PCR for viral load ($n = 6$ mice per group) *ns* $P > 0.05$; by students t-test.
Cardiac glycosides inhibit RSV at an early stage of infection

We next tested the hypothesis that cardiac glycosides directly target the virus. To determine if glycosides exert their anti-RSV effect by neutralizing, inactivating, or destroying the virus permanently, we tested the virucidal potency at the respective IC₉₀ concentration. In contrast to Virox™, none of the cardiac glycosides exhibited virucidal activity (Figure 3.7A) indicating they do not exert their anti-RSV activity directly on the RSV particle. Previously, digoxin has been demonstrated to prevent entry of viruses into cells⁴¹⁴. However, we found that digoxin and digitoxin had no impact on RSV attachment/entry (Figure 3.7B). Heparin however was able to completely inhibit RSV entry (Figure 3.7B). This strongly suggests that cardiac glycosides do not inhibit RSV infection at the stage of virus entry. To determine the temporal window during which ion-modulating drugs act to inhibit RSV infection, time-of-addition (ToA) and time-of-removal (ToR) experiments were performed. When digoxin or digitoxin was added 2-4 hpi, RSV infection was almost completely inhibited (Figure 3.7C). When treatment was delayed to 6-8 hpi, inhibition of RSV was reduced. When treatment was delayed to 12 hpi, RSV infection was almost completely unaffected (Figure 3.7C). Concordantly, we found that there was a complete lack of inhibition of RSV when digoxin or digitoxin was removed within the first 4 hpi (Figure 3.7D). These results suggest cardiac glycosides exert their antiviral effects at a postentry step but prior to (or during) viral replication. To test if cardiac glycosides directly altered RSV-specific mRNA transcription and RNA replication we used an RSV replicon (artificial RSV minigenome assays, containing GFP as a reporter⁴¹⁷). In this way we were able to perform a focused evaluation of the RSV RNA polymerase independently from other stages of the RSV lifecycle.
Figure 3.7: Cardiac glycoside inhibition of RSV occurs early in the infection cycle. (A) Cardiac glycosides or DMSO controls (0.0025%) in serum free medium at indicated concentrations were mixed with ~10^5 PFU of RSV-GFP and incubated for 15 min in a 37°C water bath. The mixture was serially diluted and the residual virus infectivity determined in Hep-2 cells. Activated hydrogen peroxide (Virox™) was included as a positive control. RSV-GFP was quantified by end-point dilution at 24 hpi. Values represent mean ± SEM (n = 3 independent experiments) *** P ≤ 0.001, ns P > 0.05; by one-way ANOVA (B) HEp-2 cells were pre-treated for 1 hour with indicated concentrations of cardiac glycosides or DMSO. After the preincubation period, RSV-GFP (in serum-free EMEM ± inhibitor) at an MOI of 1 was added to cells for 90 minutes. Heparin (1 mg/ml) was included as a positive control. Data is representative of reduction in GFP fluorescence normalized to DMSO treated cells. Values represent mean ± SEM (n = 3 independent experiments) ** P ≤ 0.01, **** P ≤ 0.0001; by one-way ANOVA. (C) Time-of-addition (ToA) and (D) time-of-removal (ToR) profiles for RSV inhibitors. Cells were infected with RSV-GFP for 90 minutes at an MOI of 1. For ToA assay, digoxin (260 nM), digitoxin (260 nM) or DMSO was added at the times indicated post RSV. For ToR assay, digoxin (260 nM), digitoxin (260 nM) or DMSO was added at 2 hours post infection. At indicated times, the drug containing medium was removed, and wells were washed before adding EMEM free of any compound. For both assays, RSV-GFP was quantified at 24 hours by epifluorescence microscopy. Y-axis denotes reduction in RSV-GFP normalized to DMSO treated cells. Values represent mean ± SEM (n = 2 independent experiments).
Our findings suggest that glycosides do not significantly impact the RSV RNA polymerase (Table 3.1). These results are consistent with an antiviral ion dependent effect occurring post RSV entry but prior to viral RNA synthesis.

3.5 Discussion

In the general population, RSV infection is the chief reason to be hospitalized in the first few years of life\textsuperscript{367,425,426}, the most frequent viral cause of death in infants worldwide\textsuperscript{427}, and second only to malaria amongst all causes of infant death\textsuperscript{71}. Despite multiple drug candidates in development for the treatment of RSV infection, an affordable and effective treatment remains an unmet clinical need. In this manuscript, we provide evidence that cardiac glycosides reduce RSV replication. We further demonstrate that antiviral effects of cardiac glycosides are dependent on Na\textsuperscript{+} and K\textsuperscript{+} and occur early in the RSV infection cycle.

The repurposing of existing drugs for new indications reduces the time and cost for drug development prior to clinical use and is a common goal\textsuperscript{428-430}. Cardiac glycosides have been used in humans for over 200 years, and are well tolerated when serum levels are kept within safe targets\textsuperscript{431}. In patients treated with digoxin, safe and acceptable serum concentrations range from 1 to 3 nM\textsuperscript{432}; lung parenchymal concentrations are 20 times serum levels\textsuperscript{433-435}. \textit{In vitro}, the potency of digoxin on inhibition of RSV replication is in the sub-nanomolar range, meaning translating findings to humans in the form of a pilot study is extremely feasible since we already know what doses of digoxin are safe to use in humans. Furthermore, the possibility of hypertonic saline inhalation as an adjuvant to enhance effects could be tested.
Table 3.1: Activity of cardiac glycosides in the RSV replicon assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>HeLa Replicon Level (IC$_{50}$ [µM])</th>
<th>Infectivity/Toxicity (HEp-2 [µM])</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IC$_{50}$</td>
</tr>
<tr>
<td>Digoxin</td>
<td>&gt;10</td>
<td>0.02661</td>
</tr>
<tr>
<td>Digitoxin</td>
<td>&gt;10</td>
<td>0.02058</td>
</tr>
</tbody>
</table>

Data is averaged from the results of a minimum of 3 independent experiments.
Toxicity concerns are mitigated by the ability to measure serum digoxin levels and offer an antidote if needed (digoxin immune antigen binding fragments).

However, historical concerns over digoxin toxicity may limit enthusiasm for direct use of digoxin for human viral infections. Thus, it is critical to understand the mechanisms underlying the observed digoxin effects as this understanding may lead to novel antiviral approaches. Digoxin and other cardiac glycosides are known to inhibit several viruses in culture systems, including Sindbis virus, Sendai virus, Semliki Forest virus, numerous herpesviruses, human immunodeficiency virus, porcine reproductive and respiratory syndrome virus, Coronavirus, and Chikungunya virus. Several of these studies involved drug screens with no mechanism identified. When mechanistic studies were involved, evidence for effects on RNA splicing pathways, ATP1A1-mediated Src signaling, and alteration of electrochemical gradients were identified. These viruses are extremely heterogeneous and yet all are affected by cardiac glycosides. This suggests that cardiac glycosides may target a fundamental process important for many intracellular pathogens.

Inhibition of the Na+/K+-ATPase disrupts ion transport and alters several cellular signaling, and vesicular sorting pathways. Our findings suggest cardiac glycoside anti-RSV activity is primarily dependent on changes in the intracellular Na+ and K+ composition. The ion dependent events appear to be after viral entry, however prior to the formation of RNA replication factories, as there was lack of inhibition in the RSV replicon (Table 3.1). Further evidence of this ion dependency is demonstrated with the lack of efficacy in the mouse model of RSV. Cardiac glycosides inhibit the Na+/K+-ATPase by binding to the α-1 subunit; blocking Na+ ion extrusion from treated
However, the murine α-1 subunit is resistant to inhibition by cardiac glycosides and thus ion fluxes remain unperturbed, providing a potential explanation for the observed lack of efficacy. This early change in ions by cardiac glycosides has also been shown to be inhibitory against other RNA viruses. Chikungunya virus, a single-stranded, positive sense RNA virus, is potently inhibited by digoxin. Similar to RSV, the addition of extracellular $K^+$ during treatment restores infectivity levels of control treated cells. Evidence for an early ion requirement is further supported by the function of the RSV small hydrophobic (SH) protein in forming pentameric, cation-selective channels allowing $Na^+$ and $K^+$ across infected cell membranes. These ions are specifically altered during cardiac glycoside treatment, suggesting they may play crucial role during the RSV life cycle. Inhibition of SH channel activity has been shown to inhibit RSV infection.

