Therapeutic efficacy of antiviral compounds in the neonatal lamb model of respiratory syncytial virus infection of infants with pathogenesis of secondary Streptococcus pneumoniae infection

Sarhad Alnajjar
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Therapeutic efficacy of antiviral compounds in the neonatal lamb model of respiratory syncytial virus infection of infants with pathogenesis of secondary *Streptococcus pneumoniae* infection

by

Sarhad S.A. Alnajjar

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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Major: Veterinary Pathology

Program of Study Committee:
Mark R. Ackermann, Co-major Professor
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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa
2019

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DEDICATION

This dissertation is dedicated to:

My wife
My family
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ABSTRACT

Respiratory Syncytial Virus (RSV) is one of the most common causes of acute lower respiratory tract infection in humans that can cause severe infections in infants and the elderly. It is estimated that annually 33 million lower respiratory tract infections in children were associated with RSV worldwide. However, in the US alone there were 75000-125,000 hospitalizations due to RSV in infants per year. Although most RSV infections of the lower respiratory tract are caused by RSV alone, occasionally, secondary bacterial infection further increases lung damage and disease severity. Despite the widespread infections by RSV throughout the world, till now there are no approved vaccines or therapeutic compounds to treat RSV infection other than Ribavirin and prophylactic administration of Palivizumab. There are several animal models of RSV infection, but neonatal lambs infected with RSV have several advantages for modeling RSV infection in infants such as similarity in pulmonary architecture, immune response, and respiratory tract size, viral replication, and lesion development.

To further extend the neonatal lamb model to evaluate secondary bacterial infection associated with RSV, lambs coinfected with RSV and Streptococcus pneumoniae were used to determine feasibility, susceptibility and disease development. Lambs developed severe disease when coinfected with both microorganisms with more severe suppurative bronchitis and pneumonia. Streptococcus pneumonia infection enhanced the severity of RSV in lambs when lambs were coinfected with both microorganisms.

To evaluate the efficacy of some antiviral compounds, two antiviral compounds efficacy were tested in the neonatal lamb model of RSV infection. First, three regimens of
JNJ-49214698, a small molecule RSV fusion protein inhibitor were tested (prophylactic, early treatment, late treatment). JNJ-49214698 prevented RSV infection when given before infection and reduced RSV induced lung lesion when used to treated established infection. Additionally, late treatment at day 3 post RSV infection had a wide window for RSV treatment. Secondly, 3 doses of RSV-NFI, an RSV non-fusion inhibitor were tested for efficacy. RSV-NFI was well tolerated and reduced RSV induced lung lesions and viral titer.
CHAPTER 1: GENERAL INTRODUCTION

Statement of the Problem

Respiratory Syncytial Virus (RSV) is one of the leading cause of acute lower respiratory tract infection in infants. Each year there are about 33 million lower respiratory tract infection cases in children under the age of five around the world and these often lead to significant hospital admission and mortality[1]. Additionally, RSV is one of the leading cause of pneumonia in older adults and high-risk (e.g., immunosuppressed) individuals [2,3]. RSV infections can occur year round but peaks in the winter months [4,5]. RSV has similar hospitalization rate and disease burden to influenza in elderly[3]. Secondary to RSV infection, about 40% of RSV infected children develop co-infections with bacteria or at increased risk of secondary bacterial pneumonia [6]. Furthermore, RSV infection in the early childhood may have delayed sequelae such as airways hyperreactivity, wheezing, and asthma in later childhood [7,8]. There is no vaccine or antiviral agent available clinically against RSV except for prophylactic humanized monoclonal antibody Palivizumab which has questionable efficacy and is expensive and Ribavirin [9,10].

There are many investigators developing new vaccines and therapeutic compounds for RSV and many of these are in advanced human clinical trials. Some newly developed vaccines under investigation provide an acceptable level of protection against RSV [11,12]. However, formalin-inactivated vaccines developed in the 1960’s led to enhanced disease and even death upon RSV infection in infants. Therefore, there are fears of adverse side effects of any new RSV vaccination regimen [13]. Newly developed anti-RSV therapeutic compounds that have reached advanced clinical trials include those targeting several critical viral components such as RSV fusion protein, N protein, L protein, SH protein, and M2-1 protein, and are from four chemical classes including:
anti-RSV immunoglobulins, siRNA-interference, fusion inhibitors, and small molecule inhibitors [14,15]. These therapeutic strategies require animal models to evaluate their efficacy.

Creating animal models for RSV investigations is challenging. There are several animal models used to evaluate RSV infection, pathogenesis, and therapeutic efficacy and these include but are not limited to: chimpanzees, baboons, sheep, cotton rats, mice, ferrets, and cattle [16]. Of these, lambs have several features advantageous as a model of RSV infection of infants including susceptibility to the human strains of RSV, lung development, structure and cellular morphology, and lesion development and lesion composition [17,18].

**Specific Aims**

The goals of the studies done in this dissertation are to determine the extent to which RSV infection can alter the susceptibility of the lung to *Streptococcus pneumoniae* (*Spn*), and test the efficacy of novel anti-RSV compounds. The hypothesis is that RSV infection enhances *Spn* infection and/or vice versa by lung damage induced by either pathogen and that anti-RSV (fusion and non-fusion inhibitors) can reduce RSV infection and disease severity. This hypothesis was tested by: 1) a study in which lambs were infected with *Spn* 3 day after initial RSV infection (Chapter 2). 2) a study to test the efficacy of anti-RSV small molecule fusion protein inhibitor (Chapter 3). 3) a study to test the efficacy of anti-RSV non-fusion inhibitor (Chapter 4).

**Dissertation Organization**

This dissertation describes the pathogenesis of secondary bacterial pneumonia in the neonatal lamb model of RSV infection and two therapeutic approaches to RSV infection. The dissertation composed of five chapters with the 1st one as general introduction and literature review. Chapters
2, 3, and 4 are composed of the three individual manuscript prepared for the peer review journal submission, with the final chapter (Chapter 5) as the conclusion and future direction.

The first paper Spn infection in RSV infected neonatal lambs (Chapter 2) were submitted to the journal Emerging Microbes and Infection. The second paper, Therapeutic efficacy of JNJ-49214698, an RSV fusion inhibitor, in RSV-infected neonatal lambs, were partially published in the journal Nature Communications, and the full manuscript was submitted to Frontiers in Microbiology. The third manuscript, therapeutic efficacy of RSV-NFI in RSV infected lambs will be submitted to a journal publishes work on antiviral compounds at a later time.

**Literature Review**

**Respiratory Syncytial Virus Structure and Strains**

RSV is an enveloped virus with non-segmented negative-sense single-stranded RNA within the family of Pneumoviridae, genus Orthopneumovirus, [19,20]. The viral RNA is composed of 10 genes that encode 11 protein two of them non-structural protein. In general, Orthopneumovirus have three externally protruded glycoproteins, the attachment (G), fusion (F), and small hydrophobic (SH). These glycoproteins pin the outer lipid envelope obtained from the host cell plasma membrane and attached to the underlining matrix (M) protein. The G protein has carboxy-terminal located to the outside of the virion and is variable among RSV strains. The G protein mediates viral attachment to cellular membrane. The F protein has amino-terminus oriented to the outside of the viral envelope and is less variable between strains than the G protein and responsible for the viral fusion and entry into the target cell. These two proteins are critical for the viral replication and infection, although virions lacking G protein can penetrate and replicate in cell culture, but less efficiently than virion having both G and F protein[21]. The G protein is heavily glycosylated and attaches to glycosaminoglycans on the host cell surface[22]. Most neutralizing
antibodies are directed against G protein. Therefore, RSV has an immune evasion strategy by which a secreted form of G protein (sG) is produced in order to avoid inhibition by neutralizing antibody [23–25]. On the other hand, F protein is highly conserved, and upon attachment and activation, F protein undergoes conformational changes leading to fusion of the virus to the target cell membrane, which subsequently induce internalization of the virus into the cell cytoplasm [26–28]. Interestingly, one study demonstrated that RSV internalized intact into the epithelial cell by macropinocytosis initiated by the RSV attachment. This process leads to RSV virion to be present within intracytoplasmic fluid-filled macropinocytosome, then followed by F protein cleavage and viral fusion that leads to internalization of RSV to the cytoplasm and infection [29]. SH protein function and location is not fully characterized [30,31]. However, SH protein was demonstrated to have an antiapoptotic activity by inhibiting TNF-α signaling [32]. Another study demonstrated that SH protein is a small hydrophobic protein (∼100 amino acid) called Viroporin, which enhance infected cell permeability by forming a hydrophilic pore in the infected cell membrane [33]. The non-segmented single-stranded RNA associated with five structural proteins, the first 3 are nucleoprotein (N), large (L) protein, and phosphoprotein (P) to form the helical nucleocapsid. The L protein considered the RNA dependent RNA polymerase used in viral genome replication. Additionally, L protein responsible for the transcription of the positive sense viral mRNA, and possess capping enzyme activity at the 5’end and polyadenylation at the 3’ end of the viral mRNA [34]. N Protein forms a complex with the RSV RNA called ribonucleoprotein complex that acts as a template for the L protein, while P protein serves as a link between L and N protein to facilitate efficient and specific recognition of ribonucleoprotein complex by L protein [35]. The other two proteins associated with the nucleocapsid are the M2-1 and M2-2 proteins which are transcriptional
enhancer proteins [36]. These proteins bind to the RNA and P protein to prevent premature termination of transcription [37,38].

There are two RSV strains: an A and a B strain which differ substantially in the G protein and noncoding portion of the genome but have less variability in the other structural proteins[39,40]. These two RSV strains co-circulate clinically throughout the year with the dominance of A strain. Furthermore, RSV group A causes more severe infection than group B RSV [41–44].

Disease Burden

RSV is a common respiratory disease affecting all ages and especially children worldwide [1,45,46]. In most infected individuals, RSV causes mild to moderate upper respiratory tract infection characterized by fever, nasal congestion, cough and rhinorrhea that persists for several days [47]. However, RSV can lead to severe acute lower respiratory tract infection (LRTI) in infants, elderly and immunocompromised individuals [48–50].

All infants eventually become infected by RSV, and it is estimated that there were 33.8 million cases of RSV associated pneumonia worldwide in children under the age of five in 2005 [1]. Furthermore, about 3.4 million RSV associated pneumonia cases required hospital admission with 66,000-199,000 resulted in mortality. Most cases of mortality occur in developing countries [1]. Based on US National viral surveillance data from 1990-1999, RSV associated deaths in infants was nine times the number of deaths related to influenza infection in the United States and RSV was the second leading viral cause of death after influenza in children age 1-5 years and older adults [51]. While the rate of RSV associated hospitalization is 30 per 1000 in the US, Japan reported 60 hospital admissions per 1000 [47]. RSV seasonality is consistent throughout Europe which accounts for 42-45% hospital admission due do LRTI [47]. In Belgium alone and during
2000 season, RSV was associated with 63% of acute LRTI in children under the age of 5 [52]. Another study in England and Wales concluded that RSV was associated with 60-80% mortality more than that of influenza in a total of 15 winters from January 1975 to December 1990 [53]. In Australia, a retrospective study of 3 RSV seasons in 1997-1999, concluded that RSV is a significant cause of morbidity and low mortality with 11.4% of infants required admission to intensive care unit [54]. In general, temperate reigns experience RSV year round with a peak in winter months, while a specific RSV seasons were seen in tropical regions [55]. RSV considered an important nosocomial agent since RSV aerosolized particles persist for an extended time in the urgent care clinic and can be inhaled and infect other patients [56]. However, several risk factors determine the severity of RSV infection in infants.

RSV infection has considerable morbidity and mortality in infants less than 3 months and those having one or more risk factors [57]. There are several risk factors associated with the host include not limited to premature birth, congenital heart diseases, and chronic lung diseases [58,59]. However, a high proportion of infants hospitalized due to RSV are healthy without any involvement of any of these risk factors [60]. Other risk factors related to the host or the environment are gender (males), young age (less than 6 months), number of siblings, daycare attendance, and exposure to tobacco smoke[61–64]. Other risk factors that can contribute to the severity of RSV infection are related to the virus itself. There is a significant association between viral load the severity of infection [65–67]. In addition to the viral load, RSV type A is associated with more severe respiratory illness in compare to type B RSV [41,68,69]. Considering all the mentioned risk factors, RSV disease severity is an association of all these risk factors, and despite the massive burden of RSV disease, there are no fully satisfactory treatment or vaccine strategies available for RSV infection.
Only two therapeutic agents, Ribavirin and Palivizumab, were approved against RSV infection but both have limitations in efficacy. Ribavirin, a nucleoside inhibitor, developed in 1972 as a virostatic agent for both RNA and DNA viruses[70]. Even though Ribavirin had a positive impact in high-risk patients such as transplant and immune compromised patients [71,72], only marginal clinical benefits were seen in RSV lower respiratory tract infection in general patients. Also, Ribavirin has a degree of toxicity. Thus, Ribavirin is no longer recommended as anti-RSV treatment[73–75]. Palivizumab is a humanized monoclonal antibody against RSV F protein and thereby prevents/block virus fusion to the host cell as well as cell to cell fusion [76]. Palivizumab use is restricted to premature infants 32-35 weeks gestation due to high cost and is used as prophylactic treatment [77]. Adding to Palivizumab’s high cost, a Palivizumab-resistant variant of RSV have been isolated from Palivizumab treated patients [78]. Vaccination and vaccine development has been held back by the severe RSV infection which occurred in a 1960 vaccination trial in which majority of vaccinated infants had a vaccine-enhanced disease that require hospitalization with several fatalities [79]. Animal models for RSV infection is an essential step in the investigation of RSV pathogenesis and the search for new therapeutics and vaccines.

RSV causes respiratory tract lesions that include severe bronchiolitis characterized by epithelial cell degeneration and necrosis with areas of hyperplasia in response to the cell damage along with syncytial cell formation. The bronchiolar lumen becomes partially occluded by neutrophils, occasional macrophages, seroproteinaceous fluid, mucin, and cell debris. Lymphocytes infiltrate the airway adventitia. The virus also infects ciliated epithelial cells of the upper respiratory tract and bronchi. With time after infection, lesions can resolve. However, there can be increases in Goblet cells and increased residual mast cells and eosinophils.
Animal Models of RSV Infection

Animal models are needed to study RSV pathogenesis and evaluate new therapeutics and vaccines candidates. Animal models are considered the middle stage between tissue and cell culture studies in vitro and human clinical trials. However, developing animal models for RSV infections is very challenging due to the high degree of specificity of the hRSV to its natural host and lack of virulence in other species. The specificity of hRSV is due to F protein, while lack of virulence in other species is due to the inability to block interferon response [80,81]. Ideal RSV models need to mimic several RSV disease aspects such as clinical signs and symptoms, viral replication, upper and lower respiratory pathology, and immune response. Several animal models have been developed to address some of these aspects if not all, but each model has its strengths and limitations. In general, RSV animal models are either heterologous or cognate host-virus models. hRSV can infect and replicate in heterologous host-virus models such as chimpanzees, baboons, sheep, Cotton rats, ferrets and mice, while related Orthopneumovirus specific to the model was used in the cognate host-virus models such as murine pneumonia virus in mice model and bovine RSV (bRSV) in calves [16]. In this section, several animal models of RSV infection will be discussed concluding with the neonatal lamb model.

Chimpanzees seem ideal as RSV animal model due to anatomical similarity to human and since RSV isolated originally from chimpanzees with respiratory tract infection in 1956 [82]. hRSV able to infect and replicate in the nasal sinuses and upper respiratory tract epithelium, and induce disease symptoms similar to that found in human RSV associated upper respiratory tract infection [83,84]. The drawback of this model is that chimpanzees rarely develop RSV LRTI. There are also concerns with the substantial economic, ethical and emotional burden associated with the use of chimpanzees. However, vaccine studies have benefited from chimpanzees since
chimpanzees tend to develop anti-RSV neutralizing antibody and their immune response similar to that found in human [85,86]. Baboons have been used as well and are being bred in large numbers at Oklahoma State University for use in RSV studies and other disease conditions [87,88].

Although rodents are an excellent animal model for experimental studies, they are considered semi-permissive for hRSV replication, and need a large inoculum to induce mild to moderate RSV disease[89,90]. Clinical signs are difficult to interpret in rodents, and RSV induces only mild to moderate bronchiolitis and pneumonia[91,92]. Cotton rats are more permissive than mice for RSV replication and considered the standard model for testing RSV therapeutics[93]. Mice, however, have the advantage of wide variety of transgenetic mice and the availability of molecular markers. Furthermore, mice can be used as a cognate host-virus model by using murine pneumonia virus, which develop a disease in mice similar to RSV disease in human[94]. Murine pneumonia virus targets bronchiolar epithelium and lead to severe disease with marked respiratory disease correlate positively with the viral inoculum[94,95]. The drawback of murine pneumonia virus model of hRSV is the far phylogenetic distance between the two viruses [96]. The critical disadvantage of rodents as a model for RSV disease is the difference in lung anatomy, histology and immune response between human and rodents that subsequently question the translations of studies done in these models to human.

Another cognate host-virus model for hRSV is the bRSV in calves. bRSV induce a respiratory disease in calves similar to what seen in human RSV. bRSV is more closely related to hRSV than other non-RSV viruses and share about 38-41% homology on nucleotide level[97]. bRSV induces upper and lower respiratory tract infection in calves[98]. Furthermore, calves have similar lung anatomy and histology, i.e., the presence of pharyngeal and nasopharyngeal tonsils, the presence of ciliated pseudostratified epithelium and submucosal glands, and similar innate and
adaptive immune response to human [99,100]. The drawback of the calves bRSV model is the large size of calves that need special housing and handling. Also, calves are not susceptible to human strains of RSV. Thus, bRSV and hRSV are two distinct viruses that induce similar disease process in their natural target host.

