Candidate antivirals for treatment of respiratory syncytial virus and coronavirus infections

Identification and elucidation of mode of antiviral activity

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Cover illustration: Perinuclear clusters of the double membrane vesicles induced by human coronavirus 229E in lung fibroblasts. Prepared by Anna Lundin and Sibylle Widehn

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To my family

Candidate antivirals for treatment of respiratory syncytial virus and coronavirus infections

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ABSTRACT

Respiratory syncytial virus (RSV) and coronaviruses (CoVs) are frequent causes of respiratory disease in humans. RSV can cause severe bronchiolitis and pneumonia in infants, especially in those born prematurely or with underlying cardiopulmonary chronic dysfunction. CoV respiratory illnesses can vary in severity ranging from common cold-like symptoms to severe respiratory disease with potential fatal outcome as exemplified by the pandemic-causing SARS- or MERS-CoVs. Despite the frequency and severity of RSV and CoV diseases, attempts to develop an effective and non-toxic antiviral treatment or a vaccine have so far been unsuccessful.

The aim of this thesis was to identify new antiviral candidates for treatment of RSV and CoV infections, and to elucidate their antiviral mechanism. Through screening of the ChemBioNet collection of ~17000 diverse compounds and a mini-library of polysulfated tetra- and pentasaccharide glycosides in a cell culture-based whole virus system, three promising anti-RSV and one anti-CoV candidate drugs were identified. Subsequent application of our step-by-step assay strategy for elucidation of mode-of-antiviral activity (**paper III**), revealed that anti-RSV P13 and C15 compounds displayed potent antiviral activity by targeting the heptad repeat regions of the viral F-protein essential for the virus-cell and the cell-cell membrane fusion (**paper I**). The anti-RSV lead drug PG545, identified in **paper II**, was prepared by coupling of a lipophilic cholestanol group to the synthetic sulfated oligosaccharide. This modification of the oligosaccharide enhanced the anti-RSV activity and conferred virucidal properties on PG545, a feature absent in native sulfated oligosaccharide inhibitors (**paper II**). PG545 exhibited dual antiviral mechanisms including (i) reduction of the RSV attachment to cells due to targeting of the highly conserved region and the receptor-binding region of the viral attachment G-protein, and (ii)

direct inactivation of viral particles. The anti-CoV candidate drug K22 potently inhibited 229E-CoV infectivity by targeting the membrane-bound viral RNA synthesis in the cytoplasm (**paper IV**). Analysis of viral variants resistant to K22 in addition to the preparation of specific 229E-recombinant viruses, revealed that K22 targets the viral nonstructural protein 6 (nsp6). This protein is involved in the recruitment and modification of host cellular membranes to create sites for the virus membrane-bound RNA synthesis. This is the first report of nsp6 as a druggable target for CoV intervention. K22 was also shown to be active against many other CoVs including the newly emerged MERS-CoV. In conclusion, P13, C15, PG545, and K22 are promising candidates for further development as new anti-RSV and anti-CoV drugs.

Keywords: antivirals, respiratory syncytial virus, coronavirus, antiviral screening, fusion inhibitors, sulfated oligosaccharides, nsp6, membrane vesicles

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SAMMANFATTNING PÅ SVENSKA

Respiratoriskt syncytialt virus (RSV) och coronavirus (CoV) utgör två av våra vanligaste orsaker till luftvägsinfektion hos människa. RSV kan orsaka lunginflammation hos spädbarn och då speciellt hos barn som är för tidigt födda eller har en underliggande kronisk hjärt- eller lungsjukdom. Även äldre drabbas i hög utsträckning av RSV, också här ofta i form av lunginflammation. Luftvägsinfektioner orsakade av CoV kan vara mer eller mindre allvarliga och orsaka allt från vanliga förkylningssymptom som kroppen själv läker ut till mer omfattande infektioner som i de svåraste fallen kräver sjukhusvård och ibland även leder till dödsfall. Exempel på CoV som kan resultera i sådana kraftigare luftvägsinfektioner är det pandemiska SARS-CoV, samt det nyligen identifierade MERS-CoV.

Det finns i nuläget ingen godkänd antiviral behandling mot infektioner orsakade av CoV och endast ett läkemedel finns tillgängligt för behandling av RSV-infektion. Detta läkemedel är relativt dyrt och ger dessvärre inte tillräcklig klinisk effekt. Trots att båda dessa virusgrupper frekvent orsakar allvarliga luftvägsinfektioner har försöken att för att ta fram nya antivirala behandlingsmöjligheter hittills inte resulterat i något nytt läkemedel.

Syftet med den här avhandlingen var att hitta nya kandidat-substanser för behandling av infektioner orsakade av RSV och CoV. Ett cellkultur-baserat metodologiskt flerstegssystem upprättades för att systematiskt undersöka de mest lovande kandidaterna samt för att ta reda på hur de hämmar virusinfektionen och vilken del av virus eller dess infektionscykel som blockeras, dvs. för att definiera deras antivirala mekanism. Genom att utgå ifrån molekylära bibliotek testades, ”screenades”, en stor mängd substanser, dels från ChemBioNet biblioteket på ca 17000 kemiska strukturer och även ett mini-bibliotek bestående av högsulfaterade kolhydrater. Studierna genomfördes inledningsvis i en modell där levande celler med ursprung från luftvägarna infekterades med virus i närvaro av en substans för att se om denna kunde skydda cellerna mot infektion. Den etablerade strategin för att utvärdera virushämmande mekanism beskrivs utförligt i arbete III. Projektet ledde till att vi här rapporterar om fyra nya lovande substanser, tre riktade mot RSV och en mot CoV, som effektivt hämmade dessa virusinfektioner *in vitro*.

I det första arbetet (I) identifierade vi substanserna P13 och C15 som hämmade RSV-infektion genom att blockera funktionen av höljeproteinet F, som virus använder för att ta sig in i värdcellen, och även för att sprida sig

mellan celler. Ytterligare en RSV-hämmande substans, PG545, identifierades i arbete II bland en grupp högsulfaterade kolhydrater som modifierats med olika lipofila grupper. När en specifik tetrasackarid kopplades till en lipofil kolestanolgrupp påvisades en starkt förbättrad blockering av RSV-infektion jämfört med den okonjugerade ursprungssubstansen. PG545 påverkade virusets förmåga att binda till celler genom att störa funktionen hos RSVs höljeprotein G som förmedlar cellbindning, men även genom att interagera med andra virusspecifika strukturer. Den lipofila gruppen medförde även att substansen kunde inaktivera viruspartiklarna, en egenskap som helt saknades hos de okonjugerade sulfaterade kolhydraterna. Anti-CoV-substansen K22 identifierades i arbete IV och hämmade effektivt infektion av CoV-stammen 229E genom att störa virus membranbundna RNA-syntes i cellens cytoplasma. Virusvarianter resistenta mot K22 uppvisade genförändringar i det icke-strukturella proteinet nsp6 vilket indikerade att denna viruskomponent var målet för den virushämmande effekten. Med revers genetik konstruerades tre rekombinanta CoV-229E, där resistensmutationerna introducerades, och funktionella studier av dessa virus bekräftade att nsp6 utgjorde det antivirala målet. Virusproteinet deltar i skapandet av ”fabriker” för CoV membranbundna replikation. Fyndet, som är det första i sitt slag beskriver nsp6 som ett nytt mål för antiviral intervention riktad mot infektioner orsakade av CoV. Vidare hämmade K22 cellulär infektion av flera andra CoVs, däribland SARS-CoV och det nyligen upptäckta MERS-viruset. Sammantaget utgör P13, C15, PG545 och K22 lovande kandidater för vidare utveckling av nya antiviraler riktade mot CoV- och RSV-orsakade infektioner.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Lundin A**, Bergström T, Bendrioua L, Kann N, Adamiak B, Trybala E. Two novel fusion inhibitors of human respiratory syncytial virus. *Antiviral Res* 2010; 88(3):317-24.

II. **Lundin A**, Bergström T, Andrighetti-Fröhner CR, Bendrioua L, Ferro V, Trybala E. Potent anti-respiratory syncytial virus activity of a cholestanol-sulfated tetrasaccharide conjugate. *Antiviral Res* 2012; 93(1):101-9.

III. **Lundin A**, Bergström T, Trybala E. Screening and evaluation of anti-respiratory syncytial virus compounds in cultured cells. *Methods Mol Biol* 2013; 1030:345-63.

IV. **Lundin A**, Dijkman R, Bergström T, Kann N, Adamiak B, Hannoun C, Kindler E, Jónsdóttir HR, Muth D, Kint J, Forlenza M, Müller MA, Drosten C, Thiel V, Trybala E. Targeting membrane-bound viral RNA synthesis reveals potent inhibition of diverse coronaviruses, including the Middle East respiratory syndrome virus. *Submitted.*

CONTENT

ABBREVIATIONS

aa	Amino acids
CM	Convoluted membranes
CPE	Cytopathic effect
DMV	Double membrane vesicle
DNA	Deoxyribonucleic acid
dsRNA	Double stranded ribonucleic acid
FCoV	Feline Coronavirus
EM	Electron microscopy
ER	Endoplasmic reticulum
GAG	Glycosaminoglycan
HAE	Human airway epithelium
HBD	Heparin binding domain
HCoV	Human coronavirus
hMPV	Human metapneumovirus
HR	Heptad repeat
HS	Heparan sulfate
HTS	High throughput screening
IBV	Infectious bronchitis virus
IC_{50}	50% inhibitory concentration
IF	Immunofluorescence
KS	Keratan sulfate
MERS	Middle East respiratory syndrome
MHV	Mouse hepatitis virus

mRNA	Messenger ribonucleic acid
Nsp6	Nonstructural protein 6
PFU	Plaque forming unit
RC	Replication complex
RdRp	RNA-dependent RNA polymerase
RSV	Respiratory syncytial virus
RTC	Replication/transcription complex
RT-PCR	Reverse transcriptase polymerase chain reaction
SARS	Severe acute respiratory syndrome
TM	Transmembrane
VP	Vesicle packets

1 INTRODUCTION

1.1 Virus respiratory infections

Acute respiratory infections have a significant health impact on individuals of all ages and represents one of the major disease burdens worldwide which led to 3.2 million fatalities in 2011 [1, 2]. Furthermore, acute respiratory infections are frequent causes of mortality in children under the age of five accounting for approximately 20% of all deaths [3]. WHO predicts that lower respiratory tract infections will still be among the top five leading causes of child death in the period of 2010 -2030 [4, 5]. A substantial portion of the reported cases of severe respiratory tract infections are caused by viruses [6-8]. There are a wide range of different respiratory viruses that constantly circulate in the population causing respiratory illness of varying severity. The common cold is a collection term for a similar set of symptoms including sneezing, nasal obstruction, sore throat and coughing, caused by a range of viruses belonging to several different families. In humans, common colds are frequently caused by RNA viruses with rhinoviruses being among the major causative agents [9-11]. In addition, viruses such as respiratory syncytial virus (RSV), human metapneumovirus (hMPV), influenza virus, parainfluenza viruses, and some adeno-, corona-, and enteroviruses, can to a varying degree be associated with common cold symptoms.

In spite of the generally mild symptoms and self-clearing illness caused by respiratory viruses, these pathogens can spread into the lower respiratory tract and other organs causing severe, frequently fatal disease. Due to the preferential tropism of these pathogens for respiratory epithelium, the physical barriers of the respiratory tract and competent innate and adaptive immunity are crucial for preventing the infection or restricting its progression into severe respiratory disease. An example of this is infants and children born prematurely, who due to narrow and not fully developed airways are vulnerable to their obstruction following infection and inflammation. In addition, their immature innate and adaptive immunity, especially that mediated by the T helper 1 (Th1) cells, lacks the strength and capacity to withhold respiratory pathogens and the infections tend to be prolonged and more severe compared to those occurring in older children [12-14].

RSV is considered the sole most important viral pathogen causing lower respiratory tract infections in infants and one of the major causes of mortality in children under the age of five [7, 15]. It was estimated that in the year

2005 RSV accounted for 33.8 million, 22%, of all acute lower respiratory tract infections in children less than five years of age worldwide [7]. A local study of the incidence of RSV in Sweden coincides with the observation that the age group of 0-4 years has the highest prevalence of RSV infections accounting for almost 20% of all the positive cases in the children studied [11]. There are two subgroups of RSV, A and B, that cause respiratory infection in humans. Several studies indicate that infection with subtype A results in a more severe disease outcome [16, 17]. Members of both subgroups circulate annually and can appear simultaneously or most often with dominance of one subtype [18, 19].

