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Research update

Drug candidates and model systems in respiratory syncytial virus antiviral drug discovery

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ABSTRACT

The development of antiviral strategies to prevent or treat respiratory syncytial virus (RSV) infections is of great importance, especially considering the fact that RSV is one of the most important causes of pediatric respiratory infections. However, despite intense efforts, there is no antiviral or vaccine approved for the prevention or treatment of RSV infections. Several inhibitors, targeting different RSV proteins have been discovered over the past decade. We here review the most important chemical series as well as recent developments in understanding which viral proteins and/or host cell factors are good targets for inhibition of viral replication. In addition, we highlight the current *in vitro* and *in vivo* model systems of the disease. A number of molecules are currently in (advanced) preclinical or clinical development. Significant breakthroughs in the field may be expected in the upcoming years.

1. RSV burden of disease, epidemiology and transmission

The respiratory syncytial virus (RSV) is a pneumovirus that belongs to the family of the *Paramyxoviridae*. The virus infects virtually all children by the age of three, making it the most important viral agent causing severe respiratory illness in infants and children worldwide. More specifically, RSV infections are responsible for one-third of acute lower respiratory infection related deaths in infants less than one year of age and are particularly problematic in premature babies as well as in children with underlying cardiac and respiratory problems [1]. The virus causes also significant disease in elderly [2,3] as well as in transplant recipients.

RSV is transmitted by contact with oral or nasal secretions, causing repeated infections throughout life. An RSV infection elicits both innate and adaptive immune responses, however immunity against the virus is not long-lived. The fusion (F) and attachment (G) surface glycoproteins have the ability to induce neutralizing antibodies, which wane quickly after infection [4]. RSV shows seasonal circulation in more temperate climate regions, with highest detection rates in late fall and early spring, in contrast to tropical regions were RSV is detectable year round, although the latter is not a consistent finding [5,6].

Currently, infected patients mainly receive symptomatic treatment consisting of bronchodilators and mucolytic agents, while high-risk young pediatric patients (<29 weeks gestation without chronic lung disease) also receive prophylactic treatment with monoclonal antibodies (Palivizumab) [7]. The observation that a higher viral load on day three of hospitalization was associated with a requirement for intensive care and respiratory failure in children under two years of age indicates there is a potential therapeutic window for the use of antivirals in this patient population [8]. Ribavirin is currently the only small molecule that has been approved for treatment of serious RSV infections, but its use is limited because of side effects, lack of efficacy, difficult route of administration and high cost.

2. Genetic diversity and genome organization

RSV is an enveloped, non-segmented, single stranded negative sense RNA virus with a genome size of approximately 15.2 kb. The ten genes encode for eleven proteins named NS1, NS2, N, P, M, SH, G, F, M2-1, M2-2 and L. RSV can be divided into two subgroups RSV-A and RSV-B, which contain different genotypes based on the nucleotide sequence of the carboxy-terminal ectodomain of the G protein [9]. A genotype classification system based on a gene segment of 629 bp for RSV-A and 724–762 bp for RSV-B, comprising both the conserved region and the hypervariable regions of the G protein has been developed [10,11]. Currently, for RSV-A the

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genotypes BE/A1, GA1, GA2, GA4 and GA5 have been defined, while 13 genotypes (GB1 to GB13) have been distinguished for RSV-B [12].

Evolutionary rates were determined for both RSV-A and RSV-B, showing a much higher substitution rate for the attachment of G protein (1.83×10^{-3} and 1.95×10^{-3} substitutions/site/year for RSV-A and RSV-B respectively), compared to the genomic evolutionary rate based on the complete genome (6.47×10^{-4} and 7.76×10^{-4} substitutions/site/year for RSV-A and RSV-B respectively) [10,13,14].

3. *In vitro* and *in vivo* model systems

3.1. Cell lines and human airway epithelial cells

In vitro, RSV replicates most efficiently in immortalized cell lines of human epithelial origin, although the virus also replicates in a rather wide range of cell lines, derived from various tissues and hosts. RSV tends to remain cell associated, making it necessary to dislodge the virus from the cells by freeze-thawing, sonication, or vortexing, in order to generate high titer virus stocks. The most commonly used cell line to grow RSV is the human HEp-2 cell line. However, the purity of this cell line has not been fully established, making it less suited for the production of virus for *in vivo* applications [15]. Vero cells are also known to be susceptible to grow RSV, although it has been reported that the progeny virus contains a truncated attachment protein that reduces its infectivity and the loss of virus G-protein binding to cell surface glycosaminoglycans [16].

Cell lines have several limitations, such as their cancerous origin, non-respiratory origin and often also inability to grow clinical viral isolates. These problems can be overcome by the use of isolated airway epithelial cells grown at an air-liquid interface. This results in the generation of a pseudostratified, mucociliary airway epithelium that displays similar morphologic and phenotypic characteristics of the *in vivo* human cartilaginous airway epithelium [17]. Studies in this human airway epithelium (HAE) have shown that RSV preferentially infects ciliated cells located at the apical surface and uses CX3CR1 as a receptor [18,19]. This technique has been successfully used to adapt clinical RSV isolates to cell culture [20].