Neonatal lambs infected with RSV has several similarities to a human infant advantageous for comparison. The most important criterion is that lambs are naturally susceptible to human, ovine and bovine strains of RSV. hRSV replicates mostly in the lower part of the respiratory tract of lamb lungs (bronchioles and bronchi) which models well bronchiolitis in infants and reduces airflow to alveoli. hRSV replicates well in neonatal lamb respiratory tract airways with a peak of viral replication at day 6 after intratracheal inoculation with A2 hRSV strain then declines with time [17,18]. However, another study using hRSV M37 strain showed that peak viral replication was at day 3 and persisted until day 6 post nebulization [101]. As in human infants, lambs have variable clinical signs associated with hRSV infection. Clinical symptoms vary from mild such as nausea, fever, reluctant to move, and reduce milk consumption to moderate and severe such as cough, wheezing, expiratory efforts. Signs of infection appear as early as 2 days post infection and progress till day 6 [17,101,102]. hRSV infected lambs develop lower respiratory tract infection characterized by moderate to severe bronchiolitis and interstitial pneumonia. There is modest thickening of alveolar septae due to edema, type II cell hyperplasia and, leukocytes infiltration in the alveolar wall, and neutrophils and macrophages infiltration into the alveolar and bronchiolar lumens. In addition, hRSV incites epithelial cell necrosis, hyperplasia of nearby epithelial cells and syncytial cell formation in lambs [103] as in infants.

Additionally, immunological response to hRSV in neonatal lambs were characterized by the Th1 proinflammatory response as it characterized by the elevated level of IFN-γ and TNFα
cytokine and decrease in the level of TGF-β and IL-10. Leukocyte recruitment to the lung were associated with IL-8, MCP-1, and MIP-1α increase in the lung [18]. IL-10 increased to the highest level in lambs lung at day 3 post-infection while other cytokine increase later in the course of the disease [101,104]. Lambs have many similarities to human infant that make it ideal for studying infant respiratory diseases. Lung development is similar between lambs and infants in terms of alveolarization which occurs prenatally, similar airways size and branching (dichotomous branching), presence of submucosal glands, and similar percent of club cells in the airways[105–108]. Lambs can be born preterm similar to human. Since there is no transfer of immunoglobulin from ewes to their lambs in utero, lambs can be colostrum deprived to avoid transmission of maternal immunoglobulin to the lambs. Finally, the size of lambs allows for better evaluation of the clinical signs, sampling, and lung evaluation.

**Immune Response to RSV Infection:**

Cell-mediated immune response guided by the proliferation of cytotoxic T cell is the preferred response to clear many viral infections, although both cell-mediated and humoral response needed for the ideal antiviral response. The innate immune response, however, has a huge effect in limiting RSV replication and respiratory infection in infants.

The innate immune response is a nonspecific response aiming to prevent and reduce initial infection to allow time for more specific acquired immune responses to develop. Innate barriers in the respiratory tract such as mucociliary system act to prevent attachment of RSV to the airway epithelial cell, which is the target cell for RSV. Additionally, in response to infection, infected or neighboring cell upregulate genes, and produce cytokines and antimicrobial peptides and proteins, which limit the microbial proliferation. There are several common cells in the bronchoalveolar tree: ciliated bronchial epithelial cells, club cells, non-ciliated cells of the bronchiolar epithelium,
and pneumocytes (Type II and Type I). These cells sense the presence of RSV through pattern recognition receptor most importantly TLR-2, TLR-4, TLR-6, TLR-7, TLR-8, Retinoic acid-inducible gene I-liKe receptor, and MDA-5[109,110]. Activation of the TLRs eventually leads to the production of cytokines that shape up the immune response and attract more immune cells to the lung. Such cytokines include IL-8, IL-10, and IL-6 that induced by TLR4 activation, RANTES, which produced in response to TLR3 activation, and IFN-α, IFN-β and IP10 production in response to RIG-I activation[109,111–114]. IL-8, which is a neutrophils chemoattractant, is produced in the respiratory tract during RSV infection and higher levels of IL-8 is associated with more severe RSV bronchiolitis in human[115,116]. IL-10 and IL-6 are associated with the Th2 response. RANTES is a chemoattractant for T cells and eosinophils and associated with severe RSV bronchiolitis and induction of allergic cellular response [117–119]. IL-10 is an anti-inflammatory cytokine produced by inflammatory cells mainly macrophages and elevated during acute RSV infection[120,121]. IFN-α and IFN-β are type I interferons that act on reducing viral replication and promote MHC type I upregulation and subsequently killing of viral infected cells. Although IFN-α is elevated in RSV infected infants, its level is markedly lower than influenza-infected patients[122–124]. That is may be due to the ability of RSV to resist Interferon through the function of NS1 and NS2 proteins [125–127]. IFNγ, which is type II interferon, is associated with Th1 response, and it is produced in part by natural killer and macrophages. Low level of IFNγ is associated with severe RSV lower respiratory tract infection [128]. On the other hand, high level of type III interferon, IFN-λ, is associated with increased RSV severity. Type III interferon has similar activity to type I IFN[129].

Mucus and fluid covering the respiratory epithelium contain other innate antimicrobial substances that act on deactivating microorganisms such as secretion of the submucosal glands
and antimicrobial peptides. Submucosal glands, which are present in both human and ruminant lung airways, secrete lactoperoxidase, lactoferrin, and lysozyme into the mucosal surface. Lactoperoxidase acts on thiocyanate and hydrogen peroxide present on the mucosal surface to produce oxythiocyanate which has antimicrobial activity against bacteria and viruses[130,131]. Antimicrobial peptides present in the respiratory system include alpha and beta-defensins, and cathelicidin. Significant antimicrobial proteins include surfactant protein A and D. Both SP- A and D are produced by type II pneumocytes and club cells into the airways lumen. Surfactant protein A and D are globular mannose-binding C-type lectins that bind to RSV F protein and enhance clearance[132–134]. A higher level of SP-A and SP-D are associated with severe RSV LRTI[135]. Beta-defensins are antimicrobial peptides secreted by respiratory epithelial cells either constitutively such as HBD1 or inducible upon infection of the cell such as HBD2, 3, and 4. Inducible HBD bind to RSV envelop preventing the virus from infecting the cell[136]. Sheep produce Sheep beta defensins 1 and 2. In contrast, the antimicrobial peptide Cathelicidin is secreted by leukocytes and stored in neutrophils and can be upregulated during viral infection[137]. All these innate immune responses are directed towards carbohydrate or other moieties rather than specific antigens and some are upregulated with RSV lower respiratory tract infection. In addition to the ability to deactivate RSV, some of these molecules act as a chemoattractant for other inflammatory and immune cells.

Several inflammatory cells are associated with severe RSV lower respiratory tract infection. However, neutrophils are the predominant inflammatory cell that increased with the RSV both in peripheral blood and in the respiratory system [138,139], and the increased number of neutrophils in blood correlated with the severity of the disease and the peak of the viral load[140]. These neutrophils are activated and producing neutrophil elastase[141], with neutrophils apoptosis and
NETosis to trap virions and prevent further spread of virions[142]. In contrast, eosinophils also are activated during RSV infection, but they are associated with the healing process[143]. Eosinophils are increased following some RSV infections and contribute to asthma development later in life and peripheral eosinophils are increased in children hospitalized for RSV LRTI along with an increase in the Leukotriene C4, eosinophil-derived neurotoxin, and eosinophil cationic protein in the respiratory tract [85,144,145]. Other inflammatory/innate immune response associated cells are macrophages, Natural killer cells, and Dendritic cells.

Although there is a predominance of neutrophil in the airway lavage fluid obtained from severe RSV infected infant, changes in other cell type can be associated with the disease outcomes[146,147]. Alveolar macrophages have an essential role in phagocytizing foreign bodies and microorganisms leading to the subsequent microbial killing and antigen presentation. In addition to the expression of RSV glycoproteins, alveolar macrophages associated with RSV infection express immune modulatory molecules such as HLA-DR, interleukin-1β, and TNF as a response to lung injury[148,149]. RSV immunoreactive staining were seen in alveolar macrophages in lambs infected with hRSV[150]. Similar to macrophages, dendritic cells (DC) internalize proteins, process and present antigen to other immune cells, and both conventional and plasmacytoid DC are recruited to the respiratory system early in RSV infection. These DC are activated and express a proinflammatory phenotype[151–153]. Most of these cells are nonspecific and respond to RSV infection to either eliminate the virus or stimulate other type of immune cells such as lymphocytes.

Lymphocytes are critical for RSV infection since they determine the magnitude and type of the acquired immune response. However, T lymphocyte number in the blood is decreased in patients with RSV associated LRTI, and the reduction in T lymphocytes number correlated with
the severity of RSV infection [138,154]. In contrast, there is an increase in the B lymphocytes number in circulation during severe RSV bronchiolitis [155]. Both T and B lymphocytes are needed to resolve RSV disease.

During RSV infection, there is a decrease in the number of all T lymphocyte population in the blood, which is more pronounced in infants, and these peripheral lymphocytes are not activated as is shown by the low level of expression of CD11a and CTL-4 markers[138,156,157]. Although there is a predominance of CD4 T cell in the BALF obtained from early severe RSV infected infants, there is a higher expansion of effector CD8 T cell with the course of infection[146,158]. Most research in RSV immunobiology suggests an imbalance in Th1/Th2 response, which determines the severity of RSV infection. While Th1 responses associated with cytotoxic T lymphocytes activation and IgG type immunoglobulin production result in resolution of RSV infection, Th2 shifted responses are associated with increased mucus secretion, cellular infiltration and atopic type reaction characterized by eosinophilia and eosinophil infiltration in the lung. Th1 response characterized by elevation of IFN-γ, IL-1, IL-2, IL-12, IL-18, and TNF-α, while TH2 response associated with the increase in IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13[159]. Since TH1 and TH2 mutually inhibit each other by IFN-γ to suppress TH2 and IL-4 to inhibit TH1, INF-γ/IL-4 ratio is used as an indicator for the TH2 bias[160]. There is lower IFN-γ and higher IL-4 leading to higher IFN-γ/IL-4 ratio in RSV associated bronchiolitis, in addition to the higher IL-10/IL-12 ratio[161]. This high IFN-γ/IL-4 ratio indicates either poor TH1 response or enhanced TH2 response. The fact that IFNγ (TH1 cytokine) level in RSV infected infants is lower than what found in Pneumovirus infected infants and that the IL-4 level (TH2 cytokine) is higher in RSV infected infants, it suggests that the TH2 response is enhanced in RSV infected infants[162]. Another study
in RSV infected infant indicted the predominance of the Th2 cytokines in the nasopharyngeal fluid [163]. Thus, Th2 enhanced response might be associated with the severe RSV bronchiolitis.

There is an increase in B-lymphocytes in the blood of RSV lower respiratory tract infected infants. Humoral immune response to RSV infection includes IgM, IgA, and IgG production within 5-10 days post infection. However, lower immunoglobulin responses are detected in children under 6 months old. Both free and cell bounded anti-RSV IgA is present in the nasopharyngeal secretion of RSV infected patients. Anti-RSV IgG is increased in RSV infected patients in both IgG1 and IgG3 subclasses. Antibodies directed to F protein crossreact with different RSV strains, while anti-G protein antibodies are strain specific [164,165]. Although both anti-RSV IgG and IgA are associated with protection against RSV infection, nasal IgA is more protective than serum neutralizing IgG antibody[166,167]. A similar finding was seen in children where high IgA level seemed to be associated with recovery[168]. Another study indicated that lack of IgA RSV-specific memory B cells in the blood of experimentally infected adults and this may explain the susceptibility to recurrent infection with RSV [169].

**Secondary Bacterial Infection**

Secondary bacterial infection is a challenging potential sequel to viral pneumonia. There are few reports of bacterial infections secondary to RSV infections due to the limitation in the diagnosis of such infections i.e., the bacterial infection masked by the pathological changes induced by the virus with difficulty in obtaining noninvasive specific diagnostic samples[170]. Thus, there are few reports on the magnitude/extent of bacterial infection secondary to RSV infection. One study indicates approximately 40% of patients hospitalized due to viral respiratory tract infection were associated with bacterial co-infection[171], while another study showed that 40% of children infected with RSV have bacterial coinfection or in high risk of bacterial
pneumonia [6]. The most common bacteria associated with secondary bacterial pneumonia are 
Streptococcus pneumoniae, Staphylococcus aureus, Streptococcus pyogenes, and Haemophilus influenza[172,6,173,174]. Because of a high percent of viral-bacterial coinfections, a synergistic
relationship between the two microbial agents that leads to enhanced host susceptibility to each
pathogen has been proposed.

Several in vitro and in vivo models investigated the relationship between viral and bacterial
respiratory infection and how these two microorganisms enhance virulence and persistence of both
pathogens. One study determined that IFN elevation induced by RSV infection enhanced
Pseudomonas aeruginosa biofilm formation in human bronchial epithelial cell culture along with
an increase in iron and Iron transport protein (Transferrin) secretion in the apical epithelium
promoting P. aeruginosa biofilm both in vitro and in vivo [175]. Another study with influenza
infected mice followed by Streptococcus pneumoniae (Spn) infected challenge revealed increases
in both viral and bacterial titers. It is thought that Spn leads to enhanced viral release from infected
cells, and alveolar macrophages impairment resulting in increased Spn [176]. An In vitro study
determined a 2-10 fold increase in the adherence of Spn to RSV infected respiratory cell line in
comparison to non-infected cells[177], while another study showed that 2-2.2 fold increase in
Haemophilus influenza and Streptococcus pneumoniae attachment to cells expressing RSV G
glycoprotein than cells infected with vector only. Furthermore, this bacterial adherence was
reduced to 78-84% when cells were incubated with anti-RSV G antibody [178]. In addition to
viral-specific characteristics that enhances bacterial coinfection, the viral infection itself lead to
alterations in host susceptibility to infection.

There are several potential mechanisms by which viruses predispose to secondary bacterial
infection. The most critical factor is epithelial necrosis and damage, which give access for
pathogenic bacterial to its receptors and to obtain iron and other micronutrients [179]. Additionally, viral infection leads to disabled innate barriers such as loss of mucociliary clearance mechanisms leading to increased bacterial colonization and increased numbers of bacteria reaching deeper location within the respiratory tree[180]. Viral infection creates a microenvironment suitable for bacterial proliferation such as aggregation of fibrin, mucus, and necrotic cells, and the occlusion of small airways leading to reduced O2 and CO2 concentration[181,182]. Furthermore, bacterial coinfection increases viral titer and prolong the course of infection with some reports demonstrating that bacterial infection increases susceptibility to viral infection [180,183].

**RSV Therapeutics**

RSV is a global burden and contributes significantly to increased hospital admission rates and mortality due to severe bronchiolitis and pneumonia, and there is no effective specific therapeutics for RSV infection. There are only two approved treatments, Ribavirin, and palivizumab, although Ribavirin no longer being recommended for treatment and Palivizumab recommended as a prophylactic treatment only in high-risk individuals. Additionally, corticosteroids and bronchodilator have limited effects and benefits. The only therapeutic options available in clinical settings are supportive cares such as oxygenation and intravenous fluid[184]. There is an increased interest from pharmaceutical companies to develop direct antiviral compound suitable for treatment of RSV infection especially for infants and immunocompromised individuals. Several compounds that differ in their characteristics and molecular targets have been evaluated, and some have reach advanced clinical trials.

Four classes of anti-RSV therapeutics been developed and investigated: immunoglobulins, siRNA-interference, fusion inhibitors, and small molecule inhibitors. Several types of Immunoglobulins have been developed and most of them targeting RSV F protein. Both
polyclonal and monoclonal antibodies have been utilized as therapies with monoclonal showing the higher neutralizing effect and lower adverse effects. siRNA is used to interfere with RSV-directed protein synthesis. Anti-RSV therapeutic been developed target five different RSV proteins[15].

Since RSV F protein is critical for RSV entry and spread to adjacent cells, most of the anti-RSV therapeutics target F protein. Several F protein inhibitors are either neutralizing antibody fragments or small molecules. Two compounds in this category (ALX-0171, REGN-2222) have reached advanced clinical trials (phase 3 human clinical trials). ALX-0171 is trimeric Nanobody (camelia antibody) that binds the antigenic site II of RSV F protein [185], while REGN-2222 is a fully human monoclonal antibody[186]. Both antibodies bind to prefusion F protein preventing entry and multicyclic RSV infection. Other small molecules that target F protein have reached advanced clinical trials. These anti-F protein compounds bind to F protein preventing its functional activity and transformation to post-fusion conformation. Since the transformation of the metastable pre-fusion to the 6-helix bundle post-fusion conformation is critical for RSV entry to the cell, these anti-F small molecule inhibitors deactivate RSV and limit the infection. Several molecules in this category reached advanced clinical trails such as GS-5806, JNJ-53718678, and AK0529). One downside for F protein inhibitor is the development of resistant viral mutation (escape mutations) [187].

Another RSV target is the L-protein, which is the RNA-dependent- RNA polymerase. Previously recommended anti-RSV compound Ribavirin is in this class. ALS-008176, a nucleoside analog chain terminator, is a newly developed RSV polymerase inhibitor developed by Alios Janssen and now in advanced clinical trials[188]. Other target proteins are N protein, SH protein, and M2-1 protein. Several anti-RSV molecules developed targeting these proteins, and
they are progressing toward clinical trails [189–191]. Another possible anti-RSV infection therapeutic is to target host immune response to infection.