Human coronaviruses (CoVs) cause approximately 5 to 30% of all upper respiratory infections in adults [9, 20-24]. There are six species of the *Coronaviridae* family that are known to infect humans, and the two most common, 229E and OC43, causing generally mild respiratory symptoms, were identified in 1966 and 1967 respectively [25, 26]. In 2003 a new member of the CoV family, SARS-CoV, caused a pandemic outbreak of severe lower respiratory tract infection affecting more than 8000 individuals with subsequent 750 fatalities [27-30]. CoV NL-63 and HKU1 were identified as late as 2004 and 2005 in the aftermath of the SARS-CoV outbreak which led to an immense increase in the efforts to characterize this new pandemic virus [31, 32]. In addition to the SARS-CoV, a new potential pandemic-causing MERS-CoV was identified 2012 in a 60-year old man from Saudi-Arabia. The patient suffered from acute pneumonia and renal failure and died 18 days after the onset of symptoms. Although the MERS- and SARS-CoV share some similarities as regards zoonotic origin and pathogenicity of the disease, the former virus is clearly distinct from the other human CoVs [33]. Recently, a report of a hospital outbreak of MERS-CoV has confirmed the person-to-person spread of this virus [34, 35]. At present, there are 114 confirmed cases of MERS-CoV disease including 54 fatalities confirming that this virus can be extremely deadly for humans (CDC, MERS-CoV update 17th of September 2013) [36].

There is a clear seasonality to most respiratory virus infections in northern temperate regions with a five to six month period ranging from late autumn to early spring with peaks occurring at different times around the winter months depending on the virus. A study of the seasonal variations of 15 different respiratory pathogens over a three year period in Sweden showed that human rhinovirus was the most prevalent irrespective of the season, followed by influenza A and RSV, both most prevailing from January to March [11]. Every other year RSV tends to have a shorter peak season and the cases seem to be less severe [37].

The human CoVs 229E, OC43, NL-63 and HKU1 that circulate in the population throughout the year follow a similar seasonality as the other common respiratory agents peaking in the winter months but without the 2-year rhythm. It has also been observed that CoVs are frequent as co-infecting pathogens [37].

1.1.1 RSV disease

RSV is the predominant cause of bronchiolitis and pneumonia in infants below 6 months of age [12, 38], a condition that frequently requires hospitalization due to respiratory failure [7]. Approximately 70% of all children have had an RSV infection during their first year, and nearly all children have experienced one or several RSV infections in their second year of life [39]. Children born prematurely or with underlying disease such as bronchopulmonary dysplasia, congenital heart disease or neuromuscular disease are at higher risk of severe respiratory disease following RSV infection [40-42]. In addition to infants and small children, RSV can also affect adults with the greatest disease severity in elderly and immunodeficient individuals of any age [43, 44]. The host-to-host spread of RSV occurs through the contact with virus-containing respiratory secretions in the form of large aerosol droplets or through contaminated material that reach the mucosal surface of nose, mouth or eye [45]. The primary RSV infection usually concerns the ciliated cells of the nasal cavity, and the first symptoms of RSV disease normally appear at 4-5 days after initial infection [46].

From the nasal cavity the virus can spread to the lower respiratory tract and infect ciliated cells of the bronchi and the type II lung alveolar cells, where it recruits immune cells. Since a Th1 cell immune response, predominant in virus infections, is not fully mature in infants under the age of 6 months, it is frequent that RSV stimulates the Th2 response typical for parasites and allergic diseases resulting in airway hypersensitivity due to production of specific cytokines, IgE, and infiltration of eosinophils [Reviewed in 47]. The progression of RSV infection to the bronchial epithelial cells and the concomitant Th2 immune response results in dysfunction and subsequent damage of the mucus-transporting ciliated cells [48, 49] as well as necrosis of the bronchial epithelium. The extensive infiltration of inflammatory cells into the respiratory epithelium in combination with excessive mucus production, dysfunctional mucus transport and increased proportion of dead cells can lead to airway obstruction and progression into pneumonia. In severe cases, the obstruction can cause emphysema and/or collapse of the small distal airways [48, 50]. This becomes particularly evident in infants and young children where the lower airways are very narrow and vulnerable to obstruction [13].

Furthermore, given the substantial involvement of the allergic type Th2 immune response in RSV disease in infants, several studies indicate that the risk of developing asthmatic and obstructive respiratory conditions is higher following a severe RSV infection at an early age [39, 51, 52].

RSV produces a characteristic cytopathic effect upon infection of monolayers of cultured cells, i.e., formation of syncytia [53], a feature contributing to the name of this pathogen (Figure 1). However, the syncytium forming activity of RSV is not usually observed when studying the infection in differentiated cultures of respiratory epithelial cells, and even though it can be observed in some RSV infected patients it is not a dominant feature [54, 55]. RSV infection can also extend to other organs causing conditions like myocardial damage, cardiac arrhythmias, neurological abnormalities and hepatitis. Furthermore, the presence of virus or virus related material have also been found in samples of peripheral blood, cerebrospinal fluid, myocardium and liver [Reviewed in 56].

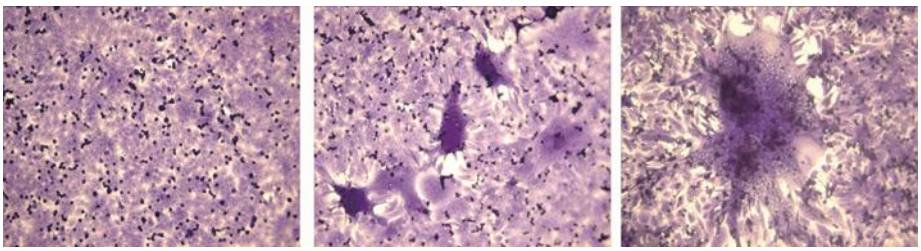


Figure 1. *Monolayer cultures of HEp-2 cells infected with RSV and stained with crystal violet. Uninfected cell monolayer (left image), the RSV-induced cytopathic effect in the form of syncytial plaques (middle), and an enlargement of syncytium formed by fusion of many cells reflected by presence of multiple nuclei seen as white spots (right image).*

1.1.2 CoV disease

Human CoVs can cause respiratory disease of varying severity. Among the six CoVs known to infect humans, the disease severity ranges from mild common cold symptoms to severe acute respiratory disease with potential fatal outcome. The human infection with CoV causing a mild common cold-like disease, i.e., 229E, OC43, NL63 and HKU1 is usually limited to epithelial cells of the nasal cavity where these viruses infects non-ciliated secretory cells (229E) or ciliated cells (OC43, NL-63, HKU1) [57]. Infections caused by the common CoVs 229E and OC43 generally include symptoms such as fever, cough, malaise, headache and sore throat that last for approximately 3-4 days but are often self-limiting [58, 59]. However, these strains as well as CoVs NL-63 and HKU1 have also been reported to cause

more severe upper and lower respiratory infections in both children and adults with symptoms of bronchiolitis and pneumonia [60, 61]. SARS-CoV exhibits a more severe course of infection leading to hospitalization in 20-30% of the cases [27] with a mortality rate of approximately 15% [62]. SARS-CoV predominantly causes pneumonia with generally less symptoms from the upper respiratory tract than in the other CoV infections, and with presence of non-respiratory symptoms such as diarrhea [27, 63]. Analysis of deceased patients indicated the involvement of multiple organs such as the lymph nodes, heart, liver and kidneys [64, 65]. Like many other respiratory viruses CoVs spread through infected respiratory secretions or fomites [58, 66].

1.2 Respiratory syncytial virus (RSV)

1.2.1 RSV particle

Human RSV belongs to the family of *Paramyxoviridae* of the order *Mononegavirales* that groups viruses such as parainfluenza, mumps and measles containing a single nonsegmented negative sense RNA genome. More specifically, pneumotropic RSV together with human MPV and their animal counterparts are classified within the subfamily of *Pneumovirinae*.

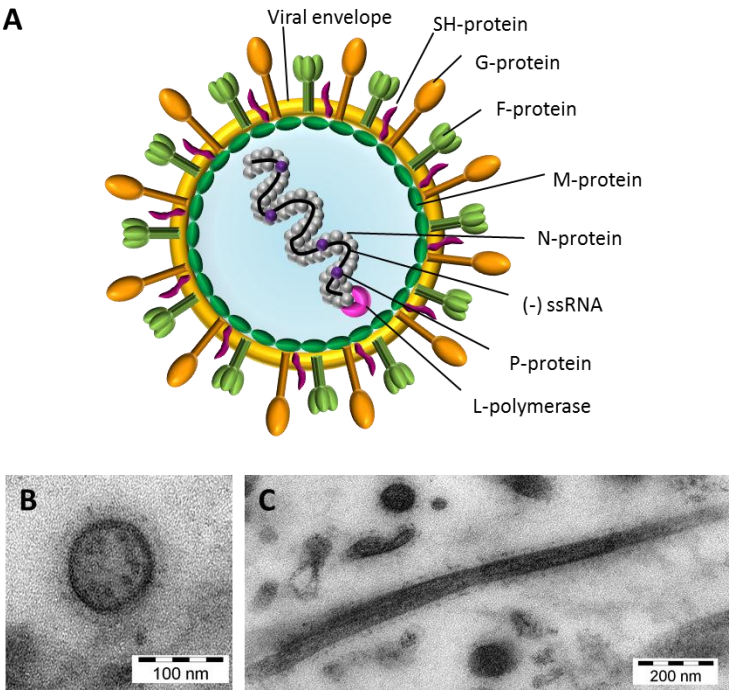


Figure 2. *The RSV virion. (A) Cartoon structure of the viral particle showing presence of the F-fusion-, G- attachment, and SH-small hydrophobic glycoproteins protruding from the virion lipid envelope; and the M-membrane scaffolding protein, and the negative-sense single strand RNA genome associated with the N-nucleocapsid, P-phosphoprotein, and the L-polymerase. (B-C) EM images of RSV particles in the spherical (B) and filamentous (C) forms.*

RSV is an enveloped pathogen that contains a genome of approximately 15 kb embedded into a nucleocapsid with a symmetric helical, "herring-bone" formation [67]. The genome carries 10 genes that encode for 11 proteins in the following 3' order: nonstructural proteins 1 and 2 (NS1, NS2), nucleocapsid-, phospho-, and matrix proteins (N, P, M), surface

glycoproteins (SH, G, F), protein M2, consisting of M2-1 and M2-2, and polymerase (L) protein. The M2 gene which partly overlaps the L protein gene is encoded from two overlapping open reading frames [68]. The RSV virions (Figure 2A) are pleomorphic and their shapes vary from a range of rounded semi-spherical forms of about 80-350 nm in diameter (Figure 2B) to the most prevalent long extended filament form with a diameter of 60-200 nm extending up to 10µm in length (Figure 2C). The budding of viral filaments from cells is frequently incomplete and these virions remain associated with the cell surface thus resembling cellular microvilli [69, 70].

1.2.2 RSV attachment to and fusion of cells – The G- and F-proteins

RSV virions possess three kinds of structural glycoproteins, known as G, F, and SH, that are embedded in the viral envelope and occur in form of surface-projecting spikes. While the biological function of the SH-protein in the RSV life cycle still requires further clarification [71], the G- and F-glycoproteins contribute essential functions in the RSV attachment and fusion events.

The G-protein is the virus attachment component

The initial interaction of RSV virions with susceptible cells occurs through binding to cell surface glycosaminoglycan (GAG) chains, a step mediated by the viral G-protein [72] discussed further in section 1.4.3, (Figure 11). The G component is a type II glycoprotein of 289-299 amino acids (aa) with a hydrophobic membrane-spanning domain at the N-terminal end that serves as a signal sequence and anchors the protein to the virion envelope [73, 74]. The ectodomain of the protein contains a central cysteine rich region (aa 173-186) flanked by the C-terminal positively charged GAG-binding region (aa 187-217) [75, 76] and the N-terminal stretch of negatively charged and hydrophobic aa at positions 160-172. Residues at positions 164-176 that partly overlap the cysteine rich region and the negatively charged domain are highly conserved among different strains of RSV. The central domain is flanked by two mucin-like regions which are referred to as such due to the high content of aa serine, threonine and proline in addition to heavy *O*-glycosylation that resembles similar structural features of mucins found at the surface of epithelial cells.