The results of our work suggest that the anti-RSV effects of cardiac glycosides are primarily dependent on changes in the intracellular $Na^+$ and $K^+$ composition. Modulation of intracellular ions, such as $Na^+$ and $K^+$, represent a novel approach to reduce RSV propagation and has the potential to be utilized as a new therapeutic modality to more broadly treat RSV infections.

**Author Contributions:**
M.J.N. conceived of, performed, and planned all experiments, analyzed the data and wrote the paper. M.M. performed initial high throughput screening assay and identified high potential hits. W.D. performed initial high throughput screening assay, assisted with plaque assay in Figure 3.6., provided guidance on statistics and edited the paper. H.O. expanded and maintained the primary human nasal epithelial cell cultures. A.G.
performed qPCR on clinical isolates in Figure 3.4. J.G. provided the RSV clinical isolates and assisted with the qPCR quantification. J.M. conceived of and supported the initial high throughput screen. T.J.M. conceived of in vivo and in vitro experiments, analyzed data, wrote and edited the paper, supervised and provided support for the project.
4. Targeting intracellular ion homeostasis for the control of RSV

4.1 Abstract

RSV is one of the most important causes of pediatric lower respiratory infections. The development of novel antiviral strategies to control RSV is of great importance. However, despite intense efforts, there is no antiviral or vaccine approved for the treatment or prevention of RSV infections. Here, we show for the first time that ionophoric antibiotics, which include drugs used in veterinary medicine and recently used as human anticancer agents, potently inhibit RSV replication. Treatment of both HEp-2 cells and primary nasal epithelial cells with salinomycin, monensin, or valinomycin, resulted in a dose-dependent decrease in RSV infection. This anti-RSV activity was dependent on changes in intracellular sodium and potassium. RSV replication also appeared to be highly sensitive to the ionic strength of the culture medium. Ion modulation inhibited events shortly after virus entry but prior to viral RNA synthesis/replication, suggesting highly controlled intracellular sodium and potassium levels are required for RSV uncoating.

4.2 Introduction

RSV is the leading causative agent of lower respiratory tract infections, including bronchiolitis and pneumonia, in infants and young children worldwide. Despite the
disease burden presented by RSV, treatment is non-specific and directed towards maintaining hydration and oxygenation\textsuperscript{297}. Given the lack of specific RSV therapies, there is an urgent and unmet need for additional safe and effective treatments for RSV.

To identify novel RSV directed therapies, we developed a live virus, high-content, high-throughput assay using cultured human epithelial cells and performed screening on the MicroSource Spectrum Library of compounds. This screen identified many approved drugs and probes with previously undocumented anti-RSV activity, including the cardiac glycosides, digoxin and digitoxin. Cardiac glycosides are generally thought to bind to and inhibit the membrane bound Na\textsuperscript{+}/K\textsuperscript{+}-ATPase. The Na\textsuperscript{+}/K\textsuperscript{+}-ATPase drives the active transport of K\textsuperscript{+} ions into cells and Na\textsuperscript{+} ions out of cells. Inhibition disrupts ion transport thereby raising the level of Na\textsuperscript{+} and decreasing the level of K\textsuperscript{+} in cells\textsuperscript{421,422}. The results of our work suggested that the anti-RSV effects of cardiac glycosides are primarily dependent on changes in the intracellular Na\textsuperscript{+} and K\textsuperscript{+} composition. With this in mind, we next wanted to explore if other drugs that modulate intracellular ion concentrations could impact RSV infection.

Here, we show that ionophoric antibiotics are potent anti-RSV agents in both HEp-2 and primary human nasal epithelial cells. Ionophoric antibiotics are characterized by their ability to form reversible, lipid soluble, cation complexes that facilitate the transport of ions across biological membranes\textsuperscript{447}. We tested various ionophores that differ in their selectivity for cations\textsuperscript{447}. Similar to cardiac glycosides, ionophoric antibiotics inhibited RSV early during the replication cycle and their anti-RSV effects were highly dependent on intracellular ion levels. We further show that a decrease in the intracellular K\textsuperscript{+} levels in the absence of pharmacological agents is sufficient to block RSV infection.
This work provides further evidence in support of the hypothesis that intracellular ions, such as Na\(^+\) and K\(^+\), play a crucial role during the life cycle of RSV. These findings also suggest that ionophoric antibiotics may represent a viable antiviral therapeutic class and that further understanding of the underlying mechanism of action may reveal modifiable therapeutic targets leading to broad antiviral strategies.

4.3 Methods

Reagents and medium

Salinomycin, valinomycin, and monensin were purchased from Sigma. Virox was purchased from Virox Technologies Inc., Oakville, Canada. Blasticidin and PrestoBlue were purchased from Thermo Fisher Scientific, Mississauga, Canada. Molecular biology grade KCl and NaCl purchased from Ambion, Burlington, Canada. N-methyl-D-glucamine (NMDG) purchased from Sigma. Eagle’s Minimum Essential Medium (EMEM) and EMEM lacking NaCl and KCl were purchased from Wisent Inc., Montreal, Canada. Basal epithelial growth medium (BEGM) for primary cell maintenance was purchased from Lonza, Walkersville, MD. Basal differentiation medium (PneumaCult) for maintenance of primary cells at an air-liquid interface (ALI) and Heparin sodium salt were purchased from StemCell Tech., Vancouver, Canada.

Cells and viruses

HEp-2 (ATCC; CCL-23) were maintained at 37°C in EMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen, Burlington, Canada), penicillin (1 U ml\(^{-1}\)), and streptomycin (1 µg ml\(^{-1}\)). Cultures were kept in a humidified incubator containing 5% CO\(_2\). The A2 strain of RSV was purchased from the American Type Culture Collection (ATCC; VR-1540). The recombinant strain of RSV expressing green
fluorescent protein (rgRSV224; here, RSV-GFP) was provided by Dr. M.E. Peeples (Children’s Research Institute, Columbus, OH) and Dr. P.L. Collins (National Institutes of Health, Bethesda, MD). RSV stocks were prepared as described.

**Viral infectivity assays**

For all inhibition experiments, HEp-2 cells were plated in black, flat, clear-bottom 96-well plates at a density of 6000 cells/well in a total volume of 200 µl per well and incubated overnight at 37°C, 5% CO2. The next day, cells were washed twice with serum free EMEM followed by the addition of 50 µl of RSV-GFP at the indicated multiplicity of infection (MOI). Plates were incubated at 37°C, 5% CO2 for 90 minutes under constant agitation. After this incubation unbound virus was removed and 200 ul of fresh medium containing serum ± drug treatment was added to cells. The plates were further incubated at 37°C, 5% CO2 until 48 hours post infection, when the amount of GFP in each well of the infected plates was determined. To assess the effect of extracellular K+ concentration on RSV replication, HEp-2 cells infected with RSV were cultured for 24 hours in serum free EMEM lacking NaCl and KCl supplemented with either 5 mM LiCl, 5 mM, 1 mM, 0.75 mM, 0.5 mM, 0.25 mM or without any KCl added. Osmolarity was maintained by isosmotic substitution of KCl with NaCl. At 24 hpi, the K+ specific medium was replaced with serum containing commercial EMEM and RSV infectivity was measured at 48 hpi. To assess the effect of extracellular Na+ substitution on RSV replication in the presence or absence of ionophoric antibiotics, RSV infected HEp-2 cells were cultured in medium containing 120 mM NaCl substituted with equimolar concentrations of N-methyl-D-glucamine (NMDG) and RSV infectivity was measured at 48 hpi. RSV-GFP infected cells were imaged on an inverted epifluorescent microscope.
(Nikon TE-2000) using a Hamamatsu C4742-80-12AG camera and Perkin Elmer
Volocity software. The total fluorescence intensity was determined from 4 random fields
per well (4X magnification) using CellProfiler\textsuperscript{415} software. The antiviral activity was
calculated by the inhibition of GFP compared to vehicle control-treated cells.