The severe inflammatory and cellular infiltration associated with severe RSV infection lead to the belief of bidirectional approach in developing anti-RSV lower respiratory tract infection therapeutic, i.e., specific anti-RSV molecules and immunomodulatory therapeutic such as chemokines and anti-leukotriene[192]. Blocking either of CCL3 (MIP-1α) or CCL5 (RANTES) in RSV infected animal model showed a significant reduction in the recruitment of inflammatory cells and increased survival[193,194]. Anti-leukotriene is also investigated since leukotriene is prominent during RSV bronchiolitis and showed that anti-leukotriene administration reduces inflammatory cellular infiltrate, reduced airway blockage, and reduced bronchiolitis in RSV infected mice and infants [195,196]. Other immunomodulating agents such as surfactant protein, vascular endothelial growth factor are also investigated and reduced RSV-associated pathology [197].

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CHAPTER 2: *STREPTOCOCCUS PNEUMONIAE INFECTION IN RESPIRATORY SYNCYTIAL VIRUS INFECTED NEONATAL LAMBS*

A paper submitted to *Emerging Microbes and Infections*

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**Abstract**

**Background:** Respiratory syncytial virus (RSV) is the primary cause of viral bronchiolitis resulting in hospitalization and the most frequent cause of secondary respiratory bacterial infection especially by *Streptococcus pneumoniae* (*Spn*) in infants. While murine studies have demonstrated enhanced morbidity during a viral/bacterial co-infection, human meta-studies have conflicting results. Moreover, less is known about the pathogenesis of *Spn* serotype 22F and especially the co-pathologies between RSV and *Spn* dual infections.
**Objective:** The objective of this study was to determine interactions and mechanisms that contribute to co-pathogen-induced morbidity using a neonatal lamb model naturally permissive to infection by both pathogens.

**Methods:** Colostrum deprived lambs (aged 3-5 days) were randomly divided into four groups. Two of the groups were nebulized with RSV M37 (1.27 x 10^7 IFFU/mL), and the other two groups nebulized with cell–conditioned mock media. At day 3 post-infection, one RSV group (RSV/Spn) and one mock-nebulized group (Spn only) were infected with (2x10^6 cfu of Spn) intratracheally. At day 6 post-infection all lambs were humanely euthanized, and bacterial/viral pathogeneses were assessed by culture, viral infectious focus forming unit assay, RT-qPCR, immunohistochemistry, and histopathology.

**Results:** Lambs dually infected with RSV and Spn had higher RSV titers, but lower Spn than the other comparable groups. Additionally, lung lesions were observed to be more intense in the RSV/Spn group characterized by increased interalveolar wall thickness accompanied by neutrophil and lymphocyte infiltration.

**Conclusions:** Despite lower Spn in lungs, lambs co-infected with RSV demonstrated greater morbidity and tissue histopathology. Thus, the perception of enhanced disease severity may be due to observed lesion development rather than elevated bacterial pathogenesis.

**Introduction**

Respiratory Syncytial Virus is one of the leading cause of severe lower respiratory infection in infants under the age of five leading to 600,000 deaths worldwide [1]. RSV is a member of the pneumoviridae family that infects all infants by the age of two years [2]. Although mild to
moderate upper respiratory tract infection is the most common form of infection, severe lower respiratory tract can develop leading to severe pneumonia and/or bronchiolitis that leads to hospitalization and sometimes death [3–5]. Lower respiratory tract infection increases the susceptibility to secondary bacterial infection(s) leading to severe and life-threatening pneumonia [6]. *Streptococcus pneumoniae* (*Spn*) is one of the most common bacterial infections that occurs concurrently with respiratory viruses such as influenza and RSV [7].

*Spn* is a Gram-positive facultative anaerobic bacterial pathogen that causes invasive disease including sepsis, meningitis, and pneumonia. Similar to RSV, *Spn* cause severe illness and presents with higher incidence in both children and the elderly worldwide [8]. Pneumococcal pneumonia is one of the leading causes of bacterial pneumonia in children worldwide, responsible for about 11% of all deaths in children under the age of five (700,000-1 million every year). Most of these deaths occur in developing countries [9]. *Spn* vaccines are effective in reducing the incidence of pneumonia caused by the serotypes contained in the vaccine [10]. However, the emergence of non-vaccine serotypes and persistence of antibiotic-resistant *Spn* such as serotype 19A highlights the importance of more investigation into *Spn* pathogenesis and therapy. Since *Spn* has an essential role in secondary bacterial infections following viral pneumonia or viral-bacterial co-infection [7,11], animal modeling for the purpose of understanding viral-bacterial co-infections are crucial to investigating therapeutics that combat both. Moreover, most studies have concentrated on influenza and *Spn* co-infections but mainly in murine models with few mechanistic studies done in humans other than calculation of frequencies of co-infections with these two pathogens. Despite the importance of RSV/*Spn* co-infections, far fewer studies have been done in this area as compared to influenza/*Spn*. Furthermore, less is known about emergent serotype 22F pathogenesis [12,13]. We have extensively used a neonatal lamb model to mimic RSV lower respiratory tract
infection in infants as a preclinical model to evaluate the efficacy of new therapeutics [14,15] and to understand RSV pathogenesis [16–18]. Sheep are also permissive to Spn infection and have served as a model of Spn sepsis that appears to manifest clinical signs similar to human infection [19,20]. Thus, in the present study, we investigated a co-infection of neonatal lambs using RSV/Spn as an alternative large animal model to better understand viral/bacterial co-infections in the young with the objective of gaining more insight into serotype 22F pathogenesis in the lung.

Material and Methods

Experimental Design

Animals: A total of 20, 2-3 day-old, colostrum-deprived lambs, were randomly divided into four groups with 5 animals per group: RSV only, RSV-Spn co-infection, Spn only, and uninfected control. Animal use was approved by the Institutional Animal Care and Use Committee of Iowa State University. Two groups were nebulized with RSV M37 (1.27x10^7 IFFU/mL) on day 0. One of the RSV infected groups was injected intratracheally with 2 ml normal saline as a mock Spn infection (RSV group), while the second RSV-infected group was injected intratracheally with 2 ml solution containing Spn serotype 22F (2x10^6 CFU/ml) 3 days post-RSV nebulization (RSV-Spn group). The other two groups were nebulized with cell-conditioned mock media containing 20% sucrose at day 0 and injected intratracheally with either normal saline (control group) or solution containing Spn (2x10^6 CFU/ml) at day 3 post nebulization (Spn group). At day 6 post-RSV infection, all lambs were humanely euthanized. Autopsy was performed to evaluate the macroscopic lung lesions. Lung samples were collected including sterile lung tissue for bacterial isolation, frozen lung sample for RT-qPCR, bronchioalveolar lavage fluid (BALF) from right caudal lung lobe for RSV infectious focus forming unit (IFFU) assay and RT-qPCR, and lung
pieces from different lobes were fixed in 10% neutral buffered formalin for histological assessment.

**Infectious Agents**

Lambs were infected with RSV strain M37, purchased from Meridian BioSciences (Memphis, TN, USA). This strain is a wild type A RSV isolated from the respiratory secretions of an infant hospitalized for bronchiolitis [21,22]. M37 was grown in HELa cells and stored at -80°C in media containing 20% sucrose [23]. 6 mL of $1.27 \times 10^7$ IFFU/mL in media containing 20% sucrose or cell-conditioned mock media (also containing 20% sucrose) was nebulized using PARI LC Sprint™ nebulizers to each lamb over the course of 25-30 minutes resulting in the total inhalation of about 3 mL by each lamb [23]. *Spn* serotype 22F was grown overnight at 37°C in Todd Hewitt media containing 2% yeast extract, 50 µg/ml of gentamicin, and 10% bovine serum. Colony forming units (CFUs) were calculated by OD$_{600}$ with confirmation by dilution plating on Tryptic Soy Agar (TSA) plates with 5% sheep blood containing gentamicin.

**Lung RSV viral and Spn bacterial titers**

BALF collected from right caudal lobe at necropsy was used to evaluate RSV IFFU. BALF were spun for 5 minutes at 3,000g to pellet large debris. Supernatants were then spun through 0.45 am Costar SPIN-X filters (microcentrifuge 15,600g) for 5 minutes. The resulting BALF samples were applied to HEla cells grown to 70% confluence in 12-well culture plates (Fisher Scientific, Hanover Park, IL) at full strength, and three serial dilutions (1:10, 1:100, and 1:1000); all samples were tested in triplicate to determine the viral titer. Lung tissue samples were used to determine *Spn* titer. Lung tissue samples were placed in 500 µl of sterile PBS and were mechanically homogenized. Lung homogenates were pelleted at 100x g, for 5 minutes. Supernatants were serially diluted and applied to 5% sheep blood TSA plates containing gentamycin.
Immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded tissue sections were used for IHC which was performed according to a previously published protocol in our laboratory [18,24]. Briefly, after deparaffinization and rehydration, antigen retrieval was performed in 10mM TRIZMA base (pH 9.0), 1mM EDTA buffer, and 0.05% Tween 20 with boiling under pressure for up to 15 minutes. Polyclonal goat anti-RSV antibody (Millipore/Chemicon, Temecula, CA; Cat. No. AB1128) was used as the primary antibody after two blocking steps. The first blocking was with 3% bovine serum albumin in Tris-buffered saline+0.05% Tween 20 (TBS-T) and the second was 20% normal swine serum in TBS-T for 15 minutes each. Primary antibody was followed by application of biotinylated rabbit anti-goat secondary antibody (KP&L; Cat. No. 16-13-06). Signal development was accomplished using a 1:200 dilution of streptavidin-horseradish peroxidase (Invitrogen; Cat. No. 43-4323) for 30 minutes followed by incubation with Nova Red chromagen solution (Vector; Cat. No. SK-4800). Positive signal was quantified in both bronchioles and alveoli for each tissue section, and a score of 0-4 was assigned according to an integer-based scale of: 0=no positive alveoli/bronchioles, 1=1-10 positive alveoli/bronchioles, 2=11-39 positive alveoli/bronchioles, 3=40-99 positive alveoli/bronchioles, 4=>100 positive alveoli/bronchioles. IHC for Spn was performed using rabbit anti-Streptococcus pneumoniae polyclonal antibody (Thermo Fisher scientific cat. #PA-7259) followed by biotin-labeled goat anti-rabbit IgG antibody (Thermo Fisher Scientific Cat.#: 65-6140). Five random images were taken for each tissue section that were then analyzed by the quantitative Halo program.

Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

BALF and lung tissue homogenates in Trizol were used to assess RSV mRNA expression by RT-qPCR. The assay was performed as published previously in our laboratory [18,24,25]. Briefly,
RNA isolation from lung tissue and BALF was performed using the TRIzol method followed by standard DNase treatment. RT-qPCR was carried out using One-Step Fast qRT-PCR Kit master mix (Quanta, BioScience, Gaithersburg, MD) in a StepOnePlus™ qPCR machine (Applied Biosystems, Carlsbad, CA) in conjunction with PREXCEL-Q assay-optimizing calculations. Primers and probe for RSV M37 nucleoprotein were designed based on RSV accession number M74568. Forward primer: 5′-GCTCTTAGCAAGATCAAGTGAAG; reverse primer: 5′-TGCTCCGTGAGGTTGTTATT; hydrolysis probe: 5′-6FAM-ACACTCAACAAAGATCAACTTCTGTCATCCAGC-TAMRA.

**Hematoxylin-Eosin Staining and Histological Scoring of Lung Sections**

Hematoxylin-eosin stained sections were examined via light microscope. An integer-based score of 0-4 was assigned for each parameter (bronchiolitis, syncytial cells, epithelial necrosis, epithelial hyperplasia, alveolar septal thickening, neutrophils in bronchial lumen, neutrophils in alveolar lumen, alveolar macrophages, peribronchial lymphocytic infiltration, perivascular lymphocytic infiltration, lymphocytes in alveolar septa, fibrosis), with 4 as the highest score. A final score was calculated by adding up all measured scores to form a 0-48 score, with 48 as the highest, which is called the accumulative histopathological lesion score.

**Statistical Analysis**

Statistical analysis used the Wilcoxon signed-rank test for non-parametric parameters such as accumulative microscopic lesion scoring, followed by nonparametric comparisons for each pair also using the Wilcoxon method. One-way ANOVA was followed by all pairs comparison by the Tukey-Kramer HSD method for gross lesion scores and viral titer analyses by RT-qPCR and IFFU assays.
Results

Infected Lambs Showed Marked RSV and Spn Titer

RSV titers and Spn colony-forming units were measured in this study to evaluate the degree of infection by each pathogen and to investigate the possible effect(s) of co-infection in the combined RSV-Spn group on the replication of each infectious agent. As measured by IFFU, infectious RSV was detected in both RSV and RSV-Spn groups (P<0.0001) (Figure 1a). RSV titer trended (not significant) 0.15 fold higher in the RSV-Spn group than RSV alone. A similar trend was observed when assessing RSV mRNA detected in BALF by RT-qPCR. RSV mRNA was elevated in both RSV and RSV-Spn groups (7.28 and 7.31 virion/ml) (Figure 1b). Furthermore, similar to the viable virus titer increase, RSV virions measured by RT-qPCR in the lung of the RSV-Spn group were 0.6 fold higher than the RSV-only group (Figure 1c). In contrast, Spn was isolated in the lung tissue of both the Spn only and the RSV-Spn groups (56902.75 and 5456 CFU/g, respectively) (p<0.0001) (Figure 1d). In contrast to the RSV/Spn group, the bacterial titer was 9.4 fold higher in the Spn only infected group (p<0.05) (Figure 1d). Interestingly, Spn titers in lambs that died before the end of the study were the highest of their groups. Lamb number 22 that was found dead 36hr after Spn infection in the RSV-Spn group had 59,302 CFU/g, while lamb number 9 that was euthanized 48 hr after Spn infection in the Spn group had a titer of 9,302,325 CFU/g (Figure 1d). Spn was detected in the blood in both Spn infected groups - indicating bacteremia/sepsis (Figure 1e).

RSV and Spn Induce a Well-recognized Macroscopic and Microscopic Lesion

Percent of lung tissue with gross lesions related to either infectious agent was determined at necropsy coupled with post-necropsy retrospective qualitative analyses. Both RSV and Spn-related lesions were found scattered across the lung surface in all lung lobes. Pinpoint dark red areas of
lung consolidation characterized RSV lesions. These areas were obvious in RSV and RSV-Spn groups (P<0.001, and p<0.005, respectively). There were no significant differences in the percentage of lung RSV macroscopic lesions detected between RSV and RSV-Spn groups (Figure 2a). *Spn* gross lesions are characterized by larger sizes of lung consolidation with bright red color - which was seen to a lesser extent when compared to RSV lesions (Figure 2b). When combining lesions observed associated with both RSV and *Spn*, there was a significant increase in observed lesions within both RSV-infected groups in comparison to the *Spn* only group (p<0.001) (Figure 2c).

Microscopic lesions observed within the lung tissue reflected the infectious agent used and contradicted our initial expectations (i.e. microscopic lesions caused by RSV infection were multifocal areas of interstitial pneumonia and bronchiolitis scattered randomly and homogeneously throughout the lung tissue). However, *Spn* induced diffuse homogenous and subtle pathological changes in the lung tissue. Infection with either *Spn*, RSV, or both, markedly increase microscopic lesions (accumulative microscopic lesion score) associated with the disease in comparison to the control group (p<0.05) (Figure 3a-f). Additionally, the combined RSV-*Spn* infection significantly increased the severity of microscopic lesions in comparison to the *Spn* only group (p<0.05). Lesions varied among lambs, and RSV lesions consisted of thickening of the interalveolar wall with inflammatory cellular infiltrates in the airway adventitia and lamina propria (lymphocytes and plasma cells), alveolar lumen (alveolar macrophages and neutrophils), and bronchiolar lumen (neutrophils). With RSV, overall, there was a varied degree of epithelial necrosis and syncytial cell formation. On the other hand, *Spn* lesions consisted of moderate interalveolar wall thickening with inflammatory cellular infiltrate mainly in the alveolar septae. Most of the microscopic lesions seen with RSV overlapped with *Spn*-induced injury. However,
congestion of the interalveolar wall capillaries and hemorrhage was seen only in \textit{Spn}-infected lambs.

Immunohistochemistry was used to identify and localize RSV and \textit{Spn} in tissue sections. RSV were present multifocally throughout the sections with bronchial and peribronchial distribution (Figure 4c). Therefore, RSV expression was evaluated in bronchioles and alveoli separately. There were marked increases in RSV expression in bronchioles in both RSV only and RSV-\textit{Spn} groups (p<0.005 and p<0.001, respectively) with high RSV expression in the alveoli of the RSV-\textit{Spn} group (p<0.01) (Figure 4a). There were no significant differences between the RSV only and RSV-\textit{Spn} groups in the degree of RSV expression in lung tissue sections. \textit{Spn} was random and homogenously scattered throughout the lung sections with more intense signal in interalveolar walls and blood capillaries (Figure 4d). Although not significant, there was a 0.5 fold increase in \textit{Spn} in the \textit{Spn} only group when compared with the RSV-\textit{Spn} group (Figure 4b).

**Discussion**

There is a critical need for an animal model to study bacterial pneumonia secondary to an initial viral infection in the lung in order to study the mechanisms of viral-bacterial co-infection and to evaluate therapeutic interventions. There are significant advantages of using lambs to model RSV infection as a correlate for human infants - including the ability to use human viral strains without adaptation and the similarity of the pathological sequelae [16,17]. In this study, we demonstrate that \textit{Spn} readily infects the lungs of lambs and establishes active bacterial pneumonia. A previous study revealed that the peak of RSV titer and infection in lambs is around day 3 post-viral nebulization, and we used this time-frame to model early human co-infection [24]. The results of this study demonstrate consistency in the infection rate of both RSV and \textit{Spn}, as well as a good relation to the lesion development induced by either of the infectious agents. Although we
used $2 \times 10^6$ CFU of $Spn$ for infection, murine studies typically use $5 \times 10^5$ to $10^7$ CFU to establish productive infections. Moreover, the lung volume of lambs is significantly larger than mice which suggests that our inoculating dose may be more dispersed throughout the lungs than murine studies. We believe that we may also be able to reduce the infection dosage to a lower CFU or potentially use a colonization model to examine co-infection and pneumonia development.