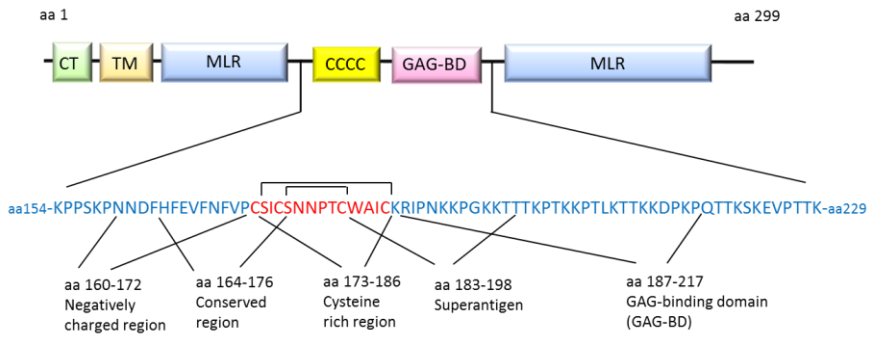


Figure 3. *Schematic structure of the RSV G-protein showing sequential order of essential regions. The aa sequence of the central region including the cysteine noose induced by cross-linking of cysteines at positions 173 and 186, and 176 and 182 are shown in red. Other regions, namely the negatively charged, the conserved, the superantigen, and the GAG-binding regions and their aa span are also indicated. CT, cytoplasmic tail endodomain; TM, N-terminal transmembrane domain; MLR, mucin-like region; CCCC, cysteine nose; GAG-BD, GAG-binding domain.*

The mucin-like regions of the G-protein are highly variable in sequence, which contribute to the G-protein being the RSV gene product with the highest sequence variability between different strains [73, 77]. Both *O*- and *N*-linked glycans constitute a substantial part of the mass of the G-protein which is synthesized as a fairly small protein precursor (32 kDa), and further modified by co-translational addition of *N*-linked sugars and subsequent *O*-glycosylation in the trans-Golgi compartments [78]. The presence of serine and threonine residues accounts for approximately 30.6% of the total aa content which infers presence of over 70 possible sites for *O*-linked glycosylation [79]. Since the mature form of the G-protein is approximately 80-90 kDa in size, the glycans contribute to approximately 60% of its molecular mass [80-82]. The mucin structures of the G-protein may stretch out the protein, shade and protect the central region and its antigenic sites from immune response or proteases, and make the protein hydrophilic and sticky.

The cysteine rich region of the G-protein is conserved and contains four cysteine residues (Figure 3) at positions 173, 176, 182 and 186 (in A2 strain). These cysteines are linked by two disulfide bridges between residues 173-186 and 176-182 respectively forming a mini-loop or "cysteine noose" [83-85]. The major function of this structure is likely to provide a tension to sequences that flank the cysteine noose, i.e., the GAG-binding and the negatively charged regions, a feature that could facilitate interaction of these

regions thus modulating the virus binding to or its removal from the cellular GAG receptors (Figure 11). Variations in charges, exposed residues and regulation of protein folding in these three central domains of the G-protein are likely to confer specificity in receptor binding [82]. Furthermore, based on the conserved nature of the cysteine rich region, this structure is also an important antigenic site in RSV of both human and animal origin [84, 85]. Apart from the virion envelope associated G-protein, RSV also expresses a truncated, secreted form of this component (Gs), where approximately 65 aa of the N-terminal end, including the CT and TM regions, are trimmed off. This Gs form accounts for about 20% of the total amount of G-protein produced, however this value may rise to approximately 80% during the first 24 h of RSV infection [86].

Gs have several suggested roles in interference with the immune response. In particular, (i) Gs may act as decoy for the host antiviral defenses including trapping of the virus-neutralizing antibodies [87]. (ii) It may impair innate immunity by inhibiting signaling from the Toll-like receptors (TLR) 2, 4, and 9 important in recognition of the viral structural envelope proteins. (iii) The central region of the G-protein possesses CX3C fractalkine-like motifs that may hamper the cellular Th1 immune response, and (iv) the central portion of the G-protein overlapping part of the cysteine noose and the GAG-binding region, i.e. the stretch 183-WAICKRIPNKKPGKKT-198 (Figure 3) [75, 76, 88] is suggested to be a superantigen that provokes hypersensitivity of airways by stimulating Th2 immune response typical for allergic diseases [68, 89, Reviewed in 90]

The F–protein mediates virus penetration into the cell

Attachment of RSV particles to cells, an event mediated by the G-protein, is prerequisite for the second step of the viral life cycle, i.e., its penetration through the cell plasma membrane into the cytoplasm. This event is mediated by the specialized fusion device of the F-protein that shows structural and functional resemblance to a wide range of other virus fusion proteins [91-94] including these of other paramyxoviruses [95]. It has been shown that the F-protein can be sufficient for all the necessary roles in RSV attachment, entry and fusion processes and hence the virus may cope without the presence of the G or SH proteins. However, in spite of the successful replication in cell culture of a mutant virus lacking G and SH, the virus infectivity was attenuated and inefficient [96, 97].

The RSV F-protein, a type I glycoprotein comprising 754 aa, is mainly responsible for the fusion between the lipids of viral envelope and the cell plasma membrane, but also for the cell-cell fusion activities during the virus

spread [98] manifested as the formation of characteristic syncytia of infected cultured cells (Figure 1, section 1.1.1) [53]. The F-protein (Figure 4) is synthesized as a single polypeptide precursor, F0, which is co-translationally glycosylated in the ER and subsequently cleaved in the Golgi compartments by the furin-like cellular endoproteases. This yields the two disulfide-linked subunits, F1 and F2, which represent the biologically active form of the F-protein inserted into the budding membrane of progeny RSV virions [99, 100]. The N-terminally located F2 subunit is smaller than the F1, it holds the signal peptide, possesses four potential sites for *N*-glycosylation [101], and is believed to have the receptor-binding activities [102]. The larger F1 subunit is a specialized fusion device and comprises an N-terminal fusion peptide hidden in the pre-fusion form of the protein [95, 103, 104], followed by the heptad repeat 1 (HR1), an intervening domain of over 250 aa [105], the heptad repeat 2 (HR2), transmembrane domain (TM), and a short cytoplasmic region [101]. Since the F-protein forms trimeric spikes in viral particles, the HR1 and HR2 fold into trimeric coiled coil structures where the α-helices are organized into an antiparallel heterodimer so that the hydrophobic surfaces face each other [106].

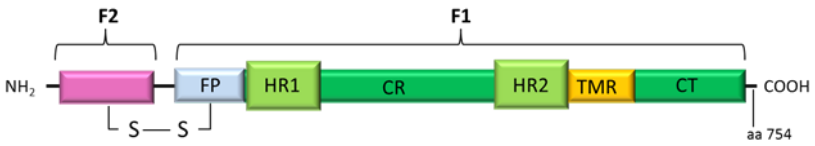


Figure 4. *Schematic structure of the disulfide linked F1 and F2 subunits of the RSV F-protein showing sequential order of the FP-fusion peptide, HR1/HR2-heptad repeats, CR-central/intermediate domain, TMR- transmembrane region, and the CT- cytoplasmic tail.*

How does the F1 protein mediate fusion between lipids of the viral and cellular membranes? The binding of the RSV particle to a host cell, an event that can be mediated by the G-protein or the F2 subunit, triggers three major changes in conformation of the prefusion "cone"-like form of the F-protein [91, 107-109] (Figure 5). First, the F1 subunit acquires an extended intermediate shape as a result of exposure of its N-terminal hydrophobic/lipophilic fusion peptide that, as mentioned above, is hidden in the prefusion form of the protein [95, 104]. The viral fusion peptide is immediately inserted into lipids of the adjacent cell plasma membrane so the virus particle and the cell are strongly bound through the F1 subunit which is anchored in the viral lipid envelope by the TM region. The next step in the virus-cell fusion is mediated by the HR1 domain which is located close to the N-terminal fusion peptide inserted into the cell, and HR2 domain that lies close to the C-terminal TM region inserted into the viral envelope. As

mentioned above the HR structures are in fact trimeric α-helices that automatically fold into the coiled coil structure. Insertion of the fusion peptide into the cell plasma membrane triggers the movement of HR1 and HR2 towards each other concluding in their automatic folding into a hexameric complex, also referred to as a six helix bundle. This conformational change is paralleled by alteration in the shape of the protein from an extended to a collapsed or hairpin intermediate. An obvious outcome of the HR1 and HR2 interaction is a tight apposition of viral and cellular membranes. This event distorts the membranes and promotes the hemifusion between the outer bilayers, followed by complete fusion, pore formation in the fused membrane and insertion of viral ribonucleocapsids into the cytoplasm [103, 107]. The basic hairpin structure of the F-protein of RSV and other paramyxoviruses is related to that of other viruses such as SARS, HIV and influenza [105] and these similarities suggest that the fusion process is a conserved mechanism [110].

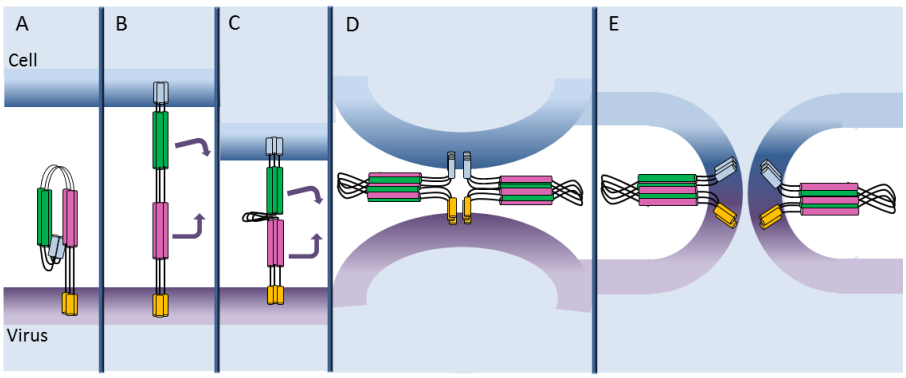


Figure 5. *Schematic representation of the RSV F-protein mediated membrane fusion. (A) In the native prefusion form the trimeric F-protein is inserted in the viral envelope with its fusion peptide (in blue) hidden inside the protein. The RSV binding to cells triggers changes in the conformation of the F-protein resulting in (B) protein elongation, and exposure and insertion of fusion peptide into cellular membrane. (C-D) Owing to the strong affinity that the two heptad repeat regions (in green and pink) have for each other, the protein collapses forcibly into the six helix bundle conformation, thus apposing the viral and cellular membranes, and (E) inducing their fusion.*

During the virus-cell fusion the F-protein undergoes a series of conformational changes resulting in several intermediate forms, and a final postfusion "lollipop" shape [109]. All these forms of the F-protein are attractive targets for antiviral development, and in fact numerous drug

candidates for RSV treatment are of the fusion inhibitor class (discussed more in section 1:6). Presence of the F-protein intermediates is also important for the vaccine development. Several antigenic sites located in the intervening domain between two HR regions of the F-protein, mainly in the central cysteine rich region near the HR2 were identified [111-113]. Structural analysis of the F-protein using EM-analysis revealed two different shapes resembling a “cone” (prefusion) and a “lollipop” (postfusion) with the location of the antigenic sites grouped on the “head” region of these protein structures [109].

1.2.3 RSV replication

Following insertion of viral ribonucleocapsid into the cell, further processing takes places entirely in the cytoplasm, more specifically in the viral replication sites known as inclusion bodies. These RSV-induced structures are not well characterized. They are clearly visible in light and electron microscope as regions of electron dense cytoplasm with several internal vacuoles and membrane-containing adjacent vesicles [114]. The viral RNA delivered to cytoplasm is specifically covered by the N protein, the aim being to protect RNA from recognition by the cellular interferon system but permit specific activity of the viral L polymerase [74, 115]. The polymerase complex proteins, i.e., the L-protein as the main RNA-dependent RNA polymerase (RdRp) in addition to the P, N, M2-1 and M2-2 constitute the core RNA replication system in RSV [116]. The phosphoprotein (P) binds to both the L polymerase and to the N protein on the ribonucleocapsid to transiently uncover the small segments of RNA thus helping the polymerase in specific recognition of viral RNA [Reviewed in 90, 117, 118]. Furthermore, the interaction of P-protein with M2-1 is important for its proper function in elongation of the newly formed RNAs [116, 119]. Later during infection the RNA transcription shifts over to replication, an event suggested to be regulated by the M2-2 protein [74]. This shift results in the generation of a full length cRNA or antigenome with opposite polarity, which is used as a template for new full length genomic RNA to be incorporated into progeny virions [68, 74]. The sequentially transcribed mRNAs are then translated into the following viral protein products NS1, NS2, N, P, M, SH, G, F, M2 (M2-1 and M2-2) and L. The N, P, M and F-proteins are required for the formation of new virus particles [120]. The nonstructural proteins NS1 and NS2 are unique to the RSV family and have no counterpart in other members of the pneumovirinae [121]. NS1 and, to a lesser extent, NS2 are known to inhibit the induction of interferon [122, 123].

Following production of progeny viral RNA and proteins an assembly of these components takes place. The M protein is critical in this event since it mediates the association of the nucleocapsid with the budding viral envelope mainly due to interaction with both the viral nucleocapsid and the F-protein of the viral envelope, a feature of importance for the morphogenesis and formation of new virus particles [120]. The M-protein is transported to the plasma membrane and partly incorporates into lipid rafts, which are the suggested sites for assembly and budding of progeny virions [124, 125]. Proteins and new genomic RNA assemble close to the membrane surface where the structural proteins are incorporated into budding host membranes after processing in the ER/Golgi [99]. The M- and the F-proteins incorporated in the lipid rafts are associated with cytoskeleton components such as actin and microtubule which in turn is significant for virus assembly and release [120, 126] and/or the formation of the filamentous viral particles associated with the cell surface [127].