**In vitro cytotoxicity**

To confirm that a decrease in fluorescence correlated with the inhibition of viral
replication and not an increase in cell death, a viability screen was run in tandem using
uninfected HEp-2 cells. Briefly, cells were seeded at a density of 6,000 cells/well in
black, flat, clear-bottom 96-well plates (Costar) and allowed to attach overnight at 37°C.
The next day, medium was replaced with 200 µl of fresh medium containing 5-fold
serially diluted compound with a concentration ranging from 3.2 nM to 10 µM. Cells
were cultured at 37°C for 2 days. Following incubation, cell viability was determined by
the addition of 20 µl of PrestoBlue viability reagent to the culture medium. The mixture
was incubated at 37°C for 1 hour and the fluorescent signal was quantified on a
Molecular Devices SpectraMax Gemini EM fluorescent plate reader.

**Virucidal activity assay**

Approximately 10\textsuperscript{5} PFU of RSV-GFP and the drug treatment at the IC\textsubscript{90} concentration
were added to serum free EMEM and mixed in a total volume of 200 ul. The virus-
compound mixture was incubated for 15 min in a 37°C water bath, then serially diluted to
the non-inhibitory concentration of test compound. The residual viral infectivity was
determined 24 hours later by standard endpoint dilution assay using GFP as an indicator
of infected cells.
Entry inhibition assay

HEp-2 cells in black, flat, clear-bottom 96-well plates were pretreated with indicated concentrations of ionophoric antibiotics in indicated solvent for 1 hour. Cells were then infected with RSV-GFP in serum free EMEM ± ion-modulating drug at an MOI of 1 for 90 minutes. After this incubation, cells were washed twice with serum containing EMEM to remove any unbound virus and drugs. RSV-GFP was quantified at 24 hpi. Heparin (1 mg/mL), an inhibitor of RSV entry, was included as a positive control44,416.

Time of drug addition and removal

HEp-2 cells were seeded at a density of 10,000 cells/well in black, flat, clear-bottom 96-well plates. Cells were infected with RSV-GFP at an MOI of 1 PFU/cell in serum free EMEM. After 90 minutes the viral inoculum was removed and cells were washed once with serum containing EMEM. Ionophoric antibiotics were added at selected time points, and the amount of GFP in each well of the infected plates was determined at 24 hpi as indicated prior. Drugs were added at a minimum of 10X the IC50 to normalize for potency differences and to ensure that efficacy was not reflected in time of addition (ToA) profiles. Time of removal (ToR) assays were done similarly, however all drugs were added at 2 hpi and replaced with serum containing EMEM at selected time points.

RSV replicon assay

HeLa cells containing a stably transformed subgenomic RSV replicon expressing a GFP reporter cassette was licensed from Apath, LLC417 and received from M. Peeples. Replicon cells were seeded at a density of 10,000 cells/well in black, flat, clear-bottom 96-well plates (Corning) and cultured overnight in growth media containing 50 µg/ml blasticidin S. The growth medium was removed and replaced with 200 µl of 5-fold
serially diluted drugs prepared in EMEM supplemented with 10% (v/v) FBS, penicillin (1 U ml\(^{-1}\)), and streptomycin (1 µg ml\(^{-1}\)) and blasticidin S (50 µg/ml). After 48 hours at 37°C, the cells were imaged on an inverted epifluorescent microscope (Nikon TE-2000) using a Hamamatsu C4742-80-12AG camera and Perkin Elmer Volocity software. Replicon gene expression was quantified using CellProfiler\(^{415}\) software by measuring the total replicon GFP fluorescence signal intensity from 4 random fields per well (4X magnification).

**Primary nasal epithelial cells**

Air-liquid interface (ALI) differentiated primary human nasal epithelial cells were cultured as described\(^{418}\). Briefly, cells are obtained from healthy volunteers (no lung disease or respiratory medication use) by lightly brushing the inferior turbinate using a sterile cytology brush (MP Corporation, Camarillo, CA). Cells are seeded on a collagen-coated flask and maintained in BEGM until 70% to 80% confluence at which point they are passaged. For experiments, cells are seeded on collagen coated Transwell inserts (6.5 mm diameter, 0.4 µm pore size, Corning) and brought to ALI by removing the media at the apical side of the cells and changing to a basal differentiation media (PneumaCult, StemCell Tech., Vancouver). By 3 weeks, cells are differentiated with a ciliated phenotype. Transepithelial resistance is quantified with an ohmmeter (World Precision Instruments, Sarasota, FL) to evaluate epithelial barrier function prior to proceeding to experiments. Cells were infected apically with RSV at an input MOI quantified by plaque forming units or infectious units required to infect ~1% of cells. For RSV-GFP, virus replication was quantified as previously stated.
Treatment of RSV-infected mice with salinomycin

Pathogen free 6-8 week old female BALB/c mice (Charles River Laboratories) were lightly sedated with isoflurane and infected intranasally with 5x10^6 plaque forming units (PFU) of RSV on day 0 as described previously. One day after inoculation with RSV, mice received 5 mg/kg of salinomycin or saline intraperitoneally (i.p.) every 24 hours until sacrifice. All mice were monitored daily for changes in weight. On day 4, the peak of viral replication, the lungs were excised, homogenized, and a standard plaque assay was performed to obtain plaque-forming units as previously described.

Statistics

Statistical significance was assessed with GraphPad Prism 7.0 software (GraphPad Software, La Jolla, California). Data are presented as means ± SEM (n ≥ 3). Student’s t test was used to determine statistical significance between the means of two groups. A one-way ANOVA was used to assess statistical significance between the mean of three or more groups, with Dunnett’s multiple comparisons test (single pooled variance). The IC50 and CC50 values of drugs were calculated using the Prism software and non-linear regression was used to fit curves. In all analyses, two-sided P values were used, and P < 0.05 was considered statistically significant.

4.4 Results

Ionophoric antibiotics inhibit RSV infection in vitro

The effects of ionophoric antibiotics on RSV replication and cell viability were examined using a dose-response study. HEp-2 cells were infected with RSV-GFP (RSV modified to express green fluorescent protein reporter gene) and treated with increasing concentrations of ionophoric antibiotics (0.0032 to 10 μM).
Figure 4.1: Ionophoric antibiotics inhibit RSV in the absence of cellular toxicity.  
(A, B, & C) Top, chemical structure of indicated drug. Bottom, dose-response curves showing the effect of drug treatment on RSV-GFP (MOI = 0.5) and cell viability in HEp-2 cells. Left y-axis denotes reduction in GFP signal (representative of RSV replication) normalized to DMSO treated cells infected with RSV-GFP. Right y-axis denotes cell death normalized to DMSO treated cells (background cell free fluorescence subtracted). Values represent mean ± SEM. (n = 3 independent experiments performed in quadruplicate). Curves represent best fit for calculating the IC\textsubscript{50} (solid line) and CC\textsubscript{50} (dotted line) values (listed below).
Inhibition of viral infection was indirectly assessed at 48 hpi by quantifying GFP fluorescence. The neutral ionophore, valinomycin, had extremely potent anti-RSV effects across all concentrations tested and demonstrated a 50% inhibitory concentration (IC$_{50}$) value of 0.0015 µM (Figure 4.1A). Similarly, the carboxylic ionophores, salinomycin and monensin, potently inhibited RSV replication with IC$_{50}$ values of 0.106 µM and 0.029 µM respectively (Figure 4.1B & C). Valinomycin resulted in HEp-2 cell death at high concentrations (CC$_{50}$ 2.705 µM) and both salinomycin and monensin had exceptionally favorable toxicity profiles with CC$_{50}$ values >10 µM suggesting the observed inhibition of infection is not due to cellular toxicity (Figure 4.1A, B, & C).