The RSV titer in our study was increased by 0.5 fold in the RSV-$Spn$ group, but bacterial presence was reduced by 9.4 fold when compared to the RSV and $Spn$ groups, respectively. These data are consistent with prior studies in mice with influenza/$Spn$ co-infections demonstrating higher viral loads [11,26], although our observed viral (RSV) increase was quite modest in comparison. Influenza co-infection studies also predict higher $Spn$ burdens in the lungs due to damaged epithelial cells serving as anchor points for the opportunistic bacteria. In other studies, RSV with $Spn$ in mice or cell culture predict that the RSV G protein on the infected epithelial surface could also serve as an anchor point for $Spn$ in the lungs [27]. In contrast to these murine models, we found lower bacterial loads in the co-infection group over the $Spn$ only group. These findings suggest that the immune response might control $Spn$ in the lungs of lambs better than mice. Importantly, in human clinical studies of co-infection, show in increase in nasal colonization numbers of $Spn$ upon viral infection but this does not translate into higher invasive lung disease [28]; suggesting that higher bacterial burdens could be a murine artifact rather than a mechanism enhancing disease. In human studies of high $Spn$ colonization, RSV disease appeared less severe [29] suggesting that further using the lamb model to explore mechanistic differences between $Spn$ colonization and pneumonia during RSV.

The only deaths that occurred in the present study were in the $Spn$-infected groups, and both lambs (lamb 11 in the $Spn$ only group, and lamb 23 in the RSV-$Spn$ group) had high lung $Spn$
colony-forming units/gram tissue. These could represent a failure to control bacterial division and subsequent septicemia.

Lesion severity was consistent with the RSV titer and *Spn* burden as is shown by the significant increase in the percent of lung tissue involved by gross lesions, and the increase in the evaluated histological parameters. Gross lesions were multifocal lesions scattered randomly in all lung lobes - which is the typical lesion distribution induced by RSV nebulization [18,24]. However, presentation of *Spn* gross lesions contradicted what was expected by the apparent development of lesions in all lobes - including the caudal lung lobe, which is not typical for bacterial pneumonia in lambs. However, the diffuse bacterial lesions and the presence of *Spn* lesions in the caudal lobe may be due to the inoculation technique used for *Spn* infection. For *Spn* infection, lambs were held vertically by one person and injected intratracheally by the second person leading to a fall of inoculum through the bronchial tree into the caudal lobe, which in this case, was favorable since it gives a bronchopneumonic distribution similar to that found in humans. It is also possible that *Spn* spreads across lung lobes after inoculation either by airflow or vascular flow. RSV-induced microscopic lesions were more prominent in comparison to *Spn*-induced lesions and subsequently lead to significant differences between the RSV-*Spn* and *Spn* only groups’ accumulative histologic lesion scores. RSV was more prominent in the bronchioles, while *Spn* was diffuse throughout the lung sections.

Although mechanisms are being assessed, the higher observed morbidity in the RSV/*Spn* group may derive from an enhanced neutrophil response present in the lungs. Evidence for this is supported by the histopathology and the lower *Spn* burdens in these animals. Likely, RSV infection served as a first activating response to neutrophils that could have then better controlled the secondary bacterial infection. It is also possible that alveolar macrophages were activated by
RSV that, in turn, secreted inflammatory mediators that enhance neutrophil activation. Enhanced neutrophil/leukocyte activation contrasts with studies in influenza co-infections in mice – which suggests innate immune exhaustion [30]. While the time of inoculation could be a reason for the observed differences, another could be the mere difference between influenza and RSV pathogenesis. In either case, the results suggest further avenues of study using this model. We are currently evaluating the neutrophil responses in these animals to ascertain how the innate antiviral response may have played a role in better limiting the bacterial infection. The observed higher RSV infection rate in the co-infection could also derive from the greater number of neutrophils in the lungs in this group. There is evidence that RSV can infect neutrophils in humans [31] including our unpublished data. Thus, if dual infection with \textit{Spn} leads to enhanced neutrophil recruitment to the lungs over RSV alone, those cells could become infected and contribute to the higher viral titer we observed in the dual infection group. The effects of RSV infection on neutrophilic antibacterial responses would be an interesting further study.

In this study, we have developed an animal model of co-infection for RSV and \textit{Spn}. We have determined enhanced disease with co-infection of both pathogens that correlates with human and murine influenza studies, but this may all be due to a complex enhanced inflammatory/immune response to co-infection rather than direct damage by either pathogen alone, although we cannot rule the culpability of co-infection causation out. Future studies will plan to utilize other serotypes of \textit{Spn} to determine whether the results we observed are unique to serotype 22F or whether they are a trend for all \textit{Spn} strains in general. Additional studies will allow refinement of this model and will include variations in inoculum volume/concentration, time between infections, and kinetic analyses.
References


Figures and Legends

**Figure 2-1:** RSV and Spn titer in lung tissue and blood

(a) number of infectious RSV particles as measured by IFFU assay, (b) RSV mRNA level in the BALF, (c) RSV mRNA level in lung tissue (d) Spn colony forming unit per 100 μg lung tissue, (e) Spn colony forming unit per 100 μl blood, all shown as average ± SEM. Animals were either infected with mock media (control), RSV, Spn, or RSV followed by Spn (RSV-Spn). *P<0.05, **P<0.01, ***P<0.005, **** P<0.001, ***** P<0.0001.
Figure 2-2: Percent of lung tissue associated with RSV and/or Spn infection.
Percent of lung tissue associated with RSV lesions (a), Spn (b), and both RSV and Spn (c), with photographic representative of each. All show average and SEM. Lambs were either infected with mock media (control), RSV, Spn, or RSV followed by Spn (RSV-Spn). *P<0.05, **P<0.01, ***P<0.005, **** P<0.001, ***** P<0.0001.
Figure 2-3: Histologic lesions associated with RSV, Spn, and RSV-Spn combined infection.

(a) Accumulative histologic lesion associated with RSV and Spn infection shown as average + SEM. (b-f) Show a representative photograph of lung tissue sections stained with Gram stain (b), H&E stained tissue section of control (c), RSV only (d), Spn only (e), combined RSV-Spn (f). Lambs were either infected with mock media (control), RSV, Spn, or RSV followed by Spn (RSV-Spn). * P<0.05, **P<0.01, ***P<0.005, **** P<0.001, ***** P<0.0001.
Number of bronchioles and alveoli express the RSV positive signal (a), surface area (mm2) occupied by Spn IHC positive staining (b), all shown as average + SEM. (c) and (d) show a photo representation of RSV (c) and Spn (d) IHC positive staining. Animals were either infected with mock media (control), RSV, Spn, or RSV followed by Spn (RSV-Spn). * P<0.05, **P<0.01, ***P<0.005, **** P<0.001, ***** P<0.0001.

Figure 2-4: Immunohistochemistry staining of RSV and Spn in FFPE lung tissue sections.
CHAPTER 3  THERAPEUTIC EFFICACY OF JNJ-49214698, AN RSV FUSION INHIBITOR, IN RSV-INFECTED NEONATAL LAMBS

A paper submitted to Frontiers in Microbiology Journal

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Authors Contributions

S.S.A., A. L., J.M.G, and M.R.A carried out several aspects of the neonatal lamb infection experiment including viral inoculation, treatment deliveries, blood draws, animal monitoring and assessment of RSV clinical signs, necropsy, IFFU, qRT-PCR, histological, IHC, and RNAscope assays. A.V. assisted with viral preparation and growth, and IFFU assays. D.R., P.R., and J.M.G performed data processing. D.R., P.R., and A.K. provided the JNJ-49214698 compound, assayed the compound for distribution in different body compartments and performed global statistical analysis of all data. D.R., P.R., A.K., S.S.A., J.M.G, and M.R.A assisted with experimental design and preparation of this manuscript.
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Conflict of Interests

Sarhad S. Alnajjar, Alejandro Larios-Mora, Albert Van-Geelen, and Jack M. Gallup worked at Iowa State University for this study and have no disclosures or conflicts. Mark R Ackermann also worked at Iowa State University for this study served as principal investigator who received funds from Janssen Pharmaceutica to conduct this study. Anil Koul, Peter Rigaux, and Dirk Roymans are employees of Janssen Pharmaceutica NV.

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Abstract

Respiratory syncytial virus (RSV) is a leading cause of respiratory infection, hospitalization, and death in infants worldwide. No fully effective RSV therapy using direct antivirals is marketed. Since clinical efficacy data from naturally infected patients for such antivirals are not available yet, studies in animals are indispensable to predict if therapeutic intervention. Here we report the impact of an RSV fusion inhibitor, JNJ-49214698, on severe RSV-associated acute lower respiratory tract infection (ALRTI) in neonatal lambs. Randomized animals were treated once-daily with 25 mg/kg JNJ-49214698, starting either before RSV infection, 1 day post-infection, or as late as peak lung viral load on Day 3 post-infection. The efficacy of treatment was assessed by scoring clinical signs of illness, development of RSV-induced gross and microscopic lung lesions, and measuring virus titers in the lungs. Treatment with JNJ-49214698 was very effective in all treatment groups. Even in animals for which treatment was delayed until peak viral load was reached, a reduced amount and severity of gross and microscopic lesions as well as RSV titers and RNA levels were found. These results strongly suggest that treatment with small-molecule fusion inhibitors is an effective strategy to treat patients who are diagnosed with an RSV-induced ALRTI.

Key Words

Respiratory Syncytial Virus, small molecule fusion inhibitor, RSV Fusion inhibitor, antiviral, neonatal lamb model, acute lower respiratory tract infection

Introduction

Respiratory syncytial virus (RSV) is one of the leading causes of acute lower respiratory tract infection (ALRTI). Globally, it is estimated that 33.8 million cases of
ALRTI associated with RSV infection occur yearly in children under the age of five. Subsequently, RSV-associated ALRTI is a major cause of hospital admissions with an estimated 3.4 million cases annually; and RSV-associated mortality is estimated to be 66,000-199,000 in children under the age of five occurring mostly in developing countries [1]. Furthermore, post-bronchiolitic development of wheezing syndrome is sometimes observed, and although still under debate, it was suggested that the development of asthma could be associated with RSV infection [2,3]. RSV is also one of the leading causes of respiratory infection in the elderly and high risk adults [4,5]. A recent study showed that 3% of pneumonia cases in human adults is caused by RSV [6] and with the widespread use of influenza vaccination, RSV has a similar disease burden in the elderly [4].

Despite decades of investigation, no market-approved vaccine exists [7]. Seasonal prophylaxis with the monoclonal antibody palivizumab, restricted to high-risk infants in developed countries, is the only specific antiviral strategy available [8]. The disease preventive effects of palivizumab in these patients were demonstrated by a significant reduction in hospitalization rate, hospital residence time, admission to intensive care unit and days requiring oxygen therapy [9]. However, the therapeutic efficacy of antibodies like palivizumab in infants hospitalized with an established RSV-associated ALRTI is questionable at best [10–12]. Other drugs like bronchodilators, corticosteroids, and antibiotics, often used but not indicated to treat RSV-associated bronchiolitis, do not seem to confer sufficient therapeutic benefit either, leaving supportive care essentially as the major treatment option [13,14].

New treatments are clearly needed to decrease the medical consequences related to RSV-associated ALRTI. Data on the impact of small-molecule direct antivirals on RSV
disease manifestation in naturally infected hospitalized patients or outpatients are not available, and as such, reliable modeling of human RSV disease remains a necessary step in the search for novel therapies.

The development of an RSV experimental human infection model has provided a new tool to obtain human proof-of-concept efficacy data of RSV direct antivirals early in the clinical development pipeline [15–17]. However, volunteers are often young, healthy adults and infection is studied only in the upper respiratory tract, limiting the information about the efficacy of therapeutics to treat ALRTI. Therefore, the recent development of a fully-replicative neonatal lamb model for RSV has presented a significant step forward to generate reliable preclinical efficacy data and to improve the selection of the proper clinical candidate molecules. Neonatal lambs are susceptible to human as well as ovine and bovine strains. Similarities between the pulmonary and immunological systems between lambs and humans make the neonatal lambs an excellent model to study human RSV disease and the impact of new potential RSV therapeutics [18,19]. Moreover, neonatal lambs infected with RSV develop clinical symptoms including but not limited to fever, tachypnea or increased expiratory effort (wheeze), lethargy, and develop mild to moderate bronchiolitis and pneumonia. Innate and adaptive immune responses by neonatal lambs are very similar to those of human infants [20,21].

A promising approach to inhibit RSV is by targeting the viral fusion (F) protein [22,23]. A few small-molecule RSV fusion inhibitors are currently being evaluated in early-stage clinical trials, but their clinical impact on RSV-associated ALRTI in hospitalized infants is yet unknown [24–26]. The purpose of this study is therefore to evaluate the therapeutic
impact of an experimental RSV fusion inhibitor, JNJ-49214698, on RSV-associated ALRTI using a lamb model of RSV infection.

**Materials and Methods**

*(see online Supplementary Data for detailed methods)*

**Compound and Dosing**

JNJ-49214698 was discovered and synthesized by Janssen Infectious Diseases (Beerse, Belgium). The compound was formulated in 10% acidified hydroxypropyl-β-cyclodextrin [10% HP-β-CD + HCl, pH 2 (vehicle)] at 6.25 mg/mL prior to dosing and stored throughout the study at 4 °C. The compound was dosed orally by catheter-mediated orogastric gavage at 4 mL/kg body weight (25 mg/kg) once daily. Dose selection in this study aimed for reaching the highest possible, safe exposure in the animals in order to maximize the likelihood to obtain efficacy while avoiding toxic side effects. A daily oral dose of 25 mg/kg was selected based on the antiviral activity of JNJ-49214698 [EC\textsubscript{50} = 0.4 ng/mL (0.8 nM) and EC\textsubscript{90} = 2.4 ng/mL (4.8 nM)], JNJ-49214698 exposure levels obtained in neonatal lambs at different doses during a separate pharmacokinetic (PK) study and an observed lack of toxicity of JNJ-49214698 at least until C\textsubscript{max} = 17,167 ng/mL and AUC\textsubscript{0-24h} = 139,993 ng.h/mL in a 5-day repeated dose rat tolerance study.

**Animals**

Twenty-one colostrum-deprived neonatal lambs (Suffolk, Polypay, Dorsett cross) aged 1-3 days and 2-7 kg body weights were obtained for this experiment. Animal use was approved by the Institutional Animal Care and Use Committee of Iowa State University. RSV-infected lambs were kept in a separate room from the non-infected animals in the Livestock Infectious Disease Isolation Facility (LIDIF). Lambs were fed iodide-free lamb
milk replacer diet (Milk Products Inc., Chilton, WI, USA) [27], and were treated with Naxcel (Ceftiofur sodium, Pfizer) intramuscular once daily to reduce/prevent secondary bacterial infections.

**Experimental Design**

Lambs were randomly assigned to five different groups. Three groups (Px, Tx-1 and Tx-2) were infected with RSV and treated with JNJ-49214698. The first group (Px, n = 4) was treated prophylactically, 1 day before RSV challenge and then daily afterward up until Day 5 post infection (p.i.). The second (Tx-1, n = 5) and the third (Tx-2, n = 5) groups were treated one day and three days after viral challenge and daily afterward up until Day 5 p.i., respectively. The vehicle group (n = 4), serving as a positive RSV control group, was infected with RSV, but received treatment with vehicle only. The No RSV group (n = 3), served as negative RSV control group, and was aerosolized with RSV-free, HEp-2 cell-conditioned media and also received vehicle. All lambs were euthanized at Day 6 p.i. and all endpoints measured after euthanasia.

**RSV Infection**

Lambs were infected with RSV strain M37, purchased from Meridian BioSciences (Memphis, TN, USA). This strain is a wild type A RSV isolated from the respiratory secretions of an infant hospitalized for bronchiolitis [28,29]. In our laboratory, M37 was grown in HEp-2 cells and stored at -80°C in media containing 20% sucrose [30]. Six mL of $1.27 \times 10^7$ Infectious Forming Unit (IFFU)/mL in media containing 20% sucrose or cell-conditioned mock media (also containing 20% sucrose) was nebulized using PARI LC Sprint™ nebulizers to each lamb over the course of 25-30 minutes resulting in the total inhalation of about 3 mL by each lamb [31].
Assessed Parameters

JNJ-49214698 exposure in plasma, BALF, and lung homogenate samples was assessed using a method based on Protein precipitation and HPLC/MS/MS analysis. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was done to measure the RSV mRNA expression in BALF and lung homogenate samples as previously done in our lab [30,32,33]. Infectious focus-forming unit (IFFU) assay was used to determine the viable RSV in BALF samples as previously described [32]. Clinical score was calculated by the number of respiratory associated signs present in each animal. For the pathological study, percent of lung parenchyma involved with RSV induced lesion were measured to evaluate the RSV gross lesion. Histologically, Hematoxylin-eosin stained sections were examined via light microscope as described previously [32] with some modification. Lung lesions were scored according to an integer-based score of 0-4 for each parameter (bronchiolitis, syncytial cells, epithelial necrosis, epithelial hyperplasia, peribronchial lymphocytic infiltration, perivascular lymphocytic infiltration, neutrophils), with 4 as the highest score. Then a final score (accumulative histo lesion score) was assigned by adding up the scores from the seven individual parameters resulting in final accumulative scores ranging from 0-28 representing the total RSV-associated lesion in each tissue section. For further evaluation of the JNJ-49214698 therapeutic efficacy, Immunohistochemical staining was performed to evaluate RSV antigen expression in lung tissue section as previously described in our laboratory [32,34,35]. Formalin-fixed paraffin-embedded tissue sections were used for the RNAscope detection of RSV mRNA in situ. A probe designed to the hRSV M37 nucleoprotein gene (accession number KM360090) was used (Probe-V-RSV-NP, Advance Cell Diagnostic, Catalog number 439866). The assay was performed according to the manufacturer’s manual (user manual document number 320497; RNAscope® 2.0 HD Detection Kit (BROWN) User
Manual PART 2). Sections were examined under light microscope, and the number of bronchioles and alveoli containing the positive signal were counted. The number of positive bronchioles and alveoli per tissue section was then assigned a score according to the simple integer-based scale of 0 = no positive alveoli/bronchioles, 1 = 1-10 positive alveoli/bronchioles, 2 = 11-39 positive alveoli/bronchioles, 3 = 40-99 positive alveoli/bronchioles, 4 = >100 positive alveoli/bronchioles.