1.3 Human respiratory coronaviruses (CoVs)

1.3.1 The CoV particle

Human respiratory CoVs, i.e., 229E, OC43, NL63, HKU1, SARS-CoV and MERS-CoV belong to the family of *Coronaviridae* in the order *Nidovirales*, and hold the largest genome of all known RNA viruses spanning between 27-32kb. CoVs are enveloped pathogens with a non-segmented positive sense single strand RNA genome associated with a nucleocapsid protein in a helical formation. The virus particle is mainly spherical of ~100-160 nm in diameter [128] (Figure 6).

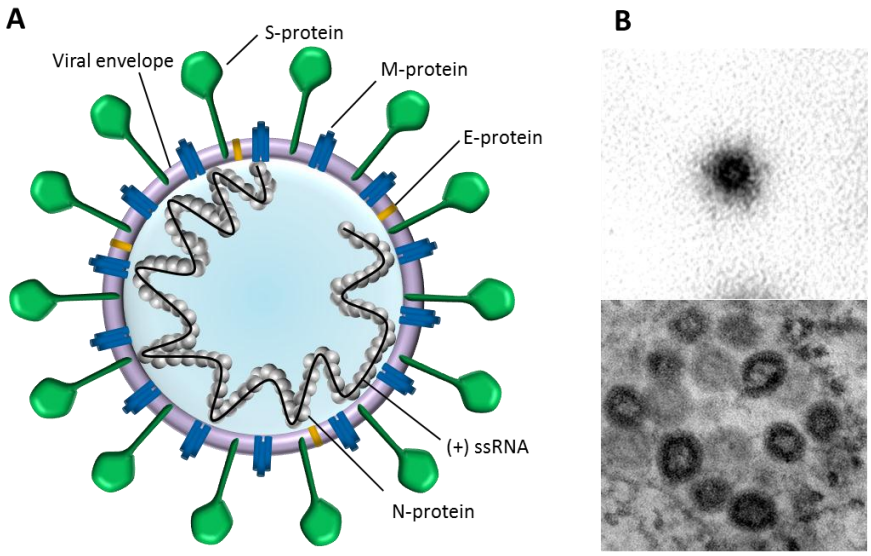


Figure 6. *Structure of the CoV virion. (A) Cartoon showing presence of the large protruding spike (S), membrane (M) and envelope (E) proteins that provide a scaffold for the viral envelope, and nucleocapsid (N) protein associated with a single strand viral RNA. (B) EM-images of a single CoV particle (top) and cluster of CoV particles (bottom).*

Since CoVs contain a positive sense RNA that could be directly translated by cellular ribosomal machinery there is no need for the presence of RNA replicative enzymes in the virions. Therefore, most CoVs hold a set of only four structural proteins, i.e., the spike (S), envelope (E), membrane/matrix (M) and nucleocapsid (N) protein while some human CoVs such as OC43 also carry an additional haemagglutinin esterase (HE) protein [128].

1.3.2 Components of the CoV particle and their activities in viral life cycle

The S-protein is a large (~200 kDa) glycoprotein protruding from the surface of the virus envelope giving it a characteristic crown (=corona)-like structure. The S-protein is heavily glycosylated and is composed of two large subunits, S1 and S2, that mediate the virus attachment to and entry into the susceptible cells respectively [129, 130]. CoVs can penetrate into the cells by direct fusion between lipids of the viral envelope and the cell plasma membrane or through the membrane of endocytic vesicles (e.g. SARS-CoV) triggered by their acidic environment. The mechanism of the S2-induced fusion is similar to that caused by the F-protein of RSV, and results in the delivery of a core structure, i.e., a complex of viral RNA with the N protein, into the cytoplasm where the entire replication process occurs. The N protein, apart from important roles in package, condensation and transcription of genomic RNA, also influences pathogenesis of CoV infections [131, 132].

Following dissociation of the nucleocapsid core, a naked viral RNA is released. As mentioned above, since CoVs have a positive strand genome it can act directly as an mRNA template for translation of proteins in ribosomes. Approximately two-thirds of CoV genome is devoted to the production of 16 viral non-structural proteins (nsps). Some of these nsps recruit and modify cellular lipid membranes to produce sites for replication of viral RNA while the other nsps which carry a range of activities such as polymerase, helicase, exoribonuclease, endoribonuclease and methyltransferase, perform and/or regulate replication of viral RNA (Figure 7). Since nsps of CoVs are the matter of this thesis (**see paper IV**) some aspects of their activities are further described in section 1.3.3.

The genomic RNA is also used to generate a new minus strand RNA for further synthesis of full length genome and for production of subgenomic mRNA. The structural proteins S, E, M and N are translated from subgenomic mRNAs, undergo processing in the Golgi and are subsequently incorporated into membranes and/or transported to the site of assembly at the ER and Golgi compartments [133, 134]. As mentioned above the N-protein binds to newly synthesized RNA forming the nucleocapsid structure which is transported to the site of assembly [132, 135]. The viral M protein, the most abundant CoV structural protein, is critical for this process. It is fairly small (~10kDa) and possesses three transmembrane domains. The protein main activity is in virion assembly and morphogenesis [136], and it performs this task by interaction with the S-protein of the viral envelope and the N-protein of the nucleocapsid. This activity helps to organize the virus budding, to

incorporate spikes into the viral envelope, and to package the nucleocapsid into virions [137-140]. CoV virions bud from the ERGIC [134, 141] where the activities of the M and another envelope component, the E protein, are required in formation of envelope curvature of progeny virions as well as in pinching the budding virions off the membrane surface [137, 142, 143]. Progeny viruses accumulate in large exocytic vesicles to be transported towards the plasma membrane and subsequently released.

1.3.3 Membrane-bound CoV RNA synthesis

An interesting feature of CoVs, that has yet to be fully resolved, is the mechanism of their replication employing the large set of 16 nsps in the intricate replication machinery characteristic for this family of viruses. The number of encoded nsps and their involvement in replication of RNA is unusual among all RNA viruses, and therefore CoVs uphold the largest RNA genome known to date. As mentioned earlier these 16 nsps are derived from the proteolytic cleavage of two large polyproteins 1a and 1 ab. The cleavage is mediated by three different proteases which are included in the polyproteins and need to be liberated by autocatalysis to cleave the rest of the polyprotein. These include the papain-like cysteine proteases PL1pro and PL2pro, located in nsp3 [144], and the chymotrypsin like main protease 3CLpro, located in nsp5. The cleavage products of these proteases and their putative functions in replication of CoV RNA are shown in Figure 7.

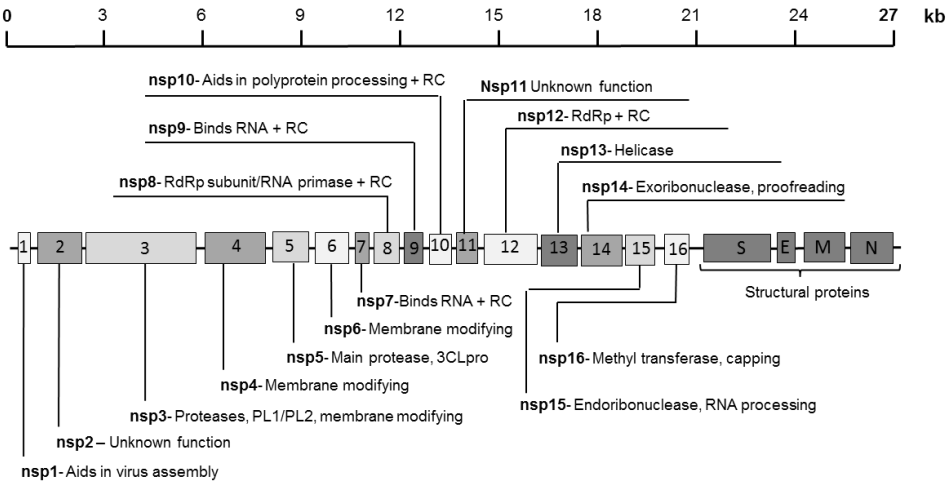


Figure 7. *Putative biological functions of the 16 nonstructural proteins (nsps) encoded by CoVs. Genomic organization of sequences coding for these 16 nsps and 4 structural proteins is also shown. RC-replication complex; RdRp, RNA-dependent RNA-polymerase.*

As seen from Figure 7, nsps 7-16 are directly involved in replication of viral RNA including its processing, proofreading, and regulation. The proofreading capacity of the nsp14 3'-5' exoribonuclease have a key function in upholding an unusually high replication fidelity of CoV and contribute to the uniquely large genomes of these RNA viruses [145, 146]. Nsp5 and part of nsp3 are proteases, while nsp3, nsp4, and nsp6, all of which are transmembrane proteins, contribute to the formation of CoV replication sites or "viral organelles" in the cytoplasm by recruiting and modifying the host cellular membranes. Nsp3 and nsp4 are believed to pair recruited membranes to form convoluted membranes (CM) while nsp6 is thought to form a set of single membrane vesicles. Concert actions of these three proteins [147] result in formation of the reticulovesicular network of paired, closely apposed membranes (Figure 8A) that includes CM, double membrane vesicles (DVM) (Figure 8C-D), and vesicle packets (VPs) all connected to ER through the outer membrane. The ~200-300 nm vesicles may appear as early as 2 h p.i. and continuously change in number and in size throughout the infection [148-151]. The CM structures are also believed to be sites for accumulation of the RNA replication complex subunits including among others nsp8 and nsp12. VP structures are formed later during infection and frequently contain multiple inner vesicles as well as large numbers of budding or fully assembled virions (Figure 8E) indicating that VPs might be created by DMV merger [150]. Although not fully resolved, the main roles of these structures during CoV replication are believed to be as follows.

(i) *Assembly and concentration of replication components*. The wide range of activities within the 16 nsps of the replicase machinery needs to be organized and concentrated for optimal function in a confined space of the modified membrane structures [152]. In addition, retaining the negative strand RNAs (an antigenome of CoV) within a limited space may increase the template specificity [153].

(ii) *Structural framework and foundation for membrane anchoring of the replication-transcription complex (RTC) of nsps*. Many of the nsps especially those that possess TM region(s) such as nsp3, nsp4, and nsp6 have a suggested scaffolding/membrane anchoring functions for the rest of RTC nsps. Furthermore, the enzyme activities for some of the subcomponents of the RTC have been shown to require tethering for optimal efficiency [144, 152] an observation that fits well to the theory of immobilized enzymes and moving templates in replication/transcription systems [154].

(iii) *Protection*. The double stranded RNA intermediate that occurs during virus replication is a very strong signal of a presence of a "foreign body" in the cells [155]. Restricting the viral replication process to the inside environment of membrane compartments delays the detection by antiviral host cell responses (interferon system) and shield the production of new genetic material. Thus, the double strand RNA intermediate product of CoV replication is hidden inside the internal vesicle of the DVM structure [150].