**RSV is dependent on intracellular K$^+$ for infection**

Previous work with cardiac glycosides (Chapter III) and now ionophoric antibiotics have indicated that drugs altering intracellular ion concentrations, including a reduction in intracellular K$^+$, are potent inhibitors of RSV infection. We therefore hypothesized that reduced intracellular K$^+$ would reduce RSV replication. We tested this hypothesis by using several different approaches. First, RSV-GFP infected HEp-2 cells were incubated for 24 hours in medium containing decreasing amounts of KCl (0 – 5 mM KCl). Incubating cells in medium containing low K$^+$ is known to cause K$^+$ efflux and a reduction in the intracellular K$^+$ content$^{448}$. After 24 hours post-RSV infection, the media was changed to 5 mM KCl media (commercial medium) and further incubated for an additional 24 hours. At 48 hpi GFP fluorescence and cell viability was measured. Under these conditions, low K$^+$ inhibited RSV replication with no significant change in cell viability (Figure 4.2A & B).
Figure 4.2: Alterations in cellular K⁺ inhibit RSV and anti-RSV effects of ionophoric antibiotics are K⁺ dependent.
(A) Left, representative images of HEp-2 cells infected with RSV-GFP (MOI 0.5), indicated medium was added 2 hpi and subsequently replaced with EMEM at 24 hpi, and infectivity was measured at 48 hpi. Right, y-axis denotes reduction in GFP signal normalized to RSV-GFP infected, EMEM treated cells. Values represent mean ± SEM (n = 3 independent experiments) ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001; by one-way ANOVA with a Tukey's post hoc test for group comparisons. (B) HEp-2 cells incubated in medium containing decreasing concentrations of KCl for 24 hours. y-axis denotes cell death (normalized to EMEM treated cells) at 48 hours post treatment. Values represent mean ± SEM (n = 3 independent experiments) ns P > 0.05; by one-way ANOVA with a Tukey's post hoc test for group comparisons. (C) Left, representative images of RSV-GFP infected HEp-2 cells incubated in medium containing indicated concentration of LiCl iso-osmotically substituted for KCl. Medium was added 2 hpi and subsequently replaced with EMEM at 24 hpi, and infectivity was measured at 48 hpi. Right, y-axis denotes reduction in GFP signal normalized to RSV-GFP infected, EMEM treated cells. Values represent mean ± SEM (n = 3 independent experiments) ** P ≤ 0.01; by students t-test. (D) HEp-2 cells incubated in medium containing indicated concentration of LiCl iso-osmotically substituted for KCl for 24 hours. y-axis denotes cell death (normalized to EMEM treated cells) at 48 hours post treatment. Values represent mean ± SEM (n = 3 independent experiments) ns P > 0.05; by students t-test. (E) Left, representative images of HEp-2 cells infected with RSV-GFP (MOI = 1) and incubated with DMSO or valinomycin at 4.8 nM in standard medium or medium supplemented with increasing concentrations of KCl and infectivity was measured at 24 hours post infection. Right, reduction in GFP signal normalized to DMSO treated cells. Values represent mean ± SEM (n = 3 independent experiments) *** P ≤ 0.001; by one-way ANOVA with a Tukey's post hoc test for group comparisons.
In a second approach, we incubated RSV-GFP infected cells for 24 hours in medium containing LiCl iso-osmotically substituted for KCl. Lithium (Li+) is documented to displace intracellular K+ and is capable of replacing K+ in various ion dependent biochemical reactions\[^{449}\]. In RSV-GFP infected cells treated with medium containing 5 mM LiCl there was a marked reduction in the amount of RSV-GFP present compared to cells incubated in medium containing 5 mM KCl with no change in cell viability (Figure 4.2C & D). Lastly, Valinomycin is a neutral ionophore that forms charged complexes with cations (selectivity of about 10,000 to 1 for K+ over Na+) and transports them down their electrochemical gradient. In mammalian cells this would result in K+ efflux. Because of this high selectivity for K+, we sought to understand if the K+ efflux caused by valinomycin is essential for its antiviral effects. To do this we treated RSV infected cells with DMSO or valinomycin in the presence of high K+, effectively reducing the gradient for K+ efflux. We found that addition of high extracellular KCl reduced valinomycin antiviral effects (Figure 4.2E), suggesting that K+ efflux mediates valinomycin anti-RSV activity.

**RSV inhibition correlates with the influx of Na+ but is not a requirement**

Next, we sought to determine if, like cardiac glycosides, Na+ fluxes play a role in the antiviral effects of ionophoric antibiotics. We iso-osmotically substituted extracellular Na+ by the organic monovalent cation NMDG and exposed RSV infected cells to varying concentrations of valinomycin or salinomycin. Reducing the extracellular Na+ concentration resulted in a striking reduction in the anti-RSV effects of salinomycin and monensin. When RSV-GFP infected cells were incubated in NMDG-substituted media containing salinomycin or monensin the IC\(_{50}\) values shifted from 0.106 and 0.029 \(\mu M\) to
2.720 and 0.7059 µM respectively (Figure 4.3A & B). Interestingly, substitution of extracellular Na⁺ with NMDG did not impair the anti-RSV effects elicited by valinomycin as no shift in IC₅₀ values was observed (Figure 4.3C). To confirm the anti-RSV effects of valinomycin are not due to the movement of Na⁺, we treated RSV infected cells with DMSO or valinomycin in the presence of increasing concentrations of NaCl. Addition of extracellular NaCl in the presence of valinomycin had no effect on the inhibition of RSV by valinomycin (Figure 4.3D). This is consistent with the mechanism of action of valinomycin (selective ionophore for K⁺) and also demonstrates that while Na⁺ influx can modulate RSV infection, it is not a requirement for the inhibition of RSV.

**Ionophoric antibiotics inhibit RSV in primary nasal epithelial cells**

To enhance the clinical relevance of our *in vitro* observations with ionophoric antibiotics, we performed studies in well-differentiated primary human nasal epithelial cell cultures (WD-PNECs). These cells are grown in an air liquid interface, have cilia and tight junctions, and form a pseudostratified, mucociliary airway epithelium that displays similar morphological and phenotypic characteristics of the *in vivo* human cartilaginous airway epithelium. These cells are thought to better model the *in vivo* environment when compared to submerged cell cultures. After 21 days of culture, RSV-GFP was added to the apical surface and valinomycin (0.0032, 0.016, and 0.08 µM) or salinomycin (0.016, 0.08, and 0.4 µM) was added to the basal media. Dose ranges were informed by the previous submerged culture experiments (Figure 4.1A & B). There was a strong dose dependent reduction in RSV-GFP replication over 72 hours following RSV-GFP infection in valinomycin and salinomycin treated cells (Figure 4.4A & B).
Figure 4.3: Anti-RSV effects of ionophoric antibiotics are associated with Na+ influx but it is not a requirement.

(A, B, & C) Salinomycin, monensin, and valinomycin were diluted in medium containing N-Methyl-D-glucamine (NMDG) iso-osmotically substituted for NaCl to maintain physiological isotonic conditions in the absence of sodium. Left y-axis denotes reduction in GFP signal normalized to DMSO treated cells. Dotted lines indicate dose response curve of compounds in EMEM and solid lines indicate dose-response curve of compounds in NMDG media. For all assays, HEp-2 cells were infected with RSV-GFP (MOI = 0.5). Data is representative of mean ± SEM (n = 3 independent experiments). Curves represent best fit for calculating the IC50 values (listed below). (D) Left, representative images of HEp-2 cells infected with RSV-GFP (MOI = 1) and incubated with DMSO or valinomycin at 4.8 nM in standard medium or medium supplemented with increasing concentrations of NaCl and infectivity was measured at 24 hours post infection. Right, reduction in GFP signal normalized to DMSO treated cells. Values represent mean ± SEM (n = 3 independent experiments) ns P > 0.05; by one-way ANOVA with a Tukey’s post hoc test for group comparisons.
Figure 4.4: Ionophoric antibiotics inhibit RSV in primary nasal epithelial cells.

(A & B) Left, representative images of primary human nasal epithelial cells 72 hours after apical surface infection with RSV-GFP (MOI = 0.01). Indicated concentrations of drug were added to the basal medium 2 hours after exposure to RSV-GFP. Right, reduction in GFP fluorescence normalized to DMSO treated cells in RSV-GFP infected PNEC cultures treated with the indicated concentrations of valinomycin or salinomycin. Human nasal epithelial cells were obtained from brushing of nasal turbinates from healthy donors (non-CF, non-asthma, nonsmokers) (n = 5 donors). Values represent mean ± SEM * P ≤ 0.05, ns P > 0.05; by one-way ANOVA with a Tukey's post hoc test for group comparisons; # represents values slightly outside the axis limits.
The cells remained viable at all drug doses as determined by microscopic appearance and maintenance of barrier integrity (i.e. apical surface remained dry).