**Statistical Analysis**

Statistical analysis was completed by using the Kruskall-Wallis test for non-parametric parameters such as accumulative microscopic lesion scoring, immunohistochemistry and RNAscope integer-based scores, followed by Dunn’s post-hoc test for multiple comparisons. One-way ANOVA followed by Dunnett multiple comparisons test was used to compare the treated groups to the RSV-infected non-treated control group for gross lesion scores and viral titer analyses by qRT-PCR.

**Results**

**JNJ-49214698 Efficiently Distributes to Different Lung Compartments of Neonatal Lambs**

During a multicyclic RSV infection, small-molecule fusion inhibitors are thought to inhibit each new infection event at the time of viral fusion. The concentration of JNJ-49214698 was measured in BALF as well as in homogenized lavaged-lung tissue. To assess the potential of JNJ-49214698 to distribute from the blood to the lungs of the neonatal lambs, we also measured its level in plasma. JNJ-49214698 was absorbed very quickly into the circulation and tended to slightly accumulate over time during the study period. The average steady-state concentration of JNJ-49214698 in plasma was in the range of 1770 ± 435 and
3112 ± 1441 ng/mL across the different treatment groups and was reached within 24-48 hr after the first dose (Figure 1a). Twenty-four hour after the final dose, the level of JNJ-49214698 in the BALF of the group receiving prophylactic treatment (Px), or groups in which compound administration was started one (Tx-1) or three (Tx-2) days after viral inoculation was 2044 ± 648, 3289 ± 977 and 1109 ± 252 ng/mL, respectively (Figure 1b). The average lavaged-lung concentrations measured were 2867 ± 403, 5211 ± 841 and 1976 ± 175, in Px, Tx-1, and Tx-2 treatment groups, respectively, resulting in an approximate lung/plasma ratio of 1.4 to 1.8 and a good distribution of JNJ-49214698 in the BALF (Figure 1c). Together, these results indicate a good distribution of JNJ-49214698 to different lung compartments.

**Treatment with JNJ-49214698 Reduces Incidence and Duration of RSV-Associated Symptoms**

Clinical signs of RSV infection in lambs, as in human infants, can be variable and sometimes difficult to assess. Despite this, RSV-associated respiratory distress can be observed in the animals as early as one day p.i., developing further in some animals into clear external signs of RSV-associated illness such as nasal discharge, wheezing or lethargy. By Day 1 p.i., most of the lambs in this study, except for the animals in the non-infected group, had already become lethargic as characterized by decreased activity (lower frequency of getting up and movement in general). The behavior and respiratory-related symptoms in lambs of the vehicle-treated group (RSV and no treatment) steadily worsened during the following days (Figure 2a). One lamb started to produce nasal discharge and displayed episodes of wheezing as from Day 3 p.i. onwards, while its respiratory rate was clearly increased by Day 6. One lamb on Day 3 p.i. died and was found during post-mortem analysis with severe gross lesions consistent with RSV infection in its lungs. The other lambs
survived until Day 6 when they were euthanized, but by Day 4, all 4 remaining animals from this group were lethargic.

In contrast, while by Day 3 post-infection 3 lambs in the Px group showed fever, the remaining lambs in Px and Tx-1 groups were free of symptoms by Day 3 post-infection (Figure 2b and c). Although less prominently, the incidence and duration of RSV-associated symptoms was also reduced in the lambs of the Tx-2 group as compared to the vehicle-only treated animals (Figure 2d).

In summary, these findings suggest that treatment with JNJ-49214698 has the potential to reduce the incidence and duration of the clinical manifestation of RSV disease in lambs.

**Treatment with JNJ-49214698 Inhibits a Multicyclic RSV Infection in Neonatal Lambs**

The presence of nucleoprotein RNA in BALF and lung samples was quantified by qRT-PCR. In BALF, The Px group showed a significant 3.4 log_{10} (p<0.0001) reduction of the viral RNA level in comparison to the RSV-infected vehicle group (Figure 3a), while the viral RNA in the Tx-1 and Tx-2 groups was reduced less by approximately 7- and a 3-fold as compared to the vehicle only treated animals. We could not detect viral RNA in the lung tissue of prophylactically treated animals, and profound reductions in RSV RNA of approximately 23-fold in Tx-1 group (p<0.02), and 10-fold in Tx-2 in compared to the vehicle group (Figure 3b). The infectious viral titer in BALF were done and partially reported in a separate manuscript [23] in which IFFU were significantly reduced in all treated groups with no infective virus detected in Px group that receive prophylaxis with JNJ-49214698 similar to what we found in RSV mRNA in lung tissue. There were 34- and a 93-log reduction in the infectious titer as compared to the RSV-infected control group, was observed
in the Tx-1 and Tx-2 groups, respectively (p<0.001) (Figure 3c). To confirm the quantified viral titers in BALF and lavaged-lung tissue, we determined RSV (M37) antigen and RNA by immunohistochemical and RNAscope analysis, respectively. RSV antigen was present in infected airway epithelial cells of bronchi, bronchioles, and alveoli and occasionally in alveolar macrophages. However, staining of RSV antigen was markedly reduced in all drug-treated groups in comparison to the RSV-infected control group (Figure 4a, c). RSV antigen was highly decreased in the Tx-2 group as compared to the vehicle-treated group, and again, almost no RSV antigen was detected in both Px (p<0.01) and Tx-1 (p<0.05) treatment groups (Figure 4a, c). When RSV RNA (M37 nucleoprotein) expression in lungs was determined by RNAscope, concomitant with the RNA levels as determined by qRT-PCR, there was a marked reduction of RSV RNA expression in all the compound-treated groups in both bronchioles and alveoli (Figure 4b, d). Heavy brown staining of RSV RNA in RSV-infected non-treated lung sections was observed which was concentrated in the bronchioles and alveoli in the consolidated part of the lung (Figure 4d). In contrast, only minimal staining was seen in the Px (p<0.05) and Tx-1 groups (Figure 4d), aligning with immunohistochemical and qRT-PCR data.

**Treatment with JNJ-49214698 Inhibits RSV-Induced Lung Pathology and Cellular Immune Response of Neonatal Lambs**

Approximately 43% of the lung surface of infected vehicle-treated animals had RSV associated macroscopic lesions which were multifocal dark red pinpoint foci of consolidation scattered randomly over the lung surface and deeply throughout the lung tissue (Figure 5a, b). Moreover, gross lung lesions were evenly distributed over the different left and right lung lobes. Prophylaxis (Px) or treatment starting twenty-four hours after infection (Tx-1) completely abolished the formation of RSV associated gross lung lesion (Figure 5a, b). When
treatment was delayed even as late as three days after infection (Tx-2), a significant reduction in the RSV associated gross lesions was observed; dropping from 43 to 16% (p<0.0001) (Figure 5b). As a negative control, no lesions were observed in the lungs of non-infected, vehicle-treated animals (Figure 5b).

To investigate the effect of JNJ-49214698 on lung tissue microscopically, we assessed the impact of the compound on the development of bronchiolitis, syncytial cell formation, epithelial necrosis and hyperplasia, and inflammatory cell infiltration in lungs. An accumulative histological lesion score was assigned by combining all the assessed parameters. Significant reductions of the accumulative score were seen across prophylactically treated animals or animals that received early treatment starting 1 day p.i. with JNJ-49214698, (p<0.01 and p<0.05, respectively) (Figure 6a). Prophylaxis with JNJ-49214698 completely prevented the formation of microscopic lung lesions after RSV infection (Figure 6a, b). Although the effect on the accumulative histologic score was least prominent in animals that received treatment with JNJ-49214698 as late as 3 days after infection, a prominent reduction as compared to the vehicle-treated group was still observed, with some animals displaying almost no microscopic lesions at all.

Typical RSV lesions that were present in the RSV-infected control group were multifocal interstitial pneumonia and bronchiolitis. There was thickening of the interalveolar wall due to type II pneumocyte hyperplasia and lymphocyte infiltration (Figure 6b). There was neutrophil infiltration in the bronchial and bronchiolar lumens and the alveolar lumen with multifocal and segmental areas of necrosis and sloughing of the epithelial cells lining bronchioles. Some bronchioles had modest thickening of epithelium due to hyperplasia. In addition, there were occasional multinucleated syncytial cells present
throughout the lung sections. In the lesions formed in the latter Tx-1 group, the formation of syncytial cells, epithelial necrosis or hyperplasia and infiltrating neutrophils was absent, while the other parameters assessed were markedly reduced, resulting in a prominent decrease of the accumulative histological lesion scores.

**Discussion**

Currently, there are no effective direct antivirals available for the treatment of RSV-associated ALRTI. Even though a number of RSV inhibitors have reached early-stage clinical evaluation, no data are available yet demonstrating their clinical benefit in naturally infected patients suffering from severe RSV-associated ALRTI, and so uncertainty remains about the therapeutic treatment window and the impact of such molecules on severe RSV disease. Attempting to minimize the risk for intended late-stage clinical development, we therefore evaluated an experimental small-molecule RSV fusion inhibitor, JNJ-49214698, in neonatal lambs, a fully-replicative animal model of RSV infection closely mimicking infant RSV disease.

The three treatment regimens chosen in the study all reflect realistic potential clinical drug administration regimens. The first group (Px) began to receive JNJ-49214698 one day before viral nebulization to test the pre-exposure prophylactic effect of the compound, while the Day 1 p.i. treatment group (Tx-1) was used to test the effect of very early post-exposure treatment of an established infection. Previous RSV viral kinetic studies in our laboratory demonstrated that RSV replication in neonatal lambs is highest between viral inoculation and Day 3 p.i., reaching peak viral titer at Day 3, while RSV-associated lung pathology peaks at Day 6 p.i. [32]. Therefore, the Day 3 post viral challenge treatment group (Tx-2) was used
to test the effect of JNJ-49214698 on RSV-associated ALRTI when treatment is started close to peak viral titer, essentially the time patients seek first-line medical assistance and on average 1 day before they present to hospital [36–40].

The overall assessments demonstrate a strong pre-exposure protective effect of JNJ-49214698 against RSV infection. The prophylaxis (Px) group showed no gross/microscopic lung lesions and no viral antigen by IHC. In addition, viral RNA levels significantly reduced close to undetectable levels and were mirrored by no detectable IFFU[23]. Only very occasional infection of individual lung cells, as demonstrated by the presence of viral RNA (by RNAscope) in these cells was observed, consistent with earlier studies which showed that fusion inhibitors are effective in inhibiting syncytia formation by preventing transmission of the virus via cell-cell spreading [38,39]. Our data are also consistent with the prophylactic efficacy of Synagis®, a humanized monoclonal antibody inhibiting the fusion protein of RSV [8]. In contrast to Synagis®, which lacks therapeutic efficacy[8], early (Tx-1) and late (Tx-2) therapeutic administration regimens with JNJ-49214698 displayed unambiguous evidence of efficacy in the neonatal lambs. Administration of JNJ-49214698 after the establishment of a multi-cyclic ALRTI strongly reduced the severity of the infection as evidenced by the strong and statistically significant reduction of gross and microscopic changes in the lung, and decreased infectious titer in BALF as compared to vehicle-only animals [23]. Consistent with these results, a considerable reduction of the viral RNA expression in lung tissue was measured. However, the significant reduction of infectious virus in BALF of therapeutically treated animals was not mirrored by a reduction of the viral RNA to the same extent. This seeming discrepancy between viral RNA expression and infectious viral titer in the BALF may be explained by the ability of JNJ-49214698 to bind
tightly to prefusion RSV F present on the envelope of the virus, resulting in neutralization of the infectivity of virus particles present in the airways [22]. Although early administration of JNJ-49214698, starting one day after RSV infection resulted in better reduction and clearing of RSV infection as compared to the group that received late treatment starting at peak viral load, the efficacy results obtained in the latter group were clearly noticeable.

Together, the results of this study demonstrate that pre-exposure prophylaxis and treatment of an established RSV infection with a small-molecule RSV fusion inhibitor results in significant reduction of viral replication as well as improvement of RSV-associated lung pathology. Moreover, our data suggest a favorable window within which to treat RSV infections in a community or hospital setting and contribute to the de-risking of late-stage clinical compound development pathways.

References


Figures and Legends

Figure 3-1: Exposure of JNJ-49214698 in different body compartments of neonatal lambs. 

a) shows the daily plasma concentration (ng/mL) 24 hr after the first dose and daily just before the next dose (C_{trough}) to the end of the study. The compound reached a steady state concentration in the plasma within 24 h of treatment and maintained that level throughout the study. b), c) show JNJ-49214698 exposure in BALF (b) and lavaged lung (c) at necropsy (day 6 p.i.).
Figure 3-2: Clinical signs score in different groups.

Data shown as average number of RSV-associated clinical signs observed in each group (wheeze, increased respiratory rate and expiratory effort). Animals were infected with RSV and received either vehicle only (Vehicle) and animal not infected with RSV (a), prophylaxis (Px) (b) or therapeutic treatment with JNJ-49214698, starting one (Tx-1) (c) or three (Tx-2) (d) days post infection. See online supplement for detailed table of the clinical signs observed.
a) the viral RNA titer as measured by qRT-PCR in BALF, and b) the viral RNA titer as measured by qRT-PCR in lung tissue, c) number of infectious particles as measured by IFFU assay [23] with modification, all shown as average + SEM. Animals were infected with RSV and received either vehicle only (Vehicle), prophylaxis (Px) or therapeutic treatment with JNJ-49214698, starting one (Tx-1) or three (Tx-2) days post infection. Non-infected, vehicle-treated animals were indicated as No RSV. JNJ-49214698 reduces the viable viral particles in BALF, as well as RSV RNA in both BALF and lung tissue. Statistical analysis was performed by one-way ANOVA, followed by Dunnett’s post-hoc test for multiple comparison correction. *p-value < 0.02; **p-value < 0.002; ***p-value < 0.001; ****p-value < 0.0001. LLOD = lower limit of detection.
Figure 3-4: Effect of JNJ-49214698 on viral antigen and RNA in lung tissue at Day 6 p.i.

a) Integer-based scoring of M37 antigen in lung tissue detected by immunohistochemistry, b) integer-based scoring of M37 RNA in lung tissue detected by RNAscope. Scoring on y-axis represents the number of bronchioles or alveoli stained positive for M37 antigen (a) or RNA (b) Bars represent the average score per group + SEM. Animals were infected with RSV and received either vehicle only (Vehicle), prophylaxis (Px) or therapeutic treatment with JNJ-49214689, starting one (Tx-1) or three (Tx-2) days post-infection. Non-infected, vehicle-treated animals were indicated as No RSV. c) and d) show representative images of M37 antigen (c) and M37 RNA (d) in the lung tissue of animals allocated to the different treatment groups. JNJ-49214698 reduced the RSV M37 antigen and RNA in the lung of all JNJ-49214698-treated animals. Statistical analysis was performed by Kruskall-Wallis non-parametric test, followed by Dunn’s post-hoc test for multiple comparison correction. p-value bars are representative for both bronchioles and alveoli. *p-value < 0.05; **p-value < 0.01.
Figure 3-5: Effect of JNJ-49214698 on development of gross lung lesions at Day 6 p.i.

(a) shows the average + SEM % of lung surface affected by RSV-induced gross lesions in the different treatment groups. Animals were infected with RSV M37 and received either vehicle only (Vehicle), prophylaxis (Px) or therapeutic treatment with JNJ-49214689, starting one (Tx-1) or three (Tx-2) days post infection. Non-infected, vehicle-treated animals were indicated as No RSV. JNJ-49214698 significantly reduces the RSV gross lesions in the treated groups in comparison to the vehicle group. Statistical analysis was performed by one-way ANOVA, followed by Dunnett’s post-hoc test for multiple comparison correction. ****p-value < 0.0001. (b) shows representative images of lungs from an animal infected with RSV receiving vehicle only, prophylaxis with JNJ-49214698, or therapeutic treatment, starting one (Tx-1) or three (Tx-2) days post-infection and Non-infected, vehicle-treated animals were indicated as Baseline. Yellow arrows indicate vari sized dark red consolidated areas on the lung surface.
Figure 3-6: Effect of JNJ-49214698 on RSV-induced accumulated lung histopathology at Day 6 p.i.