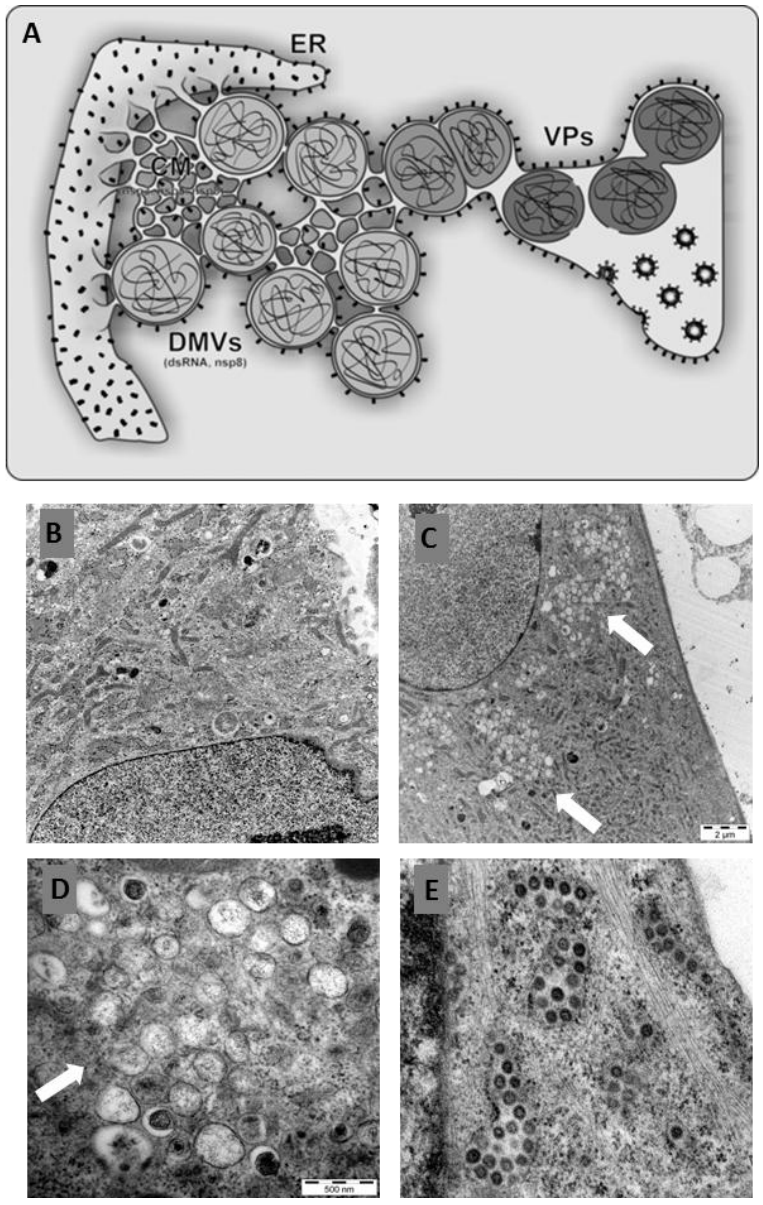


Figure 8. *(A) Schematic model of the reticulovesicular network of modified ER membranes that support CoV RNA synthesis (Figure adapted from Knoops et al PLoS Biology, 2008). (C-E) EM-images of the 229E-CoV induced clusters of DMVs (arrows) and viral particles found in MRC5 cells at 18 h p.i. Note the lack of these structures in uninfected cells (B). CM, convoluted membranes; ER, endoplasmic reticulum; DMVs, double membrane vesicles; VPs, vesicle packets.*

1.4 Respiratory epithelium, a target of RSV and CoV infections

1.4.1 Structure and basic function of the human respiratory epithelium

The conducting respiratory airways comprise the tissues that line the nasal cavity, pharynx, larynx and trachea down to the branched bronchial tree and the small alveoli. The tissues of the human airways constitute a protective barrier between the outside and internal environments of the body. In addition, the epithelial tissues of the respiratory tract comprise properties that extend far beyond having "just" a barrier function. Except for the normal regulatory and metabolic functions the epithelium has multiple roles in protection and defense of the respiratory system such as clearing out potential inhaled agents, attracting and activating inflammatory cells, and recruiting immune cells. The respiratory tract is lined with pseudostratified columnar epithelium in the nasal cavity, larynx, trachea and bronchi, and simple squamous epithelium in the small alveoli.

The major constituents of the respiratory epithelium are these schematically shown in Figure 9. The basal cells are attached to the basement membrane and anchor the superior columnar cells that can be either ciliated or secretory cells. The ciliated cells are located at the surface of the epithelium and possess up to 300 cilia/cell. Since the primary function of these cells is to transport airway fluids together with any trapped pathogens towards the nasal cavity and oropharynx via the coordinated beating of the cilia, there is an abundance of mitochondria in the cells providing the necessary energy for this process [156]. The major types of secretory cells in the respiratory epithelium include, mucus/goblet, clara, and serous cells that produce and secrete large amounts of fluids that are important for proper airway function. Mucus/goblet cells produce mucins, a major constituent of respiratory fluid covering the epithelium. Clara cells are believed to produce bronchiolar surfactants and protease inhibitors, and can be found mainly in the smaller airways of the bronchi.

1.4.2 Respiratory secretions – Physiological function and pathogen defense

Respiratory mucus is a viscous fluid that covers the surface of the epithelium and is produced by the different secretory cells mentioned above. The fluid contains a range of molecules that is carefully balanced to maintain

physiochemical properties required for function and protection of the epithelium and for facilitated transportation towards the nasal cavity and oropharynx. The viscosity of the mucus layer decreases in parallel with the reduction in the size of the airways [157, 158].

The major constituents of mucus are secreted mucins that form a network through extensive coupling of cysteine disulfide bridges. The mucins are elongated and heavily *O*-glycosylated proteins where the sugar entities account for almost 70-80% of the mucin weight [159]. Differences in the glycosylation patterns create a wide range of mucin variants that help to produce the mucin network optimal for the proper physiological and defensive function of the mucus layer [160]. The *O*-linked glycans of mucins are highly hydrophilic and their capability to bind water and ion molecules contribute to mucous viscosity [161]. The glycan chains are often terminated with negatively charged sialic acid or less frequently with sulfated monosaccharide residues [159, 162] which in addition to providing receptor sites for different pathogens [163] may contribute to the acidity of the mucin granules secreted by mucus/goblet cells, and may regulate the viscosity of the secretions [156]. The viscous mucin layer has no direct contact with the epithelial cells, and they are separated by a less viscous layer of fluid that permits undisturbed beating of cilia and thereby an easy transportation of mucus.

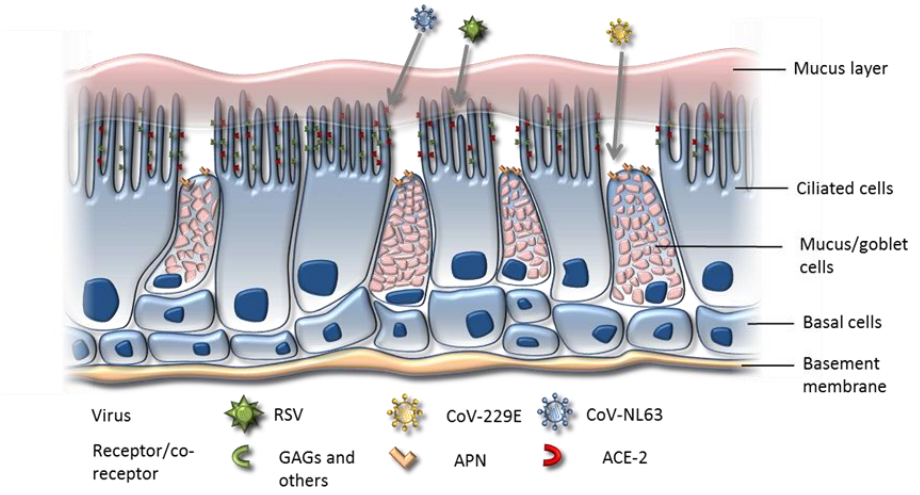


Figure 9*. Schematic structure of the airway epithelium. Note the exclusive expression of APN by goblet and ACE-2 receptor by ciliated cells, a feature associated with specific tropism of CoVs 229E and NL-63 respectively. GAGs, glycosaminoglycans; APN, aminopeptidase-N; ACE-2, angiotensin converting enzyme 2. Illustration kindly prepared by Anders Lundin.*

Antiviral properties of mucin

The mucus layer contains multiple substances with antimicrobial properties. Surfactant proteins A and D have essential roles in the airway defense including direct anti-infectious activities as well as modulation of inflammatory response by the induction of cytokines [164]. Furthermore, mucus contains a wide range of proteolytic enzymes, antiproteases, antimicrobial peptides, and antimicrobial proteins such as lactoferrin, defensins or lactoperoxidase, known for their antiviral properties [Reviewed in 165]. Moreover, respiratory secretions also contain a large number of antimicrobial lipids and sterols such as free fatty acids, cholesterol, and cholesteryl esters that contribute to the airway barrier defense function [166].

Impact of virus infection on mucus and cilia

The main function of the cilia is to transport mucus towards the oropharynx with subsequent movement to the stomach. This is an important feature of the defense system where pathogens, toxins and various particles are captured in the mucus and later destroyed in the acidic environment of the stomach. As mentioned above an optimal viscosity of the mucus and an undisturbed strength and movement of cilia beating are crucial for mucus transportation and the defense against pathogens. Infection of the ciliated cells with RSV, CoVs and other viruses can cause both functional impairment manifested as decreased frequency and in some cases dyssynchronization of ciliary beating, and morphological alterations such as loss of cilia-, and/or cell death and their release into the mucus (Figure 10) [49, 167, 168]. Furthermore, RSV has also been observed to utilize the beating cilia to spread to neighboring ciliated cells [54].

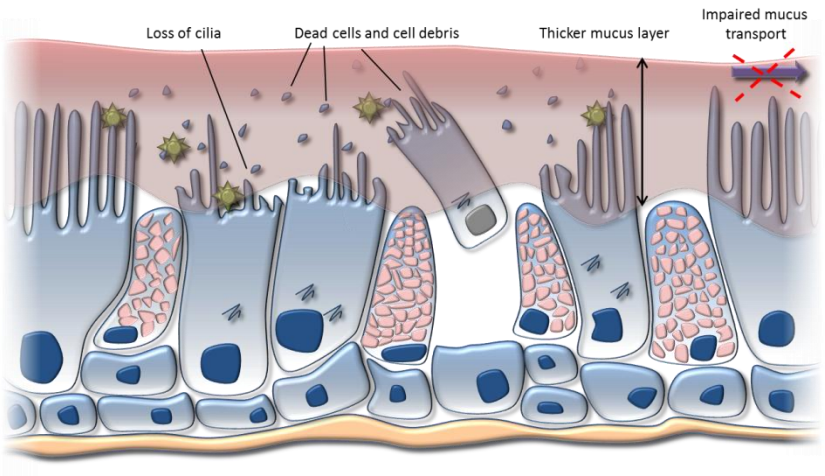


Figure 10. *Morphological and functional impairment of the airway epithelial cells following virus infection. These, among others, may include dyssynchronization of cilia beating, loss of cilia or death and fragmentation of ciliated cells, and thickening of the mucus layer that collectively results in impaired transport of mucus.* ***Illustration kindly prepared by Anders Lundin.***

1.4.3 Epithelial cell surface receptors and CoV/RSV tropism

The different proteins and/or carbohydrate components located on the surfaces of ciliated/secretory cells can provide receptor sites for initial interaction of respiratory viruses. The distribution of these proteins on a particular cell type and/or at specific parts of the respiratory epithelium, may contribute both to the viral tropism and therefore pathogenesis of infection (Figure 9) [54, 169].

RSV specifically targets ciliated cells of the respiratory epithelium and is capable to spread to all parts of the conducting airways. The infection is polarized with both initial infection and budding of progeny virions taking place at the apical surface of the epithelium [54, 170]. The identification of a "true" cellular receptor molecule for RSV has been proven to be a challenge and although a number of candidate receptors have been suggested the issue still remains to be fully resolved.

An example is nucleolin, a protein associated with cell proliferation and growth [171], and recently suggested as being a functional receptor for RSV

[172]. Although nucleolin is most abundantly expressed in the nucleus [173] it can also be found on the cell surface as a part of a protein complex [174]. However, nucleolin is expressed in most cell types and therefore unlikely to contribute to specific tropism of RSV [172].

Role of glycosaminoglycans (GAGs) in RSV infection

One class of molecules identified as initial binding sites for RSV are cell surface GAGs. Structurally, these molecules are large linear chains of repeating disaccharide units which are heavily sulfated and hence have a strong negative charge. Several different GAG classes exist which differ in the type of amino sugar, uronic acid component, glycosidic linkage, and sulfation pattern. Chondroitin sulfate, dermatan sulfate, keratan sulfate (KS), heparin and heparan sulfate (HS) represent the major classes of sulfated GAGs [175]. The GAGs are abundantly expressed on the surface of nearly all animal cells. Their function in binding and regulating activity of a plethora of different proteins is crucial and involves interactions with proteins such as growth factors, enzymes, and chemokines [176, 177]. GAGs have been shown to provide attachment sites for a range of different viruses such as herpesviruses, HIV, flaviviruses and alphaviruses [178-181]. Several studies have emphasized the importance of GAGs heparan sulfate (HS), chondroitin sulfate and heparin for RSV infection in cell culture [76, 182]. However, HS and chondroitin sulfate are poorly expressed on the apical surface of ciliated airway epithelial cells [183, 184]. Instead, based on the suggested preference of RSV for ciliated cells, GAGs such as KS, which have been shown to be extensively expressed on these cells [54], may be important for mediating the attachment of RSV in airway tissue.

The high negative charge of the GAG chains contributes to the multiple electrostatic interactions with target proteins [175]. In RSV the major GAG-binding component is the G-protein (Figure 11). A region in the G-protein ectodomain, referred to as the heparin-binding domain (HBD) or the GAG-binding domain, contains a sequence with a high content of positively charged aa such as lysine or arginine. These basic aa residues are believed to be involved in multiple electrostatic interactions with the negatively charged sulfate/carboxylate groups of GAG chains thus mediating the initial binding of RSV [75]. The RSV-GAG interaction should not be regarded as simple charge association since specific distribution of basic aa in the GAG-binding domain of the G-protein and the specific sulfation patterns of GAG chains provide specificity for this interaction. [75, 185]. Even though GAG interactions are important for RSV infection, it has been shown that mutant viruses lacking the GAG-binding domain of the G-protein was capable of

infecting mutant cell lines deficient in GAG expression [182, 186]. In addition, RSV mutants deficient in expression of the G-protein can infect cultured cells implying that the F-protein may also have the GAG-binding properties or that there are alternative mechanisms that can promote the virus attachment process.