**Ion perturbation inhibits RSV at an early stage of infection**

We next sought to determine the ion sensitive step in the RSV replication cycle. To determine if ionophoric antibiotics exert their anti-RSV effect by neutralizing, inactivating, or destroying the virus permanently, we tested the virucidal potency at the respective IC₉₀ concentration. Each ionophoric antibiotic was mixed with RSV-GFP and the residual virus infectivity was determined. In contrast to Virox™, a potent germicide, none of the ion-modulating agents exhibited virucidal activity (Figure 4.5A) indicating they do not exert their anti-RSV activity directly on the RSV particle. Previously, ionophoric antibiotics, such as valinomycin, have been demonstrated to prevent entry of viruses into cells⁴⁵⁰. We therefore sought to determine whether ionophoric antibiotics also inhibit the entry of RSV into HEp-2 cells as their mechanism of action. We found that salinomycin and monensin had no impact on RSV attachment/entry (Figure 4.5B). Heparin however was able to completely inhibit RSV entry (Figure 4.5B). This strongly suggests that carboxylic ionophores do not inhibit RSV infection at the stage of virus entry. Interestingly, valinomycin, a neutral ionophore, did show efficacy at reducing RSV infection in our attachment/entry assay (Figure 4.5B), indicating that valinomycin behaves differently from the other agents. To determine the temporal window during which ionophoric antibiotics act to inhibit RSV infection, time-of-addition (ToA) and time-of-removal (ToR) experiments were performed. Similar to cardiac glycosides (Chapter III), when valinomycin was added 2-4 hpi, RSV infection was almost completely inhibited (Figure 4.5C).
Figure 4.5: Ion perturbation inhibits early events of RSV infection.

(A) Ionophoric antibiotics or controls (DMSO or methanol) in serum free medium at indicated concentrations were mixed with \( \approx 10^5 \) PFU of RSV-GFP and incubated for 15 min in a 37°C water bath. The mixture was serially diluted and the residual virus infectivity determined in Hep-2 cells. Activated hydrogen peroxide (Virox™) was included as a positive control. RSV-GFP was quantified by end-point dilution at 24 hpi. Values represent mean ± SEM (\( n = 3 \) independent experiments) *** \( P \leq 0.001 \), ns \( P > 0.05 \); by one-way ANOVA with a Tukey's post hoc test for group comparisons (B) HEp-2 cells were pre-treated for 1 hour with indicated concentrations of ionophoric antibiotics or DMSO/methanol. After the preincubation period, RSV-GFP (in serum-free EMEM ± inhibitor) at an MOI of 1 was added to cells for 90 minutes. Heparin (1 mg/ml) was included as a positive control. Data is representative of reduction in GFP fluorescence normalized to DMSO treated cells. Values represent mean ± SEM (\( n = 3 \) independent experiments) ** \( P \leq 0.01 \), **** \( P \leq 0.0001 \); by one-way ANOVA with a Tukey's post hoc test for group comparisons. (C) Time-of-addition (ToA) and (D) time-of-removal (ToR) profiles for RSV inhibitors. Cells were infected with RSV-GFP for 90 minutes at an MOI of 1. For ToA assay, valinomycin (32 nM), 0 mM KCl medium, 5 mM LiCl medium, DMSO, or EMEM was added at the times indicated post RSV. For ToR assay, valinomycin (32 nM) or DMSO was added at 2 hours post infection. At indicated times, the medium was removed, and wells were washed before adding EMEM free of any compound. For both assays, RSV-GFP was quantified at 24 hours by epifluorescence.
microscopy. For valinomycin y-axis denotes reduction in RSV-GFP normalized to DMSO treated cells and for 0 mM KCl medium and 5 mM LiCl medium y-axis denotes reduction in RSV-GFP normalized to EMEM treated cells. Values represent mean ± SEM (n = 2 independent experiments).
When treatment was delayed to 6-8 hpi, inhibition of RSV was reduced. When treatment was delayed to 12 hpi, RSV infection was almost completely unaffected. We also examined the kinetics of low K+ medium or medium containing 5 mM LiCl and found that the addition of low- K+ medium (0 mM KCl) or 5 mM LiCl medium could be delayed until 8 hpi before any anti-RSV activity was lost (Figure 4.5C). Surprisingly, removal time of valinomycin did not significantly alter inhibition of viral infection despite being removed as early as 30 minutes after addition (Figure 4.5D). These results suggest changes in ion concentration exert antiviral effects at a postentry step but prior to (or during) viral replication. To test if ion modulation directly altered RSV-specific mRNA transcription and RNA replication we used an RSV replicon (artificial RSV minigenome assays, containing GFP as a reporter\textsuperscript{417}). In this way we were able to perform a focused evaluation of the RSV RNA polymerase independently from other stages of the RSV lifecycle. Our findings suggest that ion-modulating drugs do not significantly impact the RSV RNA polymerase (Table 4.1). These results are consistent with an antiviral ion dependent effect occurring post RSV entry but prior to viral RNA synthesis.

**Salinomycin failed to reduce RSV titers in a mouse model of RSV infection**

We examined the *in vivo* effect of salinomycin treatment in a mouse model of RSV infection. Pathogen free 6-8 week old female BALB/c mice were lightly sedated with isoflurane and infected intranasally with 5x10\textsuperscript{6} PFU of RSV on day 0. One day after inoculation with RSV, mice received PBS or 5 mg/kg of salinomycin i.p. once daily until sacrifice. All mice were monitored daily for changes in weight (Figure 4.6A).
Table 4.1: Activity of ionophoric antibiotic drugs in the RSV replicon assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>HeLa Replicon Level (IC₅₀ [µM])</th>
<th>Infectivity/Toxicity (HEp-2 [µM])</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IC₅₀</td>
<td>IC₅₀</td>
<td>CC₅₀</td>
</tr>
<tr>
<td>Valinomycin</td>
<td>&gt;10</td>
<td>0.0015</td>
<td>2.705</td>
<td></td>
</tr>
<tr>
<td>Salinomycin</td>
<td>&gt;10</td>
<td>0.1064</td>
<td>&gt;10</td>
<td></td>
</tr>
<tr>
<td>Monensin</td>
<td>&gt;10</td>
<td>0.02959</td>
<td>&gt;10</td>
<td></td>
</tr>
</tbody>
</table>

Data is averaged from the results of a minimum of 3 independent experiments.
In this model, mice lose approximately 10-15% of their original body weight by day 4, corresponding to peak viral titres\textsuperscript{390}, and begin to gain weight by day 6. On day 4, the peak of viral replication, the lungs were excised, homogenized, and a standard plaque assay was performed to obtain plaque-forming units as previously described \textsuperscript{44}. Salinomycin treatment had no significant affect on peak lung viral titres 4 days post infection (Figure 4.6B).

4.5 Discussion

Despite the multitude of drug candidates in development for the treatment of RSV infection, an affordable and effective therapy has yet to make it to the clinic. The repurposing of existing drugs for new indications reduces the time and cost for drug development prior to clinical use and is a common goal\textsuperscript{428-430}. The work herein provides evidence for the first time that ionophoric antibiotics (salinomycin, valinomycin, and monensin) reduce RSV replication. Therapeutic applications of ionophoric antibiotics are typically limited to the veterinary field, where they are widely used for improving cattle feed and the treatment of poultry coccidiosis\textsuperscript{451,452}.

In addition to their uses in the veterinary field, ionophoric antibiotics are also known for their activity as antifungal, antiparasitic, antiviral, and more recently antineoplastic agents. In the past, numerous reports have highlighted their activity against human cytomegalovirus\textsuperscript{453}, human immunodeficiency virus\textsuperscript{454}, human herpes viruses\textsuperscript{455,456}, poliovirus\textsuperscript{450}, rhinovirus\textsuperscript{457}, coronaviruses\textsuperscript{458}, and hepatitis C virus\textsuperscript{459}. In the case of herpes simplex virus, treatment appeared to inhibit the transport of progeny virus to the cell surface, while protein synthesis and DNA replication were unaffected\textsuperscript{455}. 
Figure 4.6: Salinomycin fails to reduce RSV titers in lungs of infected mice.
BALB/c mice were infected i.n. with $5 \times 10^6$ PFU of RSV on day 0. Mice received 5 mg/kg of salinomycin or PBS i.p. daily beginning on day 1 until sacrifice. (A) Weight loss of infected mice was monitored daily. (B) On day 4, the peak of viral replication, lung homogenates were analyzed by standard plaque assay for viral load ($n = 6$ mice per group) $ns \ P > 0.05$; by students t-test.
Work with rhinovirus found that valinomycin depolarized the host membrane, blocking viral fusion with endosomal membranes\textsuperscript{457}. These results are consistent with our findings, as valinomycin was unique among the ionophoric antibiotics tested in that it partially blocked RSV entry into host cells and removal time did not significantly alter inhibition of virus suggesting it may partially block fusion (Figure 4.5B & D). Our data for salinomycin, monensin and at least in part valinomycin indicate they exert their anti-RSV activity at a post-entry but prior to replication stage of the viral life cycle. The inhibition at this phase of the replication cycle is in accordance with several other viruses\textsuperscript{450,453,454,459}.