3.2. In vivo models using surrogate viruses and non-human primate models

Large animal models of RSV include non-human primates, calves and lamb and their use has been reviewed recently [21,22]. Briefly, infection of calves with bovine RSV results in severe disease with a pathology and immunological response that is comparable to that of human RSV infections in children [23]. Bovine RSV infection in cattle is responsible for significant economic losses to the farming industry. Intranasal infection of lamb with human RSV results in moderate clinical signs of both the upper and lower respiratory tract. In particular Memphis 37, a clinical strain of human RSV, has been used to infect neonatal lamb and causes a disease that is comparable to that of RSV infections of human infants [24]. Infection of chimpanzee with the human RSV results in only mild disease that is limited to the upper respiratory tract [25]. The bovine, ovine and non-human primate models have advantages of sample collection, anatomical similarity of the respiratory track (non-human primates, sheep) and reliable pulmonary function measurements, but they also have obvious disadvantages. For example, bovine- and ovine RSV are genetically different from human RSV, the use of these animals poses housing and husbandry issues, limited molecular tools are available for these animals and large quantities of inhibitors are needed for such studies. Altogether these disadvantages highlight the need for robust and convenient small animal RSV infection models.

3.3. Small animal models using human RSV

Cotton rats are semi-permissive for human RSV infection with both upper and lower respiratory tract infection [26,27]. Peak pulmonary replication is observed on day 4 post infection with virus clearing from the lungs by day 7 post infection (or earlier). Cotton rats have been widely used for modeling respiratory viral infections and the peak RSV levels are ~100 x higher than in mice. Lower respiratory tract infection is already achieved in this model upon intranasal infection with 10^4 plaque-forming-units (PFU) whereas $>10^5$ PFU is needed in mice. For this reason, the cotton rat model has been the preferred animal model for studying RSV replication and testing antivirals. The pathology associated with RSV challenge in cotton rats consists of only mild to moderate bronchiolitis or pneumonia. The model was used intensively to study how a formalin-inactivated RSV vaccine candidate caused enhanced respiratory disease following natural RSV infection. However recent results highlight that also non-viral products from the vaccine and challenge preparations give rise to enhanced disease, which limits the validity of this model [28]. The cotton rat model has also been modified, using a high-dose cyclophosphamide as immunosuppressive therapy, to replicate the persistent RSV pneumonia seen in stem cell transplant recipients. In this model RSV replication remains high for at least 16 consecutive days allowing the study of therapeutic interventions [29].

Also inbred laboratory mouse strains have been explored intensely as a model for RSV infection [21]. Compared to the cotton rat model a very high intranasal inoculum (10^5 to 10^7 PFU per mouse) is often administered, in order to have some signs of lower respiratory tract infection, while the peak levels on day 4 post infection in the lungs are rather low (10^4 PFU/g tissue). As in the cotton rat model the virus is eliminated by day 7 post infection. Several attempts have been made to improve the model by immunosuppression. A 5-day cyclophosphamide pretreatment of BALB/C mice resulted only in a moderate increase in virus titers (2x) and virus-induced pathology [30]. The use of nude mice, which are athymic BALB/c mice that cannot generate mature T-lymphocytes, turned out to be more successful [31]. Infection of these animals results in peak levels that are ~100 fold higher than those in BALB/c mice and the virus remains detectable for at least 9 days after infection. Also more severe inflammation of the airways was observed in the nude mice compared to immunocompetent BALB/c mice.

More recently the effect of infection with several RSV strains, including low-passage clinical isolates, was explored in the BALB/c mice model [32]. This revealed that infection with one particular isolate (strain 2–20) was associated with more significant lung dysfunction, as measured by increased breathing effort, airway resistance and mucin expression. Interestingly the viral load in the lungs of mice infected with these isolates was comparable or even lower compared to mice infected with the laboratory strain A2. Another RSV strain (Line 19) results in an increased mucus production and pathogenicity compared to the infection with strain A2. Line 19 is an older RSV strain that has been intensively passaged in laboratory cell lines. The virulence of Line 19 was linked to the F protein through the studies of chimeric strains and more recently mutations in this protein have been explored for their role in disease pathology [33]. This shows that increased fusogenicity of the F protein of strain Line 19 correlates with many aspects of the pathology but not with the induced high levels of airway mucin expression.

Immunocompromised ferrets are also a potential model to study human RSV replication. In a recent study an intratracheal inoculation (10^5 PFU/animal) of a low passage RSV A clinical isolate in immunocompromised ferrets showed significant levels of replication (10^5–10^6 copies/mL peak levels) for >10 days. In the same experiments delay of virus peak levels could be observed when animals were pretreated with Palivizumab indicating the potential