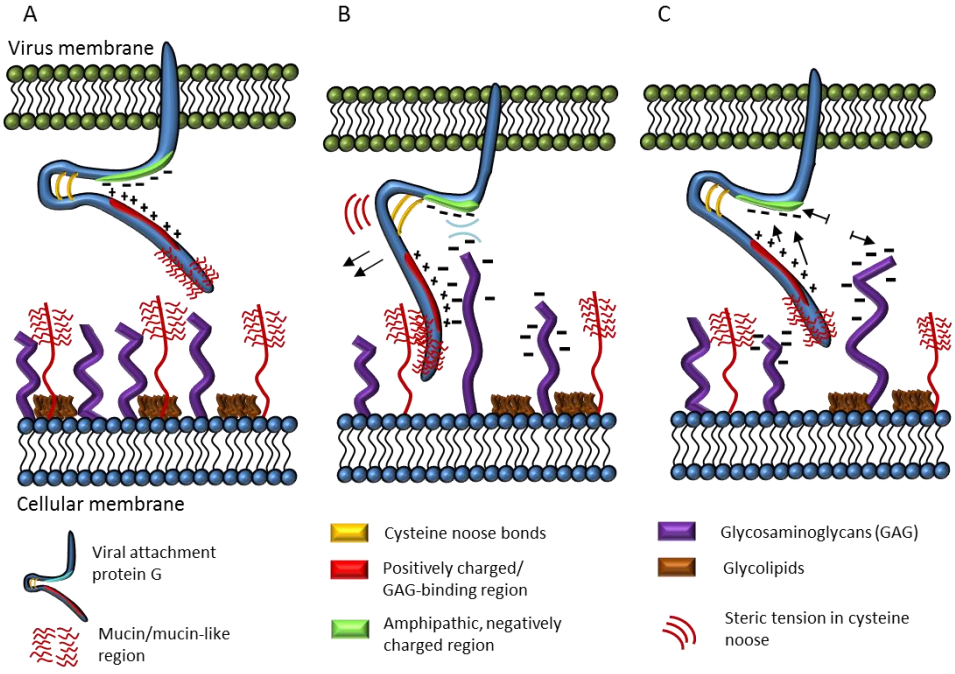


Figure 11. *Hypothetical model of the interaction of the RSV attachment protein G and cell surface GAGs. (A) Central region of the G-protein is composed of a "relaxed" cysteine noose flanked by the two oppositely charged regions i.e. the positively charged GAG-binding domain and the amphipathic negatively charged domain which interact with each other. (B) The mucin like region in the ectodomain of the G-protein may interact with mucins on the epithelial cell surface, an event that brings the positively charged GAG-binding region of the G-protein into close proximity to the negatively charged cell surface GAGs thus promoting their interaction. This binding brings GAGs in closer to the negatively charged domain of the G-protein which creates repulsion and tension in the cysteine noose, thus leading to liberation of the virion.*

CoV receptors and co-receptors

In contrast to RSV, the cellular receptor or co-receptor molecules for respiratory CoVs have been identified. Many of these receptors and their expression in different tissues have a clear connection to the viral pathogenesis. In particular, aminopeptidase N (APN) and angiotensin converting enzyme 2 (ACE2) act as functional receptors for 229E-CoV and SARS-CoV respectively [187, 188]. Both proteins are abundantly expressed in the airway epithelium consistent with predominant respiratory illness that these viruses cause. In addition, both proteins can also be found in tissues like the intestinal epithelium explaining why these viruses may also cause enteric infections [64, 65, 189]. Like SARS-CoV, the NL-63-CoV utilizes ACE 2 as its main receptor [190] while the 9-O-acetylated sialic acid [191] and the major histocompatibility complex class I C (HLA-C) [192] molecules were shown to facilitate infection of cells with OC43 and HKU1 CoVs respectively. CoV tropism, strongly associated with exclusive expression of specific receptors on certain cells, is exemplified by NL-63, OC43 and HKU1 which, similar to RSV, were found to target ciliated cells, while 229E mainly targeted secretory/goblet cells. This tropism is dependent on the expression of e.g. the CoV strain specific receptors ACE-2 and APN on ciliated and secretory cells respectively (Figure 9) [57, 193].

1.5 Antiviral strategies

Since the advent of cell culture techniques and their use for the virus propagation and antiviral testing in the 1950s, there are now more than 50 antiviral drugs available for treatment of virus infections [194]. Most of them are registered for treatment of HIV followed by those used against different herpes- and influenza viruses [195]. In addition, recent advances in development of antivirals for treatment of hepatitis C virus infection have resulted in many new potential candidate drugs [Reviewed in 196]. Efficient and non-toxic antiviral treatment against RSV and CoV infections is still lacking at present, however new antiviral strategies for the inhibition of these virus infections are continuously being developed (discussed further in section 1.6).

Numerous strategies have been established for identifying and developing new and effective antiviral drugs, one being the screening for antiviral activity of large collections of compounds. This can be conducted either on a broad, whole virus basis, which provides the possibility to identify new targets for antiviral intervention or by more target specific screening looking at the impact of a compound on a particular protein or enzyme activity

(discussed further in section 1:7). Besides the screening approach other strategies are focused on the particulars of the virus infection and interplay with the host.

1.5.1 Adverse effect of putative antiviral compounds on cells

An important issue to consider when conducting antiviral testing in cell- or tissue cultures is the possible adverse effect that the test compound in question might have on cells. This issue is of special importance when the compound can cause an unapparent cytotoxicity such as complete or partial cytostatic effect and/or an altered morphology or metabolism of otherwise viable cells. This may result in identification of "tricky" false positive antiviral hits and their subjection to expensive evaluation in experimental animals and clinics.

Since the virus life cycle is inevitably connected to a viable cell, all negative impact of a potential antiviral on cellular components may affect its replication in an unspecific manner. Notably, this mode of antiviral activity of test compounds, i.e., targeting of cellular instead of viral components, is acceptable provided that no adverse effects of potential antivirals on cells are seen (see section 1:5:2). Hence, it is essential to use several different cytotoxicity assays in antiviral discovery utilizing a live host assay system that reflect various aspects of cell viability, proliferation, and metabolism [197-200]. In an evaluation of antiviral potency of a hit compound it is important to relate its safety to the antiviral efficacy. This is often assessed by determining the selective index of the test compound, i.e. the range between the lowest concentration that results in effective antiviral activity and the highest concentration that does not cause cellular toxicity. This difference determines the window of specific antiviral activity, also referred to as "therapeutic index" in the clinical setting [201]. A compound with a narrow selective index is difficult to evaluate in experimental animals and clinics, and requires additional confirmation of antiviral potency and specificity since the risk of a false positive hit is higher if the therapeutic index is low.

1.5.2 Virus life cycle events as targets for antiviral intervention

The viral life cycle involves a number of steps such as (i) attachment and entry, (ii) uncoating and release of genetic material (iii) replication and transcription (iv) virus particle assembly (v) and budding and/or release. Each

step includes components or their activities with potential for antiviral targeting.

All viruses share the general features of the different life cycle steps described below. However, every virus has its own particular way of interacting with host cell components and metabolic pathways, largely contributing to the difficulty of identifying a broad spectrum antiviral drug. Described below and schematically illustrated in Figure 12 are examples of a few of these strategies that resulted in the development of a successful/approved antiviral drug.

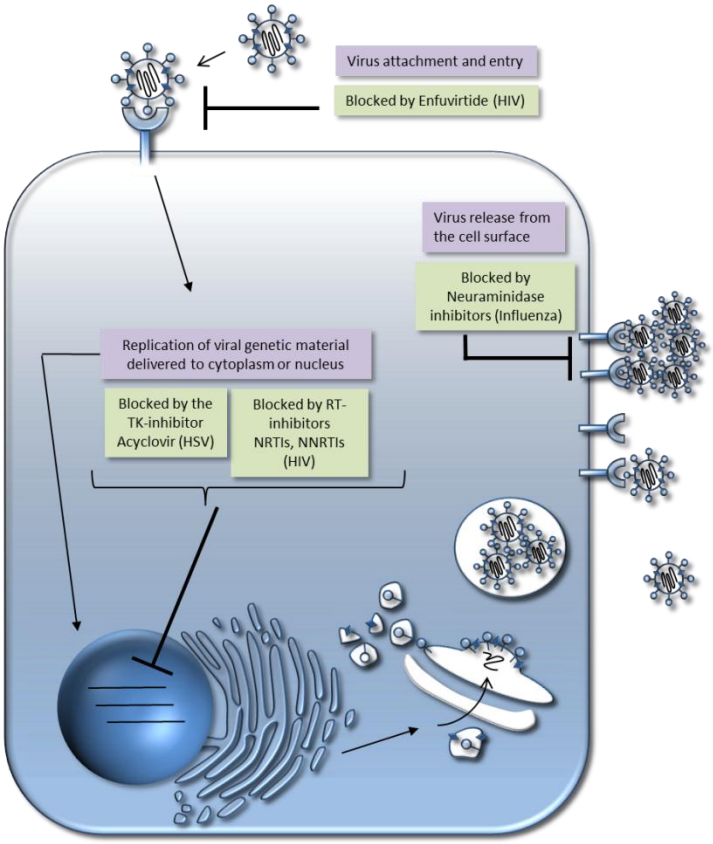


Figure 12. *Outline of the viral life cycle showing steps successfully targeted by antiviral intervention. Specific events of the viral life cycle (purple) targeted by approved inhibitors (green) are shown. TK, thymidine kinase; RT, reverse transcriptase; NRTIs, nucleoside analog reverse-transcriptase inhibitor; NNRTIs, non-nucleoside reverse-transcriptase inhibitor.*

Attachment and entry of viral particles to cells

Targeting the steps of virus attachment to and entry into the cells is often advantageous because the amount of virus present is usually quite low allowing for a potent inhibitory activity that occurs prior to the initial virus contact with cells [202]. The attachment and entry inhibitors can act through several different inhibitory mechanisms including (i) binding to specific virus attachment and entry proteins directly on the virion (ii) binding to the virus receptor molecules at the surface of susceptible cells, and (iii) binding to the intermediate, "activated", form of a viral protein triggered in the attachment or fusion process thus preventing further conformational changes necessary to complete these steps.

In spite of potent antiviral activity in cultured cells many attachment/entry inhibitors such as sulfated polysaccharides failed to protect humans in clinical trials [203]. The first and perhaps the only drug approved is enfuvirtide, a peptide derived from the HR2 region of HIV gp41 protein targeting viral fusion activities and thus the entry of HIV into the cells [Reviewed in 204].

Replication of viral nucleic acid

The viral genetic material delivered into the cell is subjected to replication/transcription events. Depending on the virus species, this involves the viral and to varying extent the cellular machinery and may take place at the nucleus, cytoplasm or both compartments. The genetic material of viruses can be either DNA or RNA, and in the case of some RNA viruses an intermediate DNA step may occur via the activity of reverse transcriptase (RT). Because of the strict virus specificity of RT this enzyme is well suited as an antiviral target, which led to the development of a new class of anti-HIV drugs referred to as RT-inhibitors. These include both nucleoside- and non-nucleoside analogs, NRTIs and NNRTIs respectively, which inhibit the RT activity and act as chain terminators in DNA elongation [205, 206].

Another group of antivirals targeting the replication process are the DNA polymerase inhibitors exemplified by the anti-herpesvirus drug acyclovir. Acyclovir was the first highly selective and non-cytotoxic antiviral drug discovered [207]. Being an analog of guanine, this drug also acts as a premature chain terminator, similar to the RT-inhibitors for HIV, but its specificity lies in a herpesviral enzyme, thymidine kinase (TK). To be accessible to viral DNA polymerase, this drug must be enzymatically activated (monophosphorylated) by TK. Acyclovir is suitable for enzymatic activation by herpesviral TKs but not cellular TKs indicating that its activity

is restricted to the virus infected cells, a quality that makes this drug highly specific with little or no cytotoxicity [207].

Budding and release of viral particles from cells

A classic example of an antiviral strategy targeting the virion release from cells is the inhibition of influenza neuraminidase. Haemagglutinin (HA) is the viral attachment protein that binds to sialic acid-containing entities on the cell surface to mediate the virus binding to and subsequent penetration into the cells. Although the sialic acid binding activity of influenza virus HA is profitable for initial events in the viral life cycle, it is an obstacle in the virus release stage since the progeny virions can be trapped at the cell surface and cross-linked to form large clumps due to virus binding to sialic acid present at the cell surface and on glycoproteins of other virions. At this stage, a second influenza virus surface protein, neuraminidase (NA), cleaves the sialic acid containing entity thus liberating trapped virions and releasing them from the cell surface. The neuraminidase inhibitors, which are analogs of sialic acid residue, exhibit tight binding to NA thus blocking the activity of this enzyme and making it inaccessible to cellular sialic acid residues [208].