(a) shows the average + SEM of the accumulative RSV-induced microscopic lesions in the different treatment groups. Animals were infected with RSV M37 and received either vehicle only (Vehicle), prophylaxis (Px) or therapeutic treatment with JNJ-49214698, starting one (Tx-1) or three (Tx-2) days post infection. Non-infected, vehicle only-treated animals were indicated as No RSV. JNJ-49214698 reduces the pathological changes associated with RSV infection. Statistical analysis was performed by Kruskall-Wallis non-parametric test, followed by Dunn’s post-hoc test for multiple comparison correction. *p-value < 0.05, **p-value < 0.01. (b) shows representative images of the lung tissue sections from animals allocated to the different treatment groups.
Supplementary Data

Materials and Methods

Compound and Dosing

JNJ-49214698 was discovered and synthesized by Janssen Infectious Diseases (Beerse, Belgium). The compound was formulated in 10% acidified hydroxypropyl-β-cyclodextrin [10% HP-β-CD + HCl, pH 2 (vehicle)] at 6.25 mg/mL prior to dosing and stored throughout the study at 4°C. The compound was dosed orally by catheter-mediated orogastric gavage at 4 mL/kg body weight (25 mg/kg) once daily. Dose selection in this study aimed for reaching the highest possible, safe exposure in the animals in order to maximize the likelihood to obtain efficacy while avoiding toxic side effects. A daily oral dose of 25 mg/kg was selected based on the antiviral activity of JNJ-49214698 [EC$_{50}$ = 0.4 ng/mL (0.8 nM) and EC$_{90}$ = 2.4 ng/mL (4.8 nM)], JNJ-49214698 exposure levels obtained in neonatal lambs at different doses during a separate pharmacokinetic (PK) study and an observed lack of toxicity of JNJ-49214698 at least until C$_{max}$ = 17,167 ng/mL and AUC$_{0-24h}$ = 139,993 ng.h/mL in a 5-day repeated dose rat tolerance study.

Animals

Twenty-one colostrum-deprived neonatal lambs (Suffolk, Polypay, Dorsett cross) aged 1-3 days and 2-7 kg body weights were obtained for this experiment. Animal use was approved by the Institutional Animal Care and Use Committee of Iowa State University. RSV-infected lambs were kept in a separate room from the non-infected animals in the Livestock Infectious Disease Isolation Facility (LIDIF). These rooms have separate ventilation units as well as separate entrances and exits to avoid any cross-contamination between infected and non-infected lambs. Lambs were fed iodide-free lamb milk replacer.
diet (Milk Products Inc., Chilton, WI, USA) [1], and were treated with Naxcel (Ceftiofur sodium, Pfizer) intramuscular once daily to reduce/prevent secondary bacterial infections.

**Experimental Design**

Lambs were randomly assigned to five different groups. Three groups (Px, Tx-1 and Tx-2) were infected with RSV and treated with JNJ-49214698. The first group (Px, n = 4) was treated prophylactically, 1 day before RSV challenge and then daily afterward up until Day 5 post infection (p.i.). The second (Tx-1, n = 5) and the third (Tx-2, n = 5) groups were treated one day and three days after viral challenge and daily afterward up until Day 5 p.i., respectively. The vehicle group (n = 4), serving as a positive RSV control group, was infected with RSV, but received treatment with vehicle only. The No RSV group (n = 3), served as negative RSV control group, and was aerosolized with RSV-free, HEp-2 cell-conditioned media and also received vehicle. All lambs were euthanized at Day 6 p.i. and all endpoints measured after euthanasia.

**RSV Infection**

Lambs were infected with RSV strain M37, purchased from Meridian BioSciences (Memphis, TN, USA). This strain is a wild type A RSV isolated from the respiratory secretions of an infant hospitalized for bronchiolitis [2,3]. In our laboratory, M37 was grown in HEp-2 cells and stored at -80°C in media containing 20% sucrose [4]. PARI LC Sprint™ nebulizers were used to administer virus or cell-conditioned control media (lacking RSV) to each lamb [5]. Six mL of $1.27 \times 10^7$ Infectious Forming Unit (IFFU)/mL in media containing 20% sucrose or cell-conditioned mock media (also containing 20% sucrose) was nebulized to each lamb over the course of 25-30 minutes resulting in the total inhalation of about 3 mL by each lamb.
Animal Monitoring for Appearance of Clinical Signs and the Clinical Score

Animals were monitored for clinical signs in the beginning of the study right before viral inoculation (Day 0: all animals scored 0) and immediately after each administration of vehicle or test article throughout the course of the study. In addition to the monitoring of their behavior, respiratory associated clinical signs (respiratory rate, wheezing, expiratory effort) were measured. Because clinical signs are variable in lambs and difficult to score in terms of severity, an accumulative clinical score to summarize the overall distress per group was applied by adding up the number of the scored clinical sings observed in each of the individual lambs and then averaging the daily obtained sums for the respective groups to normalize for group size.

Blood sampling for PK Analysis

Blood samples (1.5 - 2 mL) were collected from the jugular vein pre-dose (just before the first dose) and at 24 h following each dose until 144 h for PK analyses. Blood was dispensed into 3 mL blood collection tubes containing K$_2$EDTA anticoagulant. Blood samples were kept at room temperature prior to centrifugation. The blood samples were then centrifuged at 1,600 x g for 10 min at 4°C to obtain the plasma. Plasma was stored in 2 mL cryovials at -80°C.

Lung Collection and Processing

The thorax was opened, lungs removed and gross lesions were scored as performed previously (40). The lungs were also photographed in situ and ex vivo. After removal, percentage parenchymal involvement was scored for each lung lobe before the bronchoalveolar lavage fluid (BALF) collection procedure. Left and right lungs were then separated and each lobe excised. Tissue samples were collected from each lung lobe of all
animals. In brief, one sample from each lobe not destined for BALF collection (i.e. 4 lobes - Right Cranial, Left Cranial, Left Middle and Left Caudal) were snap-frozen in liquid nitrogen for qRT-PCR. Two samples from each of these lobes were placed in tissue cassettes and put in 10% neutral-buffered formalin (NBF) for histological and immunohistochemical analysis. Representative lung samples from each of these lobes were also placed into a cryovial and immediately snap-frozen in liquid nitrogen, then transferred to -80°C for storage and shipped on dry ice pellets for PK analysis of JNJ-53718678 compound at Janssen.

**BALF Collection**

BALF samples from each animal were collected immediately after euthanasia on Day 6 p.i. from right middle and right caudal lobes as performed previously in our laboratory [6]. Briefly, the excised lung lobes were instilled with 5 mL of cold DMIM (42.5% Iscove's modified Dulbecco's medium, 7.5% glycerol, 1% heat-inactivated FBS, 49% DMEM, and 5 μg/ml kanamycin sulfate). 100 μL of the right caudal lobe BALF was added to 1 mL TRIzol (Invitrogen) and kept in – 80 °C for the qRT-PCR assay to assess RSV mRNA, and the rest of BALF sample was placed on ice and used within 2 h for infectious focus-forming unit (IFFU) assay to assess the infectious RSV titer. The right middle lobe BALF sample placed -80°C for storage and shipped on dry ice pellets for determination of JNJ-49214698 and blood urea nitrogen concentrations at Janssen.
Quantification of JNJ-49214698 Exposure

Samples of plasma (50 µL), lung homogenate (50 µL) and BALF (50 µL) were analyzed for JNJ-49214698 using a method based on protein precipitation and HPLC/MS/MS analysis. To each sample, DMSO (50 µL) and acetonitrile (500 µL) was added. Samples were mixed thoroughly (mechanical shaking for 10 min), and then centrifuged (5000 × g for 10 min at 15°C). An aliquot (400 µL) of the resulting supernatant was transferred to a 96-well plate and assayed for JNJ-49214698 concentrations using HPLC/MS/MS employing positive-ion electrospray ionization (Sciex API 4000) and a Waters ACQUITY UPLC C18 1.7µm (50 x 2.1 mm i.d.) column. Elution was achieved at a flow rate of 0.8 mL/min with a gradient of 0.1% FA and acetonitrile. The lower limit of quantification was 1 ng/mL for plasma, 50 ng/g for lung and 1 ng/mL for BALF. The assay was linear up to 20,000 ng/mL for plasma, 5,000 ng/mL for BALF and 100,000 ng/g for lung. Samples of plasma and BALF were also analyzed for concentrations of urea in order to calculate the dilution of BALF on sample collection. To a separate sample of plasma and BALF (50 µL), 50 µL H2O/acetonitrile (50/50), 50 µL internal standard, 200 µl acetonitrile was added. Samples were mixed thoroughly (mechanical shaking for 10 min), and then centrifuged (5000 × g for 10 min at 15°C). An aliquot (200 µL) of the supernatant was transferred to a 96-well plate and evaporated at 40°C with nitrogen. An aliquot (200 µL) of camphanic chloride (1 mg/mL in acetonitrile) was added for derivatization. Samples were mixed thoroughly (mechanical shaking for 10 min) and incubated for 90 min at 37°C. After incubation, 100 µL H2O was added and assayed for urea concentrations using HPLC/MS/MS employing positive-ion electrospray ionization (Sciex API 4000) and a Waters ACQUITY UPLC HSS T3 (50 x 2.1 mm i.d.) column. Elution was achieved at a flow rate of 1 mL/min with a gradient of 0.01M Ammonium formate (pH = 3).
and acetonitrile. The lower limit of quantification was 2 µg/mL for plasma and BALF. The assay was linear up to 10,000 µg/mL.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Tissue samples from right and left cranial, left middle and left caudal lung lobes (0.3–0.4 g of each lobe) were homogenized in TRIzol (Invitrogen, Carlsbad, CA) for total RNA isolation according to manufacturer’s instructions and as previously described [4,6,7]. Briefly, RNA isolation was followed by DNase treatment (Ambion, TURBO DNase, Austin, TX) at 1:10 dilution in combination with RNaseOUT (Invitrogen) and nuclease-free water ( Gibco/Life Technologies, Carlsbad, CA). Spectrometry (Beckman Scientific, Indianapolis, IN) was used to assess each RNA sample isolate at a dilution of 1:50 to measure sample purity and quantity (A260nm/A280nm all >1.95). Agilent Bioanalyzer 2100 analyses of the RNA isolates gave RNA Integrity Number values > 8.0. qRT-PCR was carried out using One-Step Fast qRT-PCR Kit master mix (Quanta, BioScience, Gaithersburg, MD) in a StepOnePlus™ qPCR machine (Applied Biosystems, Carlsbad, CA) in conjunction with PREXCEL-Q assay-optimizing calculations [8,9]. Primers and probe for RSV M37 nucleoprotein were designed based on RSV accession number M74568. Forward primer: 5′-GCTCTTAGC-AAAGTCAAGTGAACGA; reverse primer: 5′-TGCTCCGTTGGATGGTGTATT; hydrolysis probe: 5′-6FAM-ACACTCAACAGATCATCTGTCATCCAGC-TAMRA. Each 1:10-diluted total RNA sample was further diluted so that each final qRT-PCR contained 0.784 ng total RNA/µL; as determined to be optimal by PREXCEL-Q [10]. Thermocycling conditions were 5 minutes at 50°C; 30 seconds at 95°C; and 45 cycles of 3 seconds at 95°C and 30 seconds at 60°C. Samples and standards were assessed in duplicate and each qRT-PCR quantification
cycle (Cq) was converted to a relative initial quantity ($X_0$) based on a standard curve using the following equation: $X_0 = E_{\text{AMP}}(b-Cq)$, where $E_{\text{AMP}}$ and $b$ are the PCR exponential amplification efficiency value and the y-intercept, respectively, from a sample mixture derived standard curve for RSV M37 nucleoprotein mRNA. The efficiency-corrected delta Cq ($E_{\text{AMP}}\Delta Cq$) method was used for qRT-PCR quantification calculations. Results were normalized to total tissue RNA loaded per reaction (identical for all reactions). No-RT control reactions proved negative for RSV M37. qRT-PCR was demonstrably free of inhibition based on preliminary dilution threshold analyses as per the PREXCEL-Q method for qPCR [10,11].

**Hematoxylin-Eosin Staining and Histological Scoring of Lung Sections**

Hematoxylin-eosin stained sections were examined via light microscope as described previously [6] with some modification. Lung lesions were scored according to an integer-based score of 0-4 for each parameter (bronchiolitis, syncytial cells, epithelial necrosis, epithelial hyperplasia, peribronchial lymphocytic infiltration, perivascular lymphocytic infiltration, neutrophils), with 4 as the highest score. Then a final score (accumulative histol lesion score) was assigned by adding up the scores from the seven individual parameters resulting in final accumulative scores ranging from 0-28 representing the total RSV-associated lesion in each tissue section. The scale used to assess the pathological changes briefly, for bronchiolitis scale: 0 = no remarkable lesions, 1 = minimal detectable lesion (epithelial degeneration in one or a few bronchioles per 20 X field), 2 = epithelial degeneration involving less than 10% of the airway lumen; minimal neutrophils and cell debris; adventitial lymphocytes in multiple bronchioles, 3 = epithelial degeneration involving more than 10-50% of the airway lumen with cell debris and neutrophils; adventitial
lymphocytes; multiple bronchioles. For syncytial cells scale: 0 = none, 1 = one distinct syncytial cell, 2 = up to three in three 20x fields, 3 = more than three in three fields, 4 = numerous. Epithelial necrosis and epithelial hyperplasia scale: 0 = none, 1 = minimally detectable in one or a few bronchioles per 20x per field, 2 = 10% of the bronchioles in multiple airways per field, 3 = 10-50% of the bronchioles in multiple airways per field, 4 = circumferential in multiple airways. Neutrophils scale (in bronchi, bronchioles or alveoli): 0 = none, 1 = minimally detectable, 2 = 10 or less neutrophils in one or a few airways/alveoli, 3 = 10 or more neutrophils in several airways/alveoli, 4 = 10 or more involving many or most airways/alveoli. The same scale was used for peribronchiolar lymphocytic infiltrates and perivascular lymphocytic infiltrates: 0 = none, 1 = earliest detectable lymphocytic infiltration in the adventitia, 2 = segmental to circumferential infiltration, 3 = circumferential infiltrates that expand more than three cells wide, 4 = circumferential infiltrates that form nodules.

**Immunohistochemistry (IHC) of Lung Sections**

IHC for determining the distribution of RSV antigen was performed as described previously [6,12,13]. Briefly, after deparaffinization and rehydration of the formalin fixed paraffin-embedded tissue sections, antigen retrieval was performed by placing the slides with tissue sections in pH 9.0 10mM TRIZMA base, 1mM EDTA buffer and 0.05% Tween 20 and boiling under pressure for up to 15 minutes. Polyclonal goat anti-RSV antibody (Millipore/Chemicon, Temecula, CA; Cat. No. AB1128) was used as the primary antibody after two blocking steps, the first with 3% bovine serum albumin in Tris-buffered saline + 0.05% Tween 20 (TBS-T) and the second with 20% normal swine serum in TBS-T, 15 minutes each. Primary antibody was followed by biotinylated rabbit anti-goat secondary
antibody (KP&L; Cat. No. 16-13-06). Signal development was done by using 1:200 dilution of streptavidin-horseradish peroxidase (Invitrogen; Cat. No. 43-4323) for 30 minutes followed by incubation with Nova Red chromagen solution (Vector; Cat. No. SK-4800). The positive signal was quantified in both bronchioles and alveoli for each tissue section, and a score of 0-4 was assigned according to an integer-based scale of 0 = no positive alveoli/bronchioles, 1 = 1-10 positive alveoli/bronchioles, 2 = 11-39 positive alveoli/bronchioles, 3 = 40-99 positive alveoli/bronchioles, 4 = >100 positive alveoli/bronchioles.

**RNAscope**

Formalin fixed paraffin embedded (FFPE) IHC tissue sections were used for the RNAscope detection of RSV mRNA in situ. A probe designed to the hRSV M37 nucleoprotein gene was used (Probe-V-RSV-NP, Advance Cell Diagnostic, Catalog number 439866). This probe was designed to target the 8-1111 base region of the nucleoprotein gene of accession number KM360090.1 CDS sequence (1114-2289). The assay was performed according to the manufacturer’s manual (user manual document number 320497; RNAscope® 2.0 HD Detection Kit (BROWN) User Manual PART 2). Sections were examined under light microscope, and the number of bronchioles and alveoli containing the positive signal were counted. The number of positive bronchioles and alveoli per tissue section was then assigned a score according to the simple integer-based scale of 0 = no positive alveoli/bronchioles, 1 = 1-10 positive alveoli/bronchioles, 2 = 11-39 positive alveoli/bronchioles, 3 = 40-99 positive alveoli/bronchioles, 4 = >100 positive alveoli/bronchioles.
**Infectious Focus-Forming Unit (IFFU) Assay**

Viral titers in BALF from the right caudal lobe were determined by IFFU assay [6]. BALF samples were spun down for 5 minutes in a centrifuge at 3,000 x g to pellet large debris. Approximately 800–850 μL of each supernatant was collected and then spun through 850 μL-capacity 0.45 μm Costar SPIN-X filter (microcentrifuge 15,600 x g) for 5 minutes. The resulting clear BALF samples were applied to HEp-2 cells grown to 70% confluence in 12-well culture plates (Fisher Scientific, Hanover Park, IL) at full strength and three serial dilutions (1:10, 1:100, and 1:1000), all tested in triplicate. The BALF samples in the wells were diluted with DMEM media (Mediatech, Inc., Manassas, VA) supplemented with 10% with heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA) and 50 μg/mL kanamycin sulfate (Invitrogen/Life Technologies). Plates were incubated for 1 hr at 37°C, 5% CO₂, then 1 mL of media was added to each well and plates were returned to the incubator. After a 48 hr incubation, wells were fixed with cold 60% acetone/40% methanol solution for 1 minute. Wells were then rehydrated with TBS-T, then blocked with 3% BSA solution for 15 minutes followed by overnight incubation with primary polyclonal goat anti-RSV (all antigens) antibody (EMD Millipore Corporation, Billerica, MA, USA). The next day, plates were washed with TBS-T then incubated for 1 hr with secondary antibody (Alexa Fluor® 488 F(ab’)2 fragment of rabbit anti-goat IgG (H+L) (Molecular Probes/Life Technologies). Plates were rinsed and examined under inverted fluorescence microscopy using the FITX/GFP filter (Olympus CKX41, Center Valley, PA), and total number of IFFUs (which is defined here as 3 or more fluorescing cells) were counted for each well. IFFU/mL calculations were obtained by multiplying the average number resulting from triplicate well counts by the initial BALF sample dilution factor and multiplying that value by 5 to obtain counts/mL since 1,000 μL/200 μl (the actual sample applied for each well) equals 5.
Statistical Analysis