Our data show that salinomycin, valinomycin, and monensin possess \textit{in vitro} nanomolar activity against RSV in HEp-2 cells and in primary nasal epithelial cells. This data indicates that, like cardiac glycosides, the anti-RSV activity of ionophoric antibiotics is dependent on the movement of Na\textsuperscript{+} and K\textsuperscript{+}. Importantly we also show that RSV is highly sensitive to the ionic strength of the culture medium. The selectivity and anti-RSV activity may depend on the ability of ionophoric antibiotics to induce K\textsuperscript{+} efflux from the cytosol with a consequent increase in the influx of Na\textsuperscript{+} and water\textsuperscript{447}. Little is known about the ionic milieu of RSV infected cells, however several reports have indicated that within minutes of RSV contacting the apical membrane there is a decrease in the amiloride-sensitive Na\textsuperscript{+} transport by the epithelium leading to a decrease in intracellular Na\textsuperscript{+} both \textit{in vitro} and \textit{in vivo}\textsuperscript{460-462}. Perturbation of the cation gradient and/or content in RSV infected cells may be responsible for the anti-RSV effects of ionophores. A similar mechanism has been proposed to explain the activity of ionophoric antibiotics against several parasites\textsuperscript{463-465}.
The pharmacokinetic properties of salinomycin and other ionophoric antibiotics have been well characterized in several animal models. Given most ionophoric antibiotics are highly lipophilic, they are well absorbed and distributed throughout the plasma and tissues\(^{466}\). In mice, 5 mg/kg intraperitoneally was well tolerated, and no abnormalities were observed in organ histology of liver, kidney, skeletal muscle, and heart after daily salinomycin injection for 4 weeks. Pharmacokinetic evaluation revealed a peak plasma concentration of 1.72 µM and an almost complete systemic elimination within 5 h after injection\(^{467}\). This relatively fast systemic elimination may explain the lack of efficacy in the RSV mouse model when given once daily for 3 days. Further animal studies testing alternative dosing routes and regimens following RSV infection will be critical in determining the efficacy of salinomycin and other ionophoric antibiotics \textit{in vivo}.

Ionophore toxicity has been reported in animal cases of accidental high-dose ingestion\(^{468,469}\). In non-target species, such as calves, cats, and horses, ionophoric antibiotics have been reported to cause neurotoxicity and cardiotoxicity after accidental exposure\(^{470-473}\). However, no appreciable risk for humans was demonstrated following the consumption of products from animals with low-level contamination of ionophoric antibiotics\(^{474}\). Human preclinical pharmacological and safety data remains scarce. The finding that salinomycin and other ionophores inhibit cancer stem cell growth has prompted the compassionate use of salinomycin for cancer patients. In a pilot study of metastatic cancer patients, 200 µg/kg of intravenous salinomycin given every 48 hours for 12-14 days was well tolerated and resulted in clinical regression of therapy resistant cancers\(^{475}\). In our assays, valinomycin appeared to be the most toxic for HEp-2 cells among the ionophoric antibiotics tested and both salinomycin and monensin showed no
significant toxicity at the highest tested concentrations. The narrow therapeutic index of salinomycin as an anticancer drug has already sparked the development of chemically modified derivatives and could possibly represent promising new anti-RSV therapies\textsuperscript{466,476-478}.

In conclusion our data strongly suggests a potent activity of ionophoric antibiotics against RSV. This data also suggests they exert their anti-RSV activity through the perturbation of Na\textsuperscript{+} and K\textsuperscript{+} ionic gradients in infected cells, suggesting targeting host cellular ions may be a promising strategy to control RSV.

**Author Contributions:**
M.J.N. conceived of, performed, and planned all experiments, analyzed the data and wrote the paper. M.M. performed initial high throughput screening assay and identified high potential hits. W.D. performed initial high throughput screening assay, assisted with plaque assay in Figure 3.6., provided guidance on statistics and edited the paper. H.O. expanded and maintained the primary human nasal epithelial cell cultures. A.G. performed qPCR on clinical isolates in Figure 3.4. J.G. provided the RSV clinical isolates and assisted with the qPCR quantification. J.M. conceived of and supported the initial high throughput screen. T.J.M. conceived of \textit{in vivo} and \textit{in vitro} experiments, analyzed data, wrote and edited the paper, supervised and provided support for the project.
Chapter V

5. General Discussion and Future Directions

The overall goal of this thesis was to identify novel RSV therapeutics. RSV is a common and significant pathogen that lacks effective and specific management strategies. The work presented in this thesis explored two potential strategies for the treatment of RSV. First, building on the recognized critical role of CD8 T cells in antiviral defense, I described the utility of delivering an antibody to enhance CD8 T cell costimulation. This strategy did enhance T cell numbers in the setting of RSV infection, but did not reduce RSV titres in the lung. I determined that the lack of beneficial response was associated with a failure of this strategy to enhance RSV specific CD8 T cells in the lung and concluded that caution will be required if similar approaches are considered in humans. Second, after analyzing the results of a high throughput screen, we identified that cardiac glycosides inhibit RSV replication. My work suggests that the effects of glycosides occur very early in the life cycle of RSV after viral entry and before genome replication. Third, I found that modulation of cellular potassium and sodium more broadly reduces RSV replication. This work explored novel strategies for modifying RSV replication and has the potential to inform our understanding of RSV infection and its management.

5.1 Strategies to Boost Host CD8 T Cells

CD8 T cells are critical in the control and clearance of viral infections. Strategies that enhance CD8 T cell responses may therefore prove to be effective therapeutic modalities against RSV. One such strategy is the exploitation of CD8 T cell costimulation
to enhance primary CD8 T cell responses against viruses. Antibodies that activate these costimulatory signaling pathways can enhance the expansion of virus specific CD8 T cells\textsuperscript{377,479}. The findings of our study with agonistic 4-1BB antibody suggested that administration at the time of primary RSV infection does not influence morbidity from RSV in a favorable manner. There are a few important considerations when viewing these results.

First, this work was not aimed at documenting the importance of 4-1BB signaling in RSV, but rather was focused on enhancing signaling through the 4-1BBL pathway in order to improve outcomes (reduce viral titres, reduce airway hyperresponsiveness). The lack of requirement for 4-1BB signaling could, however, explain the lack of efficacy seen against RSV. Inbred laboratory mouse strains are the most widely used model of RSV infection, however they are at best semi-permissive hosts. Even the most permissive mouse strain, BALB/c, requires a very high intranasal inoculum to achieve mild infection\textsuperscript{365}. During infection with influenza A HKx31 (X31), mice lose about 10\% of their weight, but clear the virus within 8 days, highly reminiscent of RSV infection. In this mild influenza infection, 4-1BB is only transiently upregulated on the virus-specific T cells, and 4-1BBL is dispensable for the primary CD8 T cell response and viral clearance\textsuperscript{382}. Additionally, 4-1BB/L costimulatory signals are completely dispensable for the primary CD8 T cell response to both murine gamma herpes virus (MHV-68) and vaccinia virus (VV)\textsuperscript{385,480}. It has been suggested that the requirement for 4-1BB costimulation during acute viral infection may depend on antigen load, the induced costimulatory ligand expression, the pattern recognition receptors triggered, and/or the induced inflammatory milieu. It is feasible that under the particular experimental
conditions tested here, RSV infection of BALB/c mice did not sufficiently achieve one or more of these requirements. We did not specifically study 4-1BB -/- or 4-1BBL-/- mice; other investigators have used these knockout mice to demonstrate the critical role of 4-1BB signaling in establishing a CD8 response to viral infection. It is possible that the 4-1BB/L signaling pathway is completely dispensable during RSV infection, perhaps owing to minimal expression of the receptor or due to compensatory costimulatory pathways. For instance, costimulation via CD28-B7.1/2 is essential for the induction of virus specific CD8 T cells in primary responses in several murine models of acute viral infection including those caused by influenza A virus, vesicular stomatitis virus, and vaccinia virus. Future studies should include these knockout mice to determine the role 4-1BB signaling plays during primary RSV infection as well as investigating other compensatory costimulatory pathways.