Cellular targets

In addition to the virus specific targets, there are a number of different intrinsic cellular features that are utilized by viruses such as those involved in DNA, RNA and protein synthesis [205], in intracellular signaling, innate immune mechanisms, and others. Therefore, features of the host cell exploited by the virus can also be targeted by antivirals provided that such intervention causes no adverse effects on cells. Targeting cellular processes may have the advantage of a broader spectrum of inhibition since some viruses use similar components during infection. Furthermore, antivirals aiming at cellular targets tend to be less vulnerable to the development of viral resistance. For example, ribavirin, a drug whose antiviral activity was associated with “forcing” the viral polymerases to increase the number of spontaneous errors/mutations to the level deadly for a virus, in the case of RSV was shown to act as an inhibitor of cellular inosine monophosphate dehydrogenase that depletes the cellular pool of guanosine required for replication of viruses [209]. Furthermore, a wide range of intracellular signaling pathways exemplified by RhoA used by RSV [210] and both NF-kB and Raf/MEK/ERK signaling employed by influenza virus are promising targets for antiviral intervention [Reviewed in 211].

1.5.3 Antiviral drug resistance

One of the greatest challenges in the development of antivirals is the emergence of viral resistance. Viruses have an intrinsic property of high speed replication and a remarkable capacity of adjusting to changes in their environment. In addition, virus replication is evolutionary prone to errors leading to a large number of virus variants, often called quasispecies, with a higher chance of natural drug resistance. The rate of mutation and recombination are the major determinants for emergence of drug resistant viral variants, a powerful feature of viral replication leading to the selection for resistant variants against practically all currently known antivirals [212]. The risk of resistance development is also increased in immunocompromised individuals where the duration of infection is often extended or followed by several consecutive infections requiring a prolonged drug exposure [213-215].

Since the drug resistance is a major concern in the area of antiviral intervention attempts to avoid, or at least reduce, the rate of this phenomenon have been made. One such strategy is commonly applied in the treatment of viral infections where several different classes of antivirals are available, as in the case of HIV or HCV. Simultaneous challenge of the viral pathogen with several drugs that target different viral components prevents the virus from "finding" a mutational escape route other than lethal or at least a very harmful combination of mutations for the virus. This can be exemplified by the HAART therapy for HIV infection where the simultaneous treatment with at least three antiviral drugs targeting the virus replication and transcription by different mechanisms reduces the incidence of resistance [216]. Other strategies focus on antiviral targets with available drug treatment towards which resistant strains have already appeared. Here the design of a new antiviral candidate with improved target interaction and more irreversible impact could restore the drug sensitivity in previously resistant strains, and decrease the risk for further development of resistance. An example of this strategy is the development of a novel class of neuraminidase inhibitors for treatment of influenza infections [217].

1.6 Searching for anti-RSV and anti-CoV intervention

1.6.1 RSV vaccine and prophylaxis

Early attempts to develop an RSV vaccine in 1966-1967 began with a disaster when a formalin-inactivated RSV-vaccine failed to induce a protective immune response resulting in a more severe illness upon natural reinfection in vaccinated children than in the control group. This incidence led to hesitations and large precautions in further development of RSV vaccines [218, 219].

The reason of why the formalin-inactivated RSV vaccine enhanced the disease is not completely understood, however, induction of inappropriate cell mediated immunity i.e. an exaggerated allergy-like Th2 response has been suggested. A Th2-biased response to RSV infection is associated with increased levels of white blood cells in the lungs such as pulmonary eosinophilia, in addition to IgE production and enhanced immunopathology. In addition, the Th2 response is also associated with airway hyperreactivity and increased production of mucus which can contribute to airway obstruction. Moreover, the clearance of RSV infection can be delayed by a high Th2 response since Th2 associated cytokines, such as IL-4 and IL-13, are suggested to interfere with the virus clearance function of $CD8^+$ T cells [220, 221]. Furthermore, the antibody response induced by the vaccine was altered in comparison to natural RSV infection and did not display an efficient neutralization of RSV infectivity. This indicates that immunogenic epitopes could have been damaged during vaccine preparation resulting in insufficient protective immunity [222].

Although no RSV vaccine is currently approved, a prophylactic intervention is available. Palivizumab, and its affinity-optimized variant Motavizumab, are humanized monoclonal antibodies (Mab) specific for the RSV F-protein [223, 224]. The antibodies are directed towards antigenic sites located in the F1 subunit of the protein [225] and prevent the virus-induced fusion events occurring between the viral and cellular membranes as well as in cell-to-cell spread of RSV [226]. In the large clinical studies preceding the 1998 FDA approval of palivizumab it was shown that prophylaxis with this Mab resulted in a 55% reduction in the number of children needing hospital care in comparison to control groups [227]. Palivizumab is administered by intramuscular injections once per month during the RSV season at a dose of 15mg/kg. Even though the prophylaxis can reduce the incidence of

hospitalizations the treatment is very expensive and therefore limited to infants and children at high-risk of RSV infection, meaning mainly premature infants or young children with underlying conditions such as congenital heart disease or chronic lung disease [228]. Furthermore, the cost/effectiveness of palivizumab administration is a matter of debate [229-231]

1.6.2 RSV antivirals

The only approved drug for treatment of RSV infection is the nucleoside-like analog ribavirin. The drug is administered as an aerosol for an extended period of time, and is usually provided by health care professionals in the hospital setting. Ribavirin has a broad spectrum of antiviral inhibition for a range of both DNA and RNA viruses [232, 233]. Several inhibitory mechanisms have been reported for ribavirin and the specificity of its anti-RSV activity is not entirely clear. The reported antiviral activity of ribavirin includes inhibition of inosine 5'-monophosphate (IMP) dehydrogenase that exhausts the guanine pool, blockade of the formation of the 5'cap on mRNA, and inhibition of viral RdRp. In addition, ribavirin may also play a role in enhancing antiviral immunity by shifting a Th2 response towards a Th1 response, and act as a mutagen pushing already high inaccuracy prone viral RdRp over the "error catastrophe" edge [234, Reviewed in 235]. Even though ribavirin is the only available treatment for severe RSV infection the efficiency of this drug in RSV disease is ambiguous. The activity of ribavirin is not specific for RSV and is considered to be suboptimal. In addition, the treatment is fairly expensive and the drug has an uncertain safety profile [236, 237]. Hence, due to the lack of effective and safe anti-RSV drugs, numerous attempts at identification of novel drug candidates are being made.

Because of essential functions of the F-protein in the RSV life cycle, and the presence of highly conserved structures, the fusion activity of this protein has been selected as an important target for development of antivirals. Listed in Table 1 are mostly small-molecule candidate anti-RSV drugs with antiviral potency ranging from nanomolar down to picomolar concentrations as exemplified by compounds BMS433771 and TMC-353121 respectively [238, 239]. During the virus-induced fusion the F-protein undergoes a series of conformational changes and most fusion inhibitors interfere with and prevent this protein from completing these transitions (Figure 13). The fusion inhibitors target different areas of the F-protein such as the HR domains thus preventing the formation of the six helix bundle complex (see section 1:2) [238, 240]. It is suggested that the level of antiviral inhibition is dependent on the degree of activation of the virus fusion protein. Meaning that if the antiviral compound targets the activated, fusogenic, form of the protein the

antiviral potency depends on the rate of transition of the F-protein from the inactive form into the active form [241].

Thus far, none of these compounds have progressed to late stage clinical trials. Most compounds that have reached the early phase clinical testing are later discontinued due to insufficient activity or undesirable safety- and/or pharmacokinetic profiles. These compounds can display a very short time-span of activity in vitro limited to the first hours of RSV life cycle [242]. However, recent reports of the optimized fusion inhibitor TMC353121 showed that the compound is active up to 48 hours after infection indicating that a fusion inhibitor, although time-dependent, can have an extended activity span likely caused by affecting the fusion events in the RSV cell-to-cell spread [243].

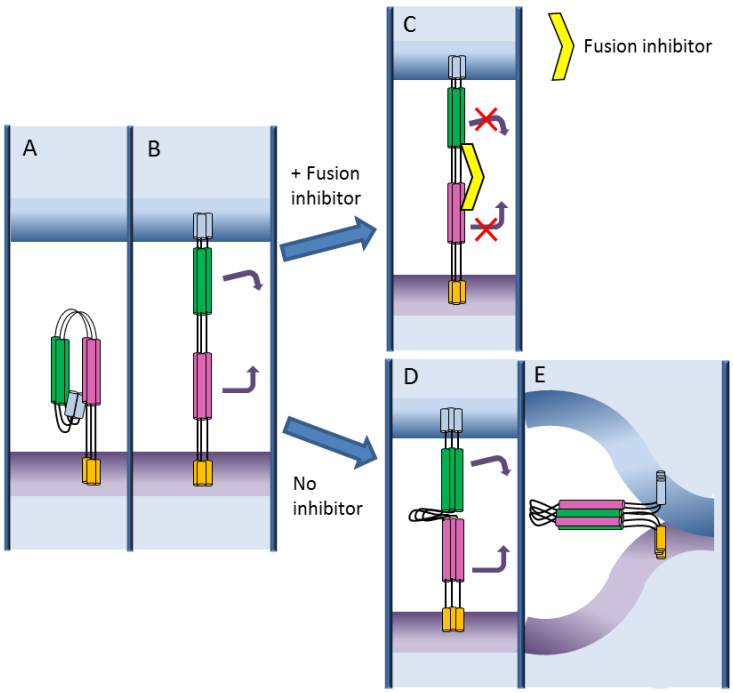


Figure 13. *Inhibitory mechanism of fusion inhibitors targeting the extended intermediate form of the F-protein. The compound binds to the F-protein in its extended form (B) thus blocking the interaction between the two HR regions (in green and purple) and/or stabilizing the intermediate form in its extended conformation (relate C to D/E steps).*

Due to the relatively high variability in the attachment G-protein aa sequence and, associated with it, decreased antigenic cross-reactivity between different RSV strains, antivirals targeting this protein are far fewer than those affecting the fusion process. Even so, the small-molecule candidate MBX-300 (NMSO3) composed of sulfated sialic acid conjugated to two alkyl chains, was found to inhibit the RSV attachment to cells through interference with the activities of the G-protein. The inhibitory effect occurred at low micromolar concentration and therefore this compound proceeded into early preclinical/clinical trials [244, 245].

Furthermore, the important interaction between the RSV G-protein and the cell surface GAG receptor during the virus attachment step have led to the development of another group of RSV inhibitors consisting of polyanionic compounds. As discussed in section 1.4 the positively charged GAG binding domain of the G-protein is important for the interaction with anionic sulfate groups of the GAG chains. Macromolecular compounds mimicking sulfation pattern and structure of the GAG chains such as dextran sulfate, heparin, pentosan sulfate, galactan sulfate and many other polysulfated compounds have been proven to be efficient inhibitors of infection of cells by RSV and many other GAG-binding viruses at low microgram/ml concentrations [246-248, Reviewed in 249]. Although this class of antiviral compounds has the potential of broad spectrum activity their interactions can be weak and reversible. Nevertheless, studies show that coupling a lipophilic entity to the GAG-mimetic could confer a stronger binding and improve antiviral activity as shown for inhibitors of HIV and HSV [250, 251]. In **paper II** the coupling of lipophilic entities to polysulfated tetra- and pentasaccharide glycosides and its effect on anti-RSV activity was evaluated.

Antiviral drug candidates targeting the post-fusion events in the RSV life cycle are mainly those interfering with the virus replication including essential activities of the N- nucleocapsid or the L-polymerase components of the RSV transcription complex. Compound YM53403 inhibited RSV infectivity with an IC_{50} value of 0.2 µM by targeting the viral polymerase [252]. The 1,4-benzodiazepine candidate drug RSV-604 was shown to target the N-protein and inhibit RSV replication if added up to six hours after infection. The compound advanced into phase II clinical trials in 2006 and was still in phase II in 2010 [253]. In addition to a small-molecule inhibitor of the N-protein there is also a small interfering RNA (siRNA) based candidate, ALN-RSV01, which inhibits RSV by interrupting the synthesis of the N protein. This compound was shown to be active if added up to 24 h p.i. and is in phase II clinical trials [254, 255].