Statistical analysis was completed by using the Kruskall-Wallis test for non-parametric parameters such as accumulative microscopic lesion scoring, immunohistochemistry and RNAscope integer-based scores, followed by Dunn’s post-hoc test for multiple comparisons. One-way ANOVA followed by Dunnett multiple comparisons test was used to compare the treated groups to the RSV-infected non-treated control group for gross lesion scores and viral titer analyses by qRT-PCR and IFFU assays.
Table 1: clinical signs observed in lambs in different groups throughout the study period

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W = wheeze, L = lethargy, R = increased respiratory rate, Ee = expiratory effort, D = Dead, ND = nasal discharge.
Panels show the average + SEM of integer-based scoring of the lung microscopic criteria induced by RSV infection. Animals were infected with RSV and received either vehicle only (Vehicle), prophylaxis (Px) or therapeutic treatment with JNJ-49214689, starting one (Tx-1) or three (Tx-2) days post infection. Non-infected, vehicle-treated animals were indicated as No RSV. Assessed criteria were a) bronchiolitis, b) syncytial cell formation, c) epithelial necrosis, and d) neutrophils. (see supplementary data for Figure 8, which shows epithelial hyperplasia, peribronchiolar nodules, and perivascular nodules. JNJ-49214698 greatly reduces all assessed criteria in all treated lambs in comparison to the RSV-infected vehicle-treated lambs. Statistical analysis was performed by Kruskall-Wallis non-parametric test, followed by Dunn’s post-hoc test for multiple comparison correction. *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

Figure 3-7: Effect of JNJ-49214698 on RSV-induced lung histopathology at Day 6 p.i.
Panels show the average + SEM of integer-based scoring of the lung microscopic criteria induced by RSV infection. Animals were infected with RSV and received either vehicle only (Vehicle), prophylaxis (Px) or therapeutic treatment with JNJ-49214689, starting one (Tx-1) or three (Tx-2) days post infection. Non-infected, vehicle-treated animals were indicated as No RSV. Assessed criteria were a) epithelial hyperplasia, b) peribronchiolar nodules, and c) perivascular nodules. JNJ-49214698 greatly reduces all assessed criteria in all treated lambs in comparison to the RSV-infected vehicle-treated lambs. Statistical analysis was performed by Kruskall-Wallis non-parametric test, followed by Dunn’s post-hoc test for multiple comparison correction. *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

Figure 3-8: Effect of JNJ-49214698 on RSV-induced lung histopathology at Day 6 p.i.
References


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CHAPTER 4 THERAPEUTIC EFFICACY OF RESPIRATORY SYNCYTIAL VIRUS NON-FUSION INHIBITOR (RSV-NFI) IN NEONATAL LAMBS INFECTED WITH A HUMAN STRAIN OF RSV

A paper will be submitted to virology journal.

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Summary

Respiratory Syncytial Virus (RSV) infects individuals of all ages and can cause severe lower respiratory tract infection in infants and elderly. Given the burden of RSV disease and the lack of any vaccine or efficacious therapeutic intervention, the discovery of new antiviral compounds are in critically need. Therefore, the efficacy of RSV-NFI, a non-fusion RSV inhibitor, was tested for efficacy in this study in the neonatal lamb model. Five groups of lambs were used to test the efficacy of this compound at different concentrations. Three groups, TX1, TX2, and TX3, were treated with RSV-NFI 24hr after RSV inoculation and once daily with 16, 4, or 1 mg/kg body weight, respectively. The two other groups were treated with vehicle only after nebulization with RSV or mock media. All lambs were euthanized at day 6 post-infection, which corresponds to the expression of severe disease features in untreated animals. Pharmacokinetic analysis indicated that RSV-NFI reached a suitable plasma concentration within 24 hr after administration, and this level persist throughout the study that helped relate the plasma level to the antiviral efficacy. All tested
doses drastically reduced the appearance of RSV-induced gross and microscopic lesions. There was a significant reduction in the viral load in the lung in both TX1 and Tx2 groups. Together; these results demonstrate efficacy of the RSV-NFI in reducing RSV infection and inhibiting RSV-induced lung pathology.

**Introduction**

Respiratory Syncytial Virus (RSV) is a common respiratory virus in all ages and leads to moderate upper respiratory tract infection. However, RSV can progress to severe acute lower respiratory tract infection (ALRTI) in children under the age of five, older adults, and immune compromised individuals[1,2]. A systematic review and meta-analysis of RSV infection worldwide estimated that 2.4 million episodes of severe RSV associated ALRTI of 33.8 million total RSV infection each year[3]. Currently, there is no fully effective treatment or vaccine available for RSV infection.

Recently, several antiviral compounds showed promising antiviral activity against RSV ALRTI and some of these have been approved for human clinical trials [4]. Only two approved treatments are commercially available including Palivizumab, which is a monoclonal antibody against RSV fusion protein administered as prophylaxis for preterm infants and high-risk individuals, and Ribavirin, a nucleoside analog antiviral drug [5,6]. Both of these compounds have limited indication and therapeutic efficacy [7–10]. Developing RSV antiviral drugs generally fall into three different categories: nucleoside inhibitors, fusion inhibitors, and non-fusion non-nucleoside inhibitors [4]. A recent study with a fusion protein inhibitor indicated the possibility of treating existing RSV infection even after peak viral load and lung pathology and demonstrate a wide therapeutic intervention window for RSV [11].
Several models for RSV infection have been developed, and of these the neonatal lamb model has some characteristics similar to those in infants with RSV lower respiratory tract infection [12]. Lambs are susceptible to the human strain of RSV that causes moderate lower respiratory tract infection [13], have similar lung size, lung anatomy, and cellular composition of human infants, with similarity in immune responses and lung development. In this study, we evaluate the therapeutic efficacy of RSV-NFI, which is a non-fusion non-nucleoside RSV inhibitor, against RSV lower respiratory tract infection in the neonatal lamb model of RSV infection.

**Materials and Methods**

**Experimental Design**

This study was completed in two parts in an experimentally identical environment. Animal use was approved by the Institutional Animal Care and Use Committee of Iowa State University. In the first part of the study, 2 high doses of RSV-NFI were tested with 4 experimental groups: RSV infected group; RSV (n=3) treated with vehicle only, TX1 (n=2) treated with RSV-NFI 16mg/kg body weight and TX2 (n=4) treated with RSV-NFI 4 mg/kg body weight. The fourth group (n=4) infected with mock media and treated with vehicle to serve as a negative control group. In the second part of the study, the efficacy of RSV-NFI low dose (1mg/kg body weight) was tested. 21 lambs were divided into 3 groups. There were two RSV infected groups: RSV (n=6) treated with vehicle only, and TX3 (n=7) treated with 1mg/kg RSV-NFI one day after viral nebulization, and a third group with no RSV (n=8) were nebulized with mock media and treated with vehicle only (Figure 1). Lambs were nebulized with 6 ml hRSV strain M37 (1.27 x 10^7 Infectious Forming Unit (IFFU)/mL) on day 0. M37 is wild-type RSV A strain isolated from human infant and commercially provided by Meridian BioSciences (Memphis, TN, USA)[14,15].
Treatments either with RSV-NFI or vehicle were started 24 hr after viral nebulization, then once daily for 5 days. Lambs were monitored daily for the presence of clinical signs, and then all lambs were humanely euthanized at day 6 post nebulization.

**Tissue and Sample Collection**

At necropsy (day 6 post nebulization), lambs were euthanized, and lungs were examined *in situ*, removed, and the percent of the lung with lesion were determined. Lung tissues were collected from right cranial, right middle, right caudal, and left cranial and placed in 10% neutral buffered formalin for histologic evaluation, or snap frozen in liquid nitrogen for qRT-PCR and pharmacokinetic study. Also, BALF samples were collected for left caudal lobe for infectious focus forming unit assay (IFFU) and RT-qPCR assays, and from left middle lobe for compound pharmacokinetic level assessment.

**Infectious Focus-Forming Unit (IFFU) Assay**

IFFU was completed on caudal lobe BALF, and homogenized lung tissue samples from the 4 collected lung lobes, and assays were performed as previously described in our laboratory [16]. Briefly, samples were pelleted and resultant supernate was spin again through a 0.45 μm Costar SPIN-X filter (microcentrifuge 15,600 x g) for 5 minutes. The filtered samples were applied to 70% confluent HEp-2 cells in a 12-well culture plates (Fisher Scientific, Hanover Park, IL) at full strength and three serial dilutions (1:10, 1:100, and 1:1000)in triplicate. After initial incubation at 37°C, 5% CO₂ for 1 hr, 1 mL of media was added to each well and plates were returned to the incubator and incubated for 48 hrs. plates, then, fixed with cold 60% acetone/40% methanol solution for 1 minute followed by immunofluorescent staining. Primary and secondary antibody were used. The primary was polyclonal goat anti-RSV (all antigens) antibody (EMD Millipore Corporation, Billerica, MA, USA), and the secondary antibody was (Alexa Fluor® 488 F(ab’)2
fragment of rabbit anti-goat IgG (H+L) (Molecular Probes/Life Technologies). Plates examined under inverted fluorescence microscopy using the FITX/GFP filter (Olympus CKX41, Center Valley, PA), and the total number of IFFUs (which is defined here as 3 or more fluorescing cells) were counted for each well. IFFU/mL calculations were obtained by multiplying the average number resulting from triplicate well counts by the initial BALF sample dilution factor and multiplying that value by 5 to obtain counts/mL since 1,000 μL/200 μl (the actual sample applied for each well) equals 5.

**Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)**

RSV RT-qPCR were completed on BALF and lung tissue. Lung samples from the 4 collected lung lobe were homogenized in TRIzol and extracted for total RNA according to the manufacturer's instruction. RNA samples were used for qRT-PCR as described previously[13,16–18] to detect RSV, IP-10, MCP-1, MIP-1α, PDL-1, IFN-λ, IL-13, and RANTES mRNA expression.

**Histologic Evaluation**

Microscopic evaluation of the tissue section were completed with hematoxylin and eosin stained tissue sections, slides stained RSV antigen by immunohistochemistry, and slides processed by RSV RNAscope to detect RSV mRNA. In H and E sections, RSV lesions were scored on a scale of 0-4. These changes include bronchiolitis, syncytial cells, epithelial cells necrosis, epithelial cells hyperplasia, neutrophils, bronchiolar nodules, and perivascular nodules. Then an accumulative histological lesion score was determined by adding up the individualized score resulting in a score of 0-28 that represent the overall pathological changes in each tissue section (FI paper). Immunohistochemistry was completed on formalin fixed paraffin embedded tissue sections and as described previously [16,19,20]. Briefly, the slides were deparaffinized and
rehydrated; then antigen retrieval step were completed using PH 9 10mM TRIZMA base, 1mM EDTA buffer and 0.05 tween 20, then boiled and put in a steamer to maintain the temperature for at least 30 minutes. Tissues were blocked with 3% BSA and 20% NSS for 15 minutes each, followed by 1:500 dilution goat anti-RSV polyclonal antibody (Millipore/Chemicone Cat. No. AB1128) and incubated in 4C overnight. The next day, tissue were washed 3 times with TBS-tw followed by 1 hr incubation with 1:300 dilution of biotinylated Rabbit anti Goat secondary antibody (KP&L Cat. No. 16-13-06). After 3 washes with TBS-tw, tissue were incubated with 3% H2O2 for 25 minutes in room temperature followed by 2 TBS-tw washes and signal detection by incubation with 1:200 dilution streptavidin-horseradish peroxidase (Invitrogen Cat. No. 43-4323) followed by Nova Red staining solution. Slides were examined under light microscope and signal were quantified in both alveoli and bronchioles. Score of 0-4 were applied according to an integer based scale (of 0 = no positive alveoli/bronchioles, 1 = 1-10 positive alveoli/bronchioles, 2 = 11-39 positive alveoli/bronchioles, 3 = 40-99 positive alveoli/bronchioles, 4 = >100 positive alveoli/bronchioles). The RNAscope assay was also done on FFPE tissue sections for the detection of RSV mRNA in situ by using a probe for RSV M37 nucleoprotein gene (Probe-V-RSV-NP, Advance Cell Diagnostic, Catalog number 439866). The assay were done as described previously (FI paper) and according to the manufacturer protocol ((user manual document number 320497; RNAscope® 2.0 HD Detection Kit (BROWN) User Manual PART 2). Microscopic tissue sections were examined, and RNA signal were counted in both alveoli and bronchioles and applied for the same integer based scale used to assess the IHC, which is 0 = no positive alveoli/bronchioles, 1 = 1-10 positive alveoli/bronchioles, 2 = 11-39 positive alveoli/bronchioles, 3 = 40-99 positive alveoli/bronchioles, 4 = >100 positive alveoli/bronchioles.).
Statistics

When appropriate and unless specified otherwise, due to the limited number of data observations in some treatment groups, the non-parametric Kruskal-Wallis test is applied to test whether there is difference between the compound groups and the vehicle group. P-values are adjusted with Bonferroni’s multiple comparisons method. For the histological analysis of RSV antigen producing cells and for the histopathological analysis, treatment group at a value of zero were remove from the analysis (on/off patterns cannot be appropriately analyzed by statistics. However, this pattern indicates the presence of a treatment effect). In the case of the histological analysis of RSV antigen producing cells, scores of at least 1 were recoded to 1 and logistic regression with random effects was applied for Slide and Animal to compare differences between treatment Tx3 and Vehicle. In the case of the histopathology analysis, scores of at least 1 are recoded to 1 and logistic regression with random effects for Animal was used to compare differences between treatments and Vehicle results.

Results

Pharmacokinetics of Different Doses of RSV-NFI in the Neonatal Lambs

Antiviral compounds need to reach an adequate and consistent exposure in the lung tissue in order to protect against multicyclic RSV infection. First, we assessed RSV-NFI levels in the plasma, then the compound distribution to the lung was evaluated. Compound absorption allowed a rapid increase in plasma exposure within 1 hr. Steady state was not reached at 24h probably due to the long half-life and slow elimination/metabolism of the compound. RSV-NFI plasma level at 24 hr after the first dose were respectively :5902ng/ml (16 mg/kg group), 871ng/ml (4 mg/kg group), and 265ng/ml (1 mg/kg group:) with more than dose proportional increase of 5.0 and 4.6 was observed when comparing 1 to 4 mg/kg doses and 4 to 16 mg/kg doses, respectively (Figure:
2a). Once-daily treatment with 1, 4 or 16 mg/kg of RSV-NFI lead to an increase of C_{trough} over time across all different dose groups (3.6 fold at 1 mg/kg, 3.5 fold at 4 mg/kg, and 3.0 fold at 16 mg/kg). At necropsy, the RSV-NFI concentration was measured in both BALF representing the airway lumen/secretion, and in the whole lung homogenate representing lung tissue (cells, interstitial tissue, and vasculature). The compound exposure in bronchoalveolar lavage fluid (BALF) and homogenized-lungs tissue were the highest in the 16 mg/kg group (24431 ng/ml, 36213 ng/g, respectively). BALF exposure in 4 and 1 mg/kg group was (2091 ng/ml, 299 ng/ml, respectively), and in the lung were (9740 ng/g, 2650 ng/g, respectively) (Figure 2 b & c). Subsequently, lung/ plasma ratio of 1.3, 0.9, 0.9 in animal treated with 1, 4, and 16 mg/kg, respectively, indicating a dose-dependent distribution of the compound to the lung compartments and optimal conditions for investigating therapeutic exposure levels.

**Viral Load in BALF and Lavaged-Lung Samples of Neonatal Lambs**

The viral titer was determined at day 6 post-infection in BALF and homogenized lung tissue samples from animals that were treated once-daily as from Day 1 post-infection with either vehicle or different concentrations of RSV-NFI by the IFFU assay. There was a strong concentration-dependent decrease of the production of infectious virions in BALF with 1.5 log_{10} reduction at 1 mg/kg, 4.2 log_{10} reduction at 4 mg/kg (p<0.01), and 4.5 log_{10} reduction at 16 mg/kg (p<0.01) (Figure 3 a). Concomitantly, lung titers of infectious virions were drastically reduced in all compound treated groups. There was a 2.0 log_{10} reduction in the viral titer in 1 mg/kg treated group (p<0.01), while full effect of 2.2 log_{10} reduction in groups treated with 4, and 16 mg/kg (p<0.01) & (p<0.05), respectively (Figure 3b). To assess whether the reduction in the viable viral particle was accompanied by a reduction in the viral RNA, a qRT-PCR was done on the same BALF samples and lung tissues representative from four lung lobes. There was a great reduction in viral RNA
copies in both BALF and lung (Figure 3c and 2d). There was a significant 1.4 log_{10} decrease in viral RNA at 1 mg/ml in BALF (p<0.0001) accompanied by 1 log_{10} reduction lung tissue. Superior effects were observed at 4 mg/kg and 16 mg/kg treatment as is shown by 2.8 log_{10} reduction in BALF(p<0.0001), accompanied by 4.4 log_{10} and 4 log_{10} reduction in lung tissue, respectively ((p<0.001) (p<0.0001) respectively). These results prove that the compound was efficient in reducing the viral load in the treated lambs.