Second, the timing of 4-1BBL signaling is relevant to the outcome; delivering the signal a few days before or after the viral infection (or antigen) may change the response seen. We chose the current model to better mimic a clinical situation where an infant or adult would present with a current infection and then may receive treatment in close temporal proximity to infection. It has been previously reported that 4-1BBL stimulation may provide a better boost to secondary responses as opposed to a primary response. Future work will focus on testing if the 4-1BB pathway can improve outcomes in RSV when used in a secondary response.

Lastly, uncoupling of antigen from the 4-1BB costimulatory signal may lead to a less specific effect of 4-1BB signaling. Previously, 4-1BBL has been coupled to antigen in the form of viral vectors. In this setting, antiviral CD8 T cell production is enhanced.
In the current model, coupling RSV to 4-1BBL would require modification of the infecting virus and is not a relevant model clinically. Thus the effect of non-coupled stimulation was investigated. The current work suggested that 4-1BB agonist antibody may reduce the numbers of some antigen specific CD8 T cells. During primary infection with influenza virus, anti-4-1BB agonist antibody appears to increase subdominant CD8 T cell responses to the virus\textsuperscript{479}. Under the conditions tested here, it is possible that non-specific, or subdominant CD8 T cells responses were induced by anti-4-1BB agonist antibody. If this antibody reduces the development of RSV specific T cells, this may lead to a worse outcome with infection. Future work will look at coupling the 4-1BB signal to an M282-90 peptide representing an immunodominant CD8 epitope in an attempt to avoid non-specific responses.

5.2 Strategies to Target Host Intracellular Ion Homeostasis

In the combined work of Chapters 3 and 4, we provide evidence that cardiac glycosides and ionophoric antibiotics reduce RSV replication. We further demonstrate that antiviral effects of cardiac glycosides and ionophoric antibiotics are dependent on Na\textsuperscript{+} and K\textsuperscript{+}. We also show that alterations in cellular Na\textsuperscript{+} or K\textsuperscript{+} independent of these drugs result in inhibition of RSV.

Direct use of digoxin or ionophoric antibiotics in humans for viral infections may not be feasible given concerns over potential toxicity. Thus, it is critical to understand the mechanisms underlying the observed Na\textsuperscript{+} and K\textsuperscript{+} ion dependence as this understanding may lead to novel antiviral approaches. We propose that RSV requires a specific ion composition to complete steps in the viral replication cycle and coordinate the functions of specific viral proteins present in the virion. Evidence for such an ion requirement is
supported by RSV internalization studies demonstrating that ethyl-isopropyl amiloride (EIPA), a potent inhibitor of the Na\(^+\)/H\(^+\) exchanger, inhibits RSV infection by 95%. As RSV entry is pH-independent, this finding suggests a high Na\(^+\) content in early macropinosomes is detrimental to RSV\(^{50}\). Additionally, several groups have documented that within minutes of RSV contacting the apical membrane there is a decrease in the amiloride-sensitive Na\(^+\) transport by the epithelium leading to a decrease in intracellular Na\(^+\) both in vitro and in vivo\(^{460-462}\). Furthermore, the SH protein of RSV is a viroporin forming pentameric, cation-selective channels allowing Na\(^+\) and K\(^+\) across infected cell membranes possibly leading to changes in cytosolic or endosomal Na\(^+\)/K\(^+\) levels\(^{345,445,446}\). Viroporins are a class of viral proteins that enhance cell membrane permeability in order to favor viral replication\(^{487}\). Our findings suggest that intracellular ions contribute to virus uncoating and/or events prior to the formation of RNA replication factories, as there was a lack of inhibition in the RSV replicon.

The reliance upon host cell ions at a post-entry stage is consistent with observations for other negative sense enveloped viruses. Post-entry uncoating of influenza virus, a segmented single stranded negative sense RNA virus, is dependent upon the influx of K\(^+\) ions into the maturing endosome for destabilization of the viral core \(^{488}\). Moreover, several screens for host proteins that support influenza infection have found cellular K\(^+\) channels to be important for viral replication\(^{489,490}\). In addition to K\(^+\) requirements, influenza, like RSV, inhibits amiloride-sensitive Na\(^+\) channels upon infection\(^{491,492}\). Influenza replication is enhanced when these Na\(^+\) channels are closed and replication is reduced when Na\(^+\) channels are open\(^{493}\). More recently, it was shown that members of the Bunyaviridae family, a group of negative sense RNA viruses, require the
activation of $K^+$ channels within the first 6 hours of infection consistent with early events in the virus life cycle such as virus uncoating. Interestingly, this study also included RSV as an unrelated negative sense RNA virus to determine if $K^+$ efflux was a requirement for infection; tetraethylammonium (TEA), a broad spectrum $K^+$ channel blocker, did not affect RSV infection. This is consistent with our findings as TEA should maintain a high intracellular $K^+$ concentration, which our results indicate is required for early events in the RSV infection cycle. Interestingly, in our initial screen for anti-RSV drugs, 10 µM diazoxide, a broad spectrum $K^+$ channel activator, reduced RSV infection by 22% (data not shown), further suggesting $K^+$ efflux can inhibit RSV.

Currently, little is known about the RSV uncoating process. After macropinocytic uptake, a second cleavage in the RSV F protein by a furin-like protease provides the cue for penetration by a pH-independent membrane fusion mechanism. After fusion, uncoating is required to release the genome into the host for viral trafficking, transcription, and replication. This process is often highly dependent on host “cues” such as receptor- and/or enzyme-based cues, chemical cues, or mechanical cues. Within the RSV virion, the viral RNA is encased in the nucleocapsid protein (N) forming the helical nucleocapsid. The polymerase (L), phosphoprotein (P), and transcription regulatory protein (M2-1) are bound to the nucleocapsid forming the ribonucleoprotein complex (RNP). The RNP, surrounded by the matrix protein (M), is connected to the membranous envelope in which the viral glycoproteins F, G, and SH are inserted. It is clear that the M protein interactions with each other and with the RNP complex must be dissolved for successful infection. It is tempting to speculate that while residing in macropinosomes, $Na^+/K^+$ ions pass through the viral ion channel SH into the virus lumen and there act to
separate the viral nucleoprotein by inducing a conformational change in the M protein. Interestingly, K\(^+\) has been found at the interface of the RSV M protein dimerization\(^9\), however is absent from the monomer\(^63\). This suggests that the interaction of the RSV matrix protein could be K\(^+\) specific and that K\(^+\) binding could function to stabilize the RSV matrix protein in a specific orientation or oligomeric state. Perhaps, like influenza\(^488\), changes in Na\(^+\) or K\(^+\) act to “prime” the viral core for uncoating by weakening the interactions between M proteins or interactions between M proteins and RNPs or both. A schematic representation of this hypothesis is illustrated in Figure 5.1.

Future studies will focus on the possibility that modulation of intracellular Na\(^+\) and K\(^+\) impact RSV uncoating. This will be investigated in several ways. Initial studies will include in vitro uncoating assays with varying concentrations of NaCl and KCl as previously described\(^488,495\). Briefly, purified RSV will be loaded on a 2-layer glycerol gradient with NP-40 on the bottom layer to remove the viral envelope. Total salt concentration will be 135 nM but the KCl and NaCl input will be varied. After ultracentrifugation, the pellet will be resuspended and subjected to SDS-PAGE for detection of viral protein. If the matrix-RNP complex remains intact, it will form a pellet and viral proteins will be detected. If the matrix-RNP complex dissociates, a pellet will not form and proteins will not be present. We will thus determine how ion concentrations affect RSV uncoating. It is possible that results from this assay may not be representative of what happens during the course of an infection. Therefore in a second approach, we will study virus infected cells by immunofluorescence and examine colocalization of the matrix protein with the RNP complex over time post infection with and without cardiac glycosides or ionophoric antibiotics present.
Figure 5.1: Proposed mechanism of inhibition of RSV uncoating.