1.6.3 CoV vaccine and prophylaxis

Before the emergence of SARS-CoV in 2003, the disease caused by CoVs was not considered to be severe enough to justify the development of a vaccine. Following the SARS-CoV outbreak there was an immediate shift in the efforts to find a vaccine for these severe infections in hopes of preventing a new outbreak. It has been reported that SARS-CoV induce the production of protective neutralizing antibodies in infected individuals [256, 257] indicating that neutralizing antibodies can be used for protective measures. In contrast, infection with a seasonally circulating CoVs such as 229E does not provide a strong protective immunity and reinfections are common [258]. Although several vaccine strategies are currently being developed no vaccine is available for immunization against any CoV.

Several studies indicate that passive administration of neutralizing antibodies specific for the S-protein can be used as effective immunoprophylaxis or immunotherapy of CoV infections [Reviewed in 259]. In spite of these efforts no preparation of CoV-neutralizing antibodies or immune serum is available for prevention/treatment of CoV infections.

1.6.4 CoV antivirals

As a consequence of the outbreak of SARS-CoV in 2003, the antiviral research focused towards this group of viruses has increased immensely. Since cellular receptors for different CoVs are known to interact with the viral S-protein [Reviewed in 260] several different antibodies, small-molecule compounds and peptides were shown to interfere, with varying efficacy, with the CoV attachment and entry steps [261-263]. Anti-CoV candidate drugs targeting other structural proteins, i.e. E-, M- and N-proteins have also been reported with focus on specific siRNAs inhibiting the expression of these proteins and preventing their important roles in the viral life cycle [Reviewed in 264].

The major portion of the anti-CoV strategies developed so far have focused on interference with important CoV enzymatic activities held by the three virus nsp proteases, i.e., the 3CLpro (nsp5), PL1pro and PL2pro (nsp3), as well as the 5'-3' helicase (nsp13) and the RdRp (nsp12) [Reviewed in 264]. The crystal structure of the main CoV protease, 3CLpro [265] revealed intricate structure details and allowed for a range of modeling techniques to be used in the search for potential antiviral compounds. The essential proteolytic activity of the 3CLpro in all CoVs and its conserved structure imply the possibility of developing a broad spectrum CoV inhibitor [266]. In

fact several candidate drugs targeting this protease such as the dipeptide inhibitor JMF1521 and others have been identified [267] and are listed in Table 1.[268, 269].

Strategies targeting the enzymatic functions of CoV helicase and RdRp have also been pursued. An example is the inhibition of SARS-CoV helicase by a group of adamantine-derived bananins with fairly good potency in the micromolar range [270]. Due to the conserved nature of these proteins their inhibitors may have a broad CoV-spectrum potential, however, so far no sufficiently strong antiviral candidate has been identified [271]. Although CoVs express 16 nsps only a few of them exhibiting enzymatic activities were targeted by antivirals, some of which are shown in Table 1. In **paper IV,** we report for the first time on the identification of an inhibitor of nsp6 activity in 229E-CoV and other human and animal CoVs.

Table 1. *Anti-RSV and anti-CoV candidates*

	Target class	Drug/ Candidate name	Development status	Target/mode of action	Ref
RSV	Prophylactic monoclonal anti-F antibody	Palivizumab	Approved 1998	Binds to epitopes on the F1 subunit	[226]
	Nucleoside analogue	Ribavirin	Approved 1980 (inhalant form)	Replication inhibitor	[272]
	Fusion inhibitor	TMC-353121	Phase II	Binds to the heptad repeat regions	[239]
	Fusion inhibitor	BMS-433771	Phase I, discontinued	Binds to epitopes on the F1 subunit	[238]
	Fusion inhibitor	VP-14637	Phase I, discontinued	HR2 and intermediate domains of the F1 subunit	[240]
	Fusion inhibitor	RFI-641	Preclinical, discontinued	Binds to epitopes on the F1 subunit	[273]
	Attachment inhibitor	MBX-300 (NMSO3)	Preclinical	Conserved regions of Protein G	[245, 274]
	Polymerase (L) inhibitor	YM-53403	Preclinical	Transcription/replication inhibitor targeting L-polymerase	[252]
	Nucleocapsid (N) protein inhibitor	ALN-RSV01	Phase II	siRNA, prevent the synthesis of N-protein	[254]
	Nucleocapsid (N) protein inhibitor	RSV-604	Phase II	N-protein inhibitor	[275]
CoV	Protease inhibitor	JMF1521	Unknown	Competitive inhibitor of SARS-CoV 3CLpro	[267]
	Protease inhibitor	Cinanserin	Unknown	SARS-CoV and 229E 3CLpro inhibitor	[268]
	Protease inhibitor	Octapeptide AVLQSGFR	Unknown	SARS-CoV 3CLpro inhibitor	[269]
	Nucleoside analogue	6-azauridine	Unknown	NL63 replication inhibitor	[276]
	Helicase inhibitor	Adamantene-derived Bananins	Unknown	SARS-CoV helicase ATPase activity inhibitor	[270]

1.7 High throughput screening

High throughput screening (HTS) is a method employed for the screening of large collections, libraries, of up to over a million compounds where the effect/activity on a biological process of interest is measured in cluster 96 to 3456 plate formats in a total assay volume of 1-100 µl. This methodology makes it possible to screen a large number of compounds or factors simultaneously under the same conditions which also helps to reduce intra- and inter sample variations making the method robust. In addition, the assays can be scaled down and hence reduce the amount of material required for the assays to a minimum. The process can be more or less automated giving rapid assessment of a large number of factors in a standardized system at a lower cost [277-282].

Libraries to be used in HTS can contain random small-molecule compounds, natural product extracts, monoclonal antibodies, siRNAs, biomarkers, enzymes etc. and can be further divided into categories e.g. based on what biological activity they target. Selection of small compounds to be included in the libraries can vary widely but emphasis is put on their "drug-like" properties and a varying degree of diversity. The compounds with potential drug-like properties are identified through comparative analysis of medicinal chemistry databases and drug-like indexes that relates molecular structures of known drugs with candidate library compounds [283]. The evaluation of the drug-like potential of a molecule in question is based on analysis of functional groups and basic chemical properties, often involving the Lipinski's rule of five. These set of rules or guidelines help to identify compounds with desirable qualities in a molecular structure suitable for a drug [284].

Based on the fact that a number of licensed drugs originate from natural products, many libraries also contain extracts from plants and microbes with both land and marine origin, some of which are used in traditional medicine [285-288]. Due to the complex process of extracting and isolating active compounds from plants, the focus has previously been on screening of synthesized molecules. However, with new preparation techniques and the need for unexplored compound sources more libraries containing natural products are still emerging [289, 290].

HTS has now developed into one of the most important tools for drug discovery including the field of antiviral research. In the case of antiviral

screening, one of the more traditional protocols for whole-virus assays include testing the effects of library compounds on virus infection of cultured cells with the use of microscopic analysis to evaluate the protection of cells from the virus-induced cytopathic effect. With the recent development of screening systems with more focus on tissue specific primary cells it is also possible to get a higher physiological relevance in the screens [291]. This method, although time consuming, allows for differentiation of "true" antiviral hits from those causing adverse effects on cultured cells. The use of viral constructs expressing so called reporter proteins such as GFP can facilitate the reading of HTS assays, however identified hits have to be reevaluated for their cytotoxicity. Apart from this empiric/random screening, the HTS can be more rational/specific [292] where the viral target such as expressed and/or purified viral proteins with enzymatic activity (e.g. polymerase or neuraminidase) or viral components possessing non-enzymatic activities (e.g. viral membrane-fusion protein) are screened in cultured cells or in cell-free system. The results are subsequently acquired with different read-out techniques, such as spectroscopy/luminescence/fluorescence, FACS or high content imaging analysis. The basic luminescence/fluorescence read-outs are commonly used in HTS and are straightforward, inexpensive and suitable for studying the activity of particular target protein and/or cellular toxicity. In comparison, HTS read-outs with high content imaging (HCI) results in large amounts of data from several variables simultaneously that can be compared across cell populations. The HCI technique has a higher complexity and efficiency and allows for the analysis of multiple factors and their correlation. Even so, due to the amount of data generated the analysis is time consuming and the costs are higher.

2 AIMS

The aim of this work was to develop new antiviral candidates for treatment of RSV and CoV infections through establishing and using a sequential assay strategy for their identification and subsequent elucidation of their mechanism of action. The specific aims were the following:

- To identify novel anti-RSV and anti-CoV candidate drugs by screening large compound collections in a whole virus cell culture-based system optimized for RSV and CoV.

- To establish and optimize an assay-strategy for the step-by-step evaluation of the mode of antiviral activity of the anti-RSV and anti-CoV hits.

- To assess/elucidate antiviral potency, cytotoxicity and mode of antiviral activity of the following hits and their analogues.

    i. Lipophile-conjugated polysulfated oligosaccharides as anti-RSV hits
    ii. Benzenesulfonamide-based P13 and diazepane-based C15 anti-RSV hits
    iii. Benzamide-based K22 anti-CoV hit

3 MATERIALS AND METHODS

3.1 Collections (libraries) of compounds for antiviral screening

The compounds screened for anti-RSV and anti-CoV activity in **paper I** and **IV** originated from the ChemBioNet library of 16671 diverse drug-like compounds obtained from the Leibniz Institute for Molecular Pharmacology (FMP, Berlin, Germany). Compounds were supplied in a 384-well plate format at 10 mM concentration in DMSO and were subsequently diluted in water to appropriate concentrations and stored frozen at -20°C until screening. Larger quantities of compound K22 were obtained from ChemDiv (San Diego, California, USA) and subsequently verified for structure and purity by ^{1}H NMR and LCMS.

The mini-library of polysulfated tetra- and pentasaccharide glycosides, evaluated in **paper II,** was obtained from Progen (Australia). Most of these glycosides were composed of α(1→3)/α(1→2)-linked mannose residues with a set of varying lipophilic groups attached to the reducing end. These glycosides and the PG545 glycoside composed of maltotetraose coupled to a cholestanol group, were all prepared and characterized by ^{1}H NMR, ^{13}C NMR, mass spectrometric, and microanalytical techniques as described previously [293, 294]. The precursor compounds for the saccharide fragments of these glycosides, except for PG545, were the sulfated di- to hexasaccharides of muparfostat (PI-88). These oligosaccharides were prepared by sulfonation of phosphomannan, derived from the yeast Pichia Holstii [295, 296], as described previously [297]. All oligosaccharides and glycosides were dissolved in water to a final concentration of 10 mg/ml and stored frozen at -20°C.

3.2 Viruses and cells

Screening of library compounds for anti-RSV activity and other cell-based assays were conducted using the laboratory strain RSV A2 [298] and human laryngeal epidermoid carcinoma (HEp-2) or in some assays baby hamster kidney (BHK-21) cells. They were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal calf serum (FCS), 60 µg/ml of penicillin, 100 µg/ml of streptomycin (PEST), and 8% tryptose–phosphate broth (for BHK-21).

Screening for anti-CoV activity and other cell-based assays were conducted using the laboratory strain of 229E-CoV [25] and human embryonic lung fibroblasts (MRC-5). The cells were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% heat-inactivated fetal calf serum, (HI-FCS), 1% L-glutamine and PEST. Antiviral testing concerning other CoVs of human and animal origin were conducted using infectious bronchitis virus Beaudette (IBV-Beau-R) strain [299] and Vero cells, SARS-CoV strain Frankfurt-1 [300] and Vero cells, MERS-CoV [33, 301] and well-differentiated cultures of human airway epithelial (HAE) cells, and recombinant Renilla luciferase expressing feline CoV (FCoV-Black-Ren) [302] and feline fetal FCWF-4 cells, Gausia luciferase expressing murine hepatitis virus (MHV-A59-Gluc) and murine L929 fibroblasts, and Renilla luciferase-expressing 229E-CoV (229E-CoV-Ren) [303] and HAE cells. Isolation of normal human bronchial epithelial cells and their subpassaging to form pseudostratified/differentiated cultures of HAE cells were performed as described previously [57, 304]. Use of CoV recombinants expressing reporter luciferases facilitated evaluation of candidate antiviral compounds in HAE cells.

3.3 Antiviral screening and initial assessment of antiviral activity and cytotoxicity of hits

Screening of the ChemBioNet library for anti-RSV and anti-CoV activity was conducted in a 384-well plate format in a total volume of 50 µl (Figure 14). HEp2 or MRC-5 cells were seeded one day prior to the experiment to a confluency of 60-90%. Appropriate RSV A2 or 229E-CoV concentration for the assay was determined by the virus stock titration in the 384-well system, i.e. at the same conditions as the screening assay. Library compounds were added to cell monolayers at final concentrations of 60 µM and 20 µM for the anti-RSV and anti-CoV screenings respectively followed by the virus addition. Cells in some wells received corresponding volumes of DMSO solvent or water with or without presence of virus to serve as controls. Plates were incubated at 37°C, 5% CO_2, for four days and inspected microscopically for presence of virus induced cytopathic effect (CPE). Compounds were evaluated based on their capability of protecting the cells from virus induced CPE which also provided the first estimation of their specific antiviral activity and possible adverse effects on cells.