**Treatment with RSV-NFI Significantly Reduced RSV-Induced Lung Lesion**

Six days after infection, lungs from animals across different study groups were removed, and the gross lesion developed by the lung upon RSV infection were scored. The RSV gross lesion which was prominent in the RSV infected vehicle-treated group characterized by multifocal dark red areas of consolidation on the lung surface and extend deep into the lung parenchyma in cut section. In lungs of non-infected vehicle-treated animals, no gross lesions compatible with RSV infection was seen. In contrast, in infected vehicle-treated animals, a significant level of the lung surface area (32% on average) had gross lesions compatible with RSV infection (Figure 4 a and b). Treatment with different doses of RSV-NFI drastically reduced the level of gross lesions observed in all treated group when compared to the RSV infected vehicle-treated group. Gross lesions were decreased to 0.76 % (p<0.05) or 0% (p<0.01) of lung surface when animals were treated with 1 mg/kg or higher doses (4 and 16 mg/kg), respectively (Figure 4 a and b).

Microscopically, different concentrations of RSV-NFI resulted in a drastic reduction of all assessed parameters and subsequently the accumulative histopathologic lesion score. The histopathological lesion observed in the RSV infected vehicle-treated group was severe interstitial pneumonia with bronchiolitis. There was inter-alveolar wall thickening due to inflammatory cell infiltration, and epithelial cell hyperplasia with occasional necrotic epithelial cells and syncytia
formation leading to an accumulative histopathologic score of 11 (figure 5 a and b and figure 8). The compound treatments significantly reduce the accumulative histopathologic lesion score in all treated group (P<0.01) (Figure 5 a and b). 16 mg/kg reduced the accumulative histopathologic score to 0.12, while 4mg/k and 1mg/kg treatments reduced the accumulative histopathologic score to 0.6 and 1.2 (Figure 5 a and b). To further evaluate the degree of RSV infection in the lung tissue, RSV immunohistochemical stain was done on FFPE tissue sections. RSV M37 antigen demonstrated a dose-dependent decrease in the density of M37 antigen in the different lung lobes. No RSV antigen detected in animals treated with 4 and 16 mg/kg (P<0.01 and P<0.05, respectively) (Figure 6 a). There was a significant decrease in the RSV antigen expression in bronchioles of animal treated with the low dose RSV-NFI (1 mg/kg)(p<0.05), with a high reduction in the alveoli (P=0.059) indicating the efficiency of treatment with the study compound to reduce RSV dissemination. Similar to what found in qRT-PCR of the BALF and lung tissue, there was a significant reduction in RNA expression in situ in all RSV-NFI treated groups (P<0.01) (Figure 7 b) as tested by RNAscope technique. The compound treatment drastically reduces RSV mRNA in all treated groups with the most reduction was in 16 and 4 mg/kg treatment. Hence, the RSV-NFI showed a strong effect in reducing RSV antigen and mRNA and subsequently RSV associated lung lesion.

**Treatment with RSV-NFI Significantly Decrease Inflammatory Chemokines**

By qRT-PCR in lung at day 6 p.i. RSV-NFI greatly reduce mRNA expression of IP-10, MCP-1, MIP-1α, PDL-1, and IFN-λ in comparison to the RSV infected vehicle-treated group in which the expression of these chemokines were elevated (Figure 9 a-e). There was no difference shown in the endogenous levels of IL-13 and RANTES mRNA expression (figure 9 f, g).
Discussion

Given the burden of RSV infection in infant and elderly, the development of active anti-RSV therapeutic is urgently needed. Several antiviral drugs that target RSV have reached advanced clinical trials. Most of these antiviral drugs are monoclonal antibodies that inhibit fusion [4]. However, RSV resistant mutants can potentially form after few passages [21] and thus, additional antiviral compounds with different mode of action that targets other viral structural component is needed. In this study, we evaluate a non-fusion RSV inhibitor RSV-NFI, which is a non-nucleoside replication inhibitor, in the neonatal lamb model. This study aims to assess the therapeutic effect of different doses of RSV-NFI on established RSV associated acute lower respiratory tract infection with treatment started one day after the viral nebulization and repeated once daily up to 5 days. Our results demonstrated a potent antiviral activity as reflected by reduced viral titer, mRNA expression, and RSV associated lesions in infected lambs.

Different concentrations of the compound resulted in different plasma exposure with more than dose proportional when comparing each treatment with the one higher. However, the plasma exposure was stable throughout the study with a tendency to increase over time. All the treatment groups reached a concentration above the EC90 after 24 hr post-treatment except for 1mg/kg treatment group which reached a concentration above the EC90 48hr after the first dose. This plasma concentration leads to adequate lung exposure (at day 6 post-infection) which reflect the concentration pattern seen in the plasma, i.e., high dose group produced a higher RSV-NFI concentration in the lung tissue. Consistent RSV-NFI presence in the lung gives the compound the ability to act on each new viral cycle reducing the RSV replication and cellular reinfection.

Lambs are permissive for hRSV and the human strains replicates well in lambs airways causing acute lower respiratory tract infection [19,20,18,22]. Lambs nebulized with hRSV have
the peak viral load by day 3 of infection with the most lung lesions developing at day 4 to 6 post nebulization [16]. RSV infected and vehicle-treated lambs in this study had high infective virus and mRNA levels with distinct lesions in lung tissue characterized by interstitial pneumonia and bronchiolitis. In contrast, treatment with different concentrations of RSV-NFI resulted in consistent and significant reductions of all the assessed endpoints such as the reduction of lung viral titers as measured by IFFU assay or qRT-PCR and reduced RSV associated lung lesions. The results of both IFFU and qRT-PCR indicate the presence of viable RSV and whole viral load, respectively, and were consistent and had the same pattern in BALF and lung tissue, and both had a great reduction in the viral load with treatment that correlated with the RSV-NFI concentration. Treatment with RSV-NFI as low as 1mg/kg caused a drastic reduction in the viral load in lung. However, the most pronounced reduction occurred in 4 and 16 mg/kg groups. Several studies indicated the positive relation between viral load and disease severity in infant [23–25]. Thus, the observed reduction in the viral load in this study predicted to have a substantial effect in reducing the pathological lesions associated with RSV by giving the lambs the ability to respond and repair the tissue damage.

RSV associated gross and microscopic lesions were significantly decreased in all treated animals. Almost no RSV lesions were seen at day 6 post-infection in the 4 and 16 mg/kg groups with a great decline the lesions in the 1 mg/kg treated group. These results were consistent with RSV immunohistochemistry and RNAascope results that had similar reduction trend in RSV antigen and RSV RNA expression. Additionally, the increased inflammatory cell infiltration in lung tissue of RSV infected vehicle treated lambs were accompanied by elevated level of MCP-1 and MIP-1α, and consistent with an earlier study in lamb indicated increase level of these chemokines in RSV infected lambs at day 6 p.i. [18]. Several studies on RSV lower respiratory
tract infection in infants indicated that elevated levels of IP10, MIP-1α, and MCP-1 act as a chemoattractant for inflammatory cells and subsequently associated with severe bronchiolitis and hospitalization [26,27]. In contrast, an elevated level of PDL-1 in RSV infected vehicle treated lambs may suggest a vital role in inhibiting lymphocytes antiviral response by modulating PDL-1 expression in infected epithelial cells [28,29]. The presence of minimal RSV antigen and mRNA in the lung tissue section of the treated group indicate the ability of RSV-NFI to halt RSV replication and infection of adjacent cells.

In summary, the compound under investigation had a strong effect on reducing RSV viral load and lesions in all treated groups in comparison to the RSV infected vehicle-treated group. This strong treatment effect occurred even when RSV-NFI was delivered to lambs one day after viral nebulization, indicating the efficacy of the compound as a post infection therapeutic modality to decrease the severity of lower respiratory tract infection after established RSV infection.

References


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Figure 4-1: Experimental design.

a) RSV-NFI dosing schedule. b) RSV nebulization, treatment, and blood draw timeline.
Figure 4-2: Exposure of RSV-NFI in different body compartments.

a) RSV-NFI daily plasma concentration (ng/ml) 24 hr post-treatment. RSV-NFI concentration in BALF (b) and homogenized lung tissue (b) at day 6 post-infection shown as average ± SEM. The compound reached a steady state concentration in the plasma and accumulated over time with subsequent high exposure in BALF and lung tissue after daily dosed with either 1, 4 or 16 mg/kg of RSV-NFI.
Figure 4-3: Effect of RSV-NFI on viral titer in BALF and lung tissue homogenates at day 6 p.i.

a and b show the infectious viral titer as measured by IFFU assay in BALF (a) and in lung tissue (b). c and d show the RSV mRNA titer as measured by qRT-PCR in BALF (c) and in lung tissue (d). All shown as individual titer and mean. Treatment with RSV-NFI reduces the infectious viral titer and RNA in BALF and Lung tissue. Animal infected with RSV and treated with either vehicle only (0) or different concentration of RSV-NFI (1, 4, 16 mg/kg). Baseline lambs are not infected and treated with vehicle only. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. n.s.: not significant.
Figure 4-4: Effect of RSV-NFI on the development of RSV associated gross lesion.

a) Show the average % of lung tissue parenchyma involved with RSV gross lesion shown as individual % and mean. b) Shows picture representative of gross lesion observed in different treatment group. RSV-NFI greatly prevent the development of RSV associated gross lesion. Animal infected with RSV and treated with either vehicle only (0) or different concentration of RSV-NFI (1, 4, 16 mg/kg). Baseline lambs are not infected and treated with vehicle only. *p<0.05, **p<0.01.
Figure 4-5: Effect of RSV-NFI on the development of RSV associated histologic lesion.

a) the accumulative histological lesion associated with RSV infection, which represents the sum of lesions scored in RSV infected lung tissue which includes Bronchiolitis, Syncytial cells, Epithelial cells necrosis, epithelial cells hyperplasia, neutrophils, Bronchiolar nodules, and perivascular nodules. b) a photo representative for each treatment group. RSV-NFI greatly reduced the RSV associated microscopic lesion in lung tissue. Animal infected with RSV and treated with either vehicle only (0) or different concentration of RSV-NFI (1, 4, 16 mg/kg). Baseline lambs are not infected and treated with vehicle only. *p<0.05, **p<0.01.
Figure 4-6: Effect of RSV-NFI on RSV antigen.

a) integer-based scoring of RSV M37 antigen in lung tissue detected by IHC had as average + SEM. Treatment with RSV-NFI greatly reduces RSV antigen expression in lung tissue. b) shows photo representation antigen distribution from different treatment group. Lung from lamb infected with RSV and treated with either vehicle only (0) or different concentration of RSV-NFI (1, 4, 16 mg/kg). Baseline lambs are not infected and treated with vehicle only. Estimated odds ratios: ***p<0.001. $: on/off pattern indicating an obvious effect. n.s.: not significant.
Figure 4-7: Effect of RSV-NFI on RSV M37 RNA expression.

a) integer-based scoring of RSV M37 RNA in lung tissue detected by RNAscope had as average + SEM. Treatment with RSV-NFI greatly reduces RSV RNA expression in lung tissue. b) Pictures depict findings representation from different treatment group. Animal infected with RSV and treated with either vehicle only (0) or different concentration of RSV-NFI (1, 4, 16 mg/kg). Baseline lambs are not infected and treated with vehicle only. *p<0.05, **p<0.01. n.s.: not significant.
Figure 4-8: Effect of RSV-NFI on the development of RSV associated histologic lesion.

Graphs show an integer-based score of Bronchiolitis (a), Syncytial cells (b), Epithelial cells necrosis (c), epithelial cells hyperplasia (d), neutrophils (e), Bronchial nodules (f), and perivascular nodules (g) RSV-NFI greatly reduce the RSV associated microscopic lesion in lung tissue. Animal infected with RSV and treated with either vehicle only (0) or different concentration of RSV-NFI (1, 4, 16 mg/kg). Baseline lambs are not infected and treated with vehicle only. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. n.s.: not significant. $.: on/off pattern indicating an obvious effect.
Figure 4-9: Effect of RSV-NFI inflammatory chemokine RNA expression in lung tissue.

Chemokine RNA expression IP-10 (a), MCP-1 (b), MIP-1α (c), PDL-1 (d), INF-λ (e), IL-13 (f), RANTES (g). All are shown as average + SEM. Treatment with RSV-NFI greatly reduces IP-10, MCP-1, MIP-1α, PDL-1, INF-λ. Animal infected with RSV and treated with either vehicle only (0) or different concentration of RSV-NFI (1, 4, 16 mg/kg). Baseline lambs are not infected and treated with vehicle only. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. n.s.: not significant.
CHAPTER 5. GENERAL CONCLUSION
Secondary Bacterial Co-Infection

The neonatal lamb model of RSV infection is well established, and many aspects of the model were investigated in our laboratory such as different viral strains, different viral cultures, different inoculation techniques, viral kinetics, immune response, and associated clinical signs [1–6]. Investigating mechanisms of bacterial infections secondary to RSV infection is the next step to understand the pathogenesis of bacterial coinfection and utilize the model to test the efficacy of therapeutic agents in complex infections which are associated with enhanced disease severity and death. This study demonstrated the susceptibility of lamb to *Streptococcus pneumoniae* (Spn) infection, and the enhanced disease severity when lambs challenged with Spn 3 days after primary RSV nebulization in comparison to lambs receiving either RSV or Spn alone. The titer of RSV were higher in the RSV-*Spn* infected group than RSV alone, while there were no differences between *Spn* infected lambs in *Spn* lung titer. There was an increase in severity of lung lesions in the RSV-*Spn* group, which was characterized by a higher number of infiltrating neutrophils, and increased epithelial necrosis and degeneration. These findings were in agreement other studies of viral-bacterial co-infection, and demonstrated the enhanced infection by both pathogens.

**Efficacy of Anti-RSV Fusion Inhibitor**

The neonatal lamb model of RSV infection which mimics RSV infection in infants provides an excellent tool to evaluate the efficacy of antiviral compound on the development of RSV-associated acute lower respiratory tract infection. This study demonstrated the efficacy of JNJ-49214698, which is an RSV small molecule fusion inhibitor that binds to the RSV prefusion conformation preventing critical F protein transformation to postfusion conformation and
subsequently preventing multicyclic RSV infection. In three treatment regimen, JNJ-49214698 had a very protective effect when given prophylactically 1 day before RSV nebulization. These lambs had no infectious RSV or mRNA in the lung, and had minimal clinical signs, and almost no RSV associated lung lesion. Although prophylactic administration of JNJ-49214698 is not its intended use, the finding demonstrates the ability of fusion inhibitor to prevent RSV infection. When JNJ-49214698 given 1 day and 3 days post-RSV nebulization, it had significant decrease in infectious RSV in the BALF with a less pronounced reduction in the mRNA. There were reductions in RSV associated clinical signs, gross and microscopic lung lesion, and RSV protein and mRNA expression in lung tissue. The effect of JNJ-49214698 was more evident when given 1 day after RSV nebulization. These data suggest a protective effect of JNJ-49214698 in reducing RSV replication and subsequently RSV associated signs and lesions. Therefore, the compound is a promising antiviral candidate against RSV infection in infants.

**Efficacy of Anti-RSV Non-Fusion Inhibitor**

Another mechanistic type of anti-RSV compounds are the non-fusion inhibitors, which bind and inhibit viral structural protein other than F protein. In this study, we evaluated the efficacy of the non-fusion inhibitor JNJ-64417184 in the neonatal lamb model of RSV infection. In this study, 3 different doses were used to treat lambs 1 day after RSV nebulization (16, 4, or 1 mg/kg body weight). The study demonstrated that JNJ-64417184 markedly reduced all RSV associated lesions in treated lambs in comparison to RSV infected vehicle-treated lambs. Additionally, the compound significantly reduced infectious RSV titer and mRNA expression in BALF and lung tissue homogenate and markedly reduced RSV antigen and mRNA expression in lung tissue sections. These results were accompanied by a reduction in immunomodulatory cytokines IP-10, MCP-1,
MIP-1α, PDL-1, and IFN-λ, and correlated with the reduction of inflammatory cells in the lung tissue observed in histological evaluation. These results indicate the ability of JNJ-64417184 to reduce RSV replication and disease progress.

**Future Directions**

The neonatal lamb model of RSV infection in infants is a well-characterized model and currently used to evaluate anti-RSV therapeutics[7,8]. We demonstrated that neonatal lambs infected with RSV are also susceptible for infection with Spn and lead to enhance disease severity in lambs infected with both RSV-Spn. Several aspects of this model can be modified and further developed in future studies of viral-bacterial co-infection such as: expanding the length of time of infection, assessing different inoculation time points of Spn following RSV, assessing different doses of Spn, and different types of Spn delivery (e.g., intranasal, intrabronchial, aerosolization). Also regarding Spn, a colonization model similar to human and mice could be tested to evaluate whether such mechanism of infection is a possibility in the lamb model. A third consideration is to try assess other bacterial pathogens or combinations of: *Staphylococcus aureus, Streptococcus pyogenes*, and *Haemophilus influenza*. These three bacterial pathogens are the most common bacteria identified secondarily to viral infections in human.

Several characteristics make the neonatal lamb model an interest and compelling option for pharmaceutical companies to assess their compounds. Although our laboratory has inoculated lambs by various routes including intra-tracheal, bronchoscopy guided intra-bronchial inoculation, nebulization of the virus has several advantages [2,5,6]. RSV nebulization provides more homogenous distribution of the virus to different lung lobes and subsequently homogenized lung pathology[5,9]. RSV nebulization is one feature of the neonatal lamb model that provides consistency for RSV associated lower respiratory tract infection and does overwhelm areas of RSV.
infection as can occur with intrabronchial deposition. We demonstrated that two antiviral compounds which target fusion protein (entry of the virus) and non-fusion protein (replication of the virus) had a strong effect in reducing RSV replication and associated pathology. In this same fashion, other viral targets could be tested in the lamb model such as RSV N, SH, and L proteins. Other potential studies could include a combination of compound that target 2 or more RSV protein and different time points for treatment administration. However, treating within 2 days post viral inoculation may be ideal because this likely is the time an infant may seek medical intervention[10].

**References**


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