(A) After macropinocytic uptake of RSV, changes in the ionic composition of the endosomal lumen occur along the endocytic pathway. The high concentrations of Na⁺ decrease along the endocytic pathway, while the amount of K⁺ ions moderately increase from that found extracellularly. This increase in K⁺ ions enter the viral lumen through the SH protein and induce a conformational change in the RSV matrix protein causing disruption of M-RNP interactions thereby priming the virion for uncoating after fusion. (B) With the addition of cardiac glycosides or ionophoric antibiotics the endosomal compartment does not increase in K⁺ ions and subsequently has an increase in Na⁺ ion levels. Na⁺ ions may stabilize the matrix protein or RNP in an oligomeric state thereby preventing the dissolution of M-RNP interactions and blocking uncoating after fusion.
As uncoating progresses, colocalization should be reduced. To address the possibility that Na$^+$ or K$^+$ changes can effect the stabilization of matrix proteins in a specific oligomeric state, we will perform fluorescence based thermo shift assays on purified M proteins$^{496}$. Varying concentrations of KCl or NaCl will be used and the respective protein melting temperature determined.

Although our data suggests modulation of intracellular ions has a direct inhibitory effect on the viral life cycle, we cannot rule out other effects on the host cell that may be inhibitory to the virus. Recent evidence has suggested that the Na$^+$/K$^+$-ATPase is a versatile signal transducer and binding of cardiac glycosides can activate multiple downstream signal transduction pathways$^{497}$. In addition, the inhibition of Wnt signaling by salinomycin may be involved in the inhibition of cancer stem cells and human cytomegalovirus replication$^{453,498,499}$. Given this multiplicity of effects of both cardiac glycosides and ionophoric antibiotics, we performed an initial proteomic analysis of RSV infected HEp-2 cells ± ion modulating drugs (digoxin, salinomycin, valinomycin, and monensin) using isobaric 10 plex TMT-MS (Tandem Mass Tag- Mass Spectrometry, ThermoFisher with SPARC - SickKids Proteomics, Analytics, Robotics & Chemical BioCentre). Based on our time of addition assays, we examined cells 6 hours post RSV infection and compared this to uninfected controls. With 10 channels and 2D analysis, ~8000 proteins were quantified and then analyzed by Scaffold Qplus (Proteome Software, Inc.). In RSV infected cells we identified a total of 36 proteins that are differentially regulated by ≥ 2 fold (24 upregulated and 12 downregulated) relative to uninfected controls. From this, we identified 19 proteins that returned to reference levels with cardiac glycoside or ionophoric antibiotic treatment (Figure 5.2).
Tandem mass tag mass spectrometry was used to identify a total of 36 proteins differentially regulated by ≥ 2 fold (24 upregulated and 12 downregulated) in RSV infected HEp-2 cells relative to uninfected controls. From this, 19 proteins were identified that returned to reference levels with ion modulating drug treatment.

**Figure 5.2: RSV infection alone or in the presence of ion modulating drugs affects the expression of host cellular proteins 6 hpi.**

Tandem mass tag mass spectrometry was used to identify a total of 36 proteins differentially regulated by ≥ 2 fold (24 upregulated and 12 downregulated) in RSV infected HEp-2 cells relative to uninfected controls. From this, 19 proteins were identified that returned to reference levels with ion modulating drug treatment.
Additionally, we further identified 10 proteins that are minimally changed with RSV infection however are upregulated by $\geq 2$-fold in the presence of cardiac glycosides or ionophoric antibiotics (Figure 5.3).

Due to the complexity of cardiac glycoside and ionophoric antibiotic-mediated effects on cells, it is possible multiple mechanisms may contribute to the inhibition of RSV. Changes in intracellular ion concentration may impact multiple cell processes leading to a reduction in viral replication. These pathways may be novel antiviral targets. Our proteomics results identified several proteins demonstrating a change in expression with RSV infection that returned to reference levels with ion modulating drug treatment. D-dopachrome tautomerase (D-DT) (Accession Number P30046) was one of the top 5 proteins upregulated in RSV infection ($\geq 4.1$ fold), and drug treatment in the setting of RSV reduced protein expression to uninfected levels. D-DT is a homolog of the macrophage migration inhibitory factor, MIF-1, and has diverse pro-inflammatory functions relevant to viral pathogenesis including a role as an alarmin and inhibiting apoptosis$^{500}$. Additionally, of the 24 upregulated proteins showing $\geq 2$-fold increased expression in infected cells that were simultaneously reduced to baseline levels with drug treatment, 3 of them were subunits of the proteasome (Accession Numbers P49720, P25789, P49721). The proteasome is known to play a role in viral infections and is not simply activated as a stress response$^{501}$. The idea of modulating proteasome activity in humans for clinical benefit is an area of active investigation$^{502}$. In addition to upregulated expression, RSV infection also resulted in reduced expression of proteins compared to uninfected reference cells.
Figure 5.3: Ion modulating drugs upregulate numerous host proteins 6 hours post RSV.
Tandem mass tag mass spectrometry identified a total of 10 proteins that are minimally changed with RSV infection however are upregulated by ≥ 2-fold in the presence of ion modulating drugs.
The voltage dependent L-type calcium channel (Accession Number Q13936-10) was reduced 2-fold with RSV infection and levels were actually increased by over one fold with drug treatment. Viruses are well known to appropriate or interrupt both Ca$^{2+}$ signaling pathways and other Ca$^{2+}$-dependent processes for replication$^{503}$. Interestingly, increasing cytosolic Ca$^{2+}$ has recently been shown to be detrimental to RSV replication$^{357}$. Lastly, drug treatment was shown to increase the expression of several proteins in the setting of RSV that remained unchanged during RSV infection alone. Thymosin $\beta$-10 (Accession Number P63313) was increased by 2.8-fold by drug treatment of RSV infection. Interestingly, Thymosin $\beta$-4 (Accession Number P62328) was reduced 2.5 fold with RSV infection and levels were restored to baseline with drug treatment. These proteins play a role in regulating actin polymerization, a process modulated by intracellular pathogens$^{504}$, and their overexpression has been linked to apoptosis$^{505}$.

Future work will aim to analyze and validate our initial proteomic data. We will then employ parallel reaction monitoring (a focused form of TMT-MS to quantify specific proteins) and western blots with time course analyses and varying doses of virus and drug to examine the effect of these proteins and pathways in viral infection. Once confirmed that drug treatment alters a specific host protein/pathway in the setting of viral infection, we will manipulate this selected protein/pathway with siRNA or small molecule inhibitors/activators/analogues to measure the impact of these identified factors on RSV replication in the presence and absence of drug. As an example, D-dopachrome tautomerase (D-DT) was upregulated over 4-fold during RSV infection, and drug treatment (both cardiac glycoside and ionophoric antibiotics) in the setting of RSV reduced protein expression to uninfected levels. First, we will deliver recombinant D-DT
in the setting of drug treatment. D-DT should reduce antiviral effects if antiviral drug effects operate through inhibition of D-DT. Second, the role of D-DT in viral infection will be tested by treating infected cells with a commercially available D-DT inhibitor or siRNA. Thus, we will test the hypothesis that drug mediated antiviral effects in part operate through D-DT inhibition.

In conclusion, RSV remains an important pathogen in young infants and children as well as in the elderly. No vaccines or antivirals are commercially available to prevent or treat RSV infection. The results of our work suggest that the anti-RSV effects of cardiac glycosides and ionophoric antibiotics are primarily dependent on changes in the intracellular Na$^+$ and K$^+$ composition. Although safety concerns must be addressed before these drug classes could be used clinically to treat RSV infection, they also remain useful as further mechanistic work may lead to development of novel antiviral drugs and may shed light on the role intracellular ions play during RSV infection. Modulation of intracellular ions, such as Na$^+$ and K$^+$, represent an innovative approach to reduce RSV propagation and has the potential to be utilized as a new therapeutic modality to treat RSV infection.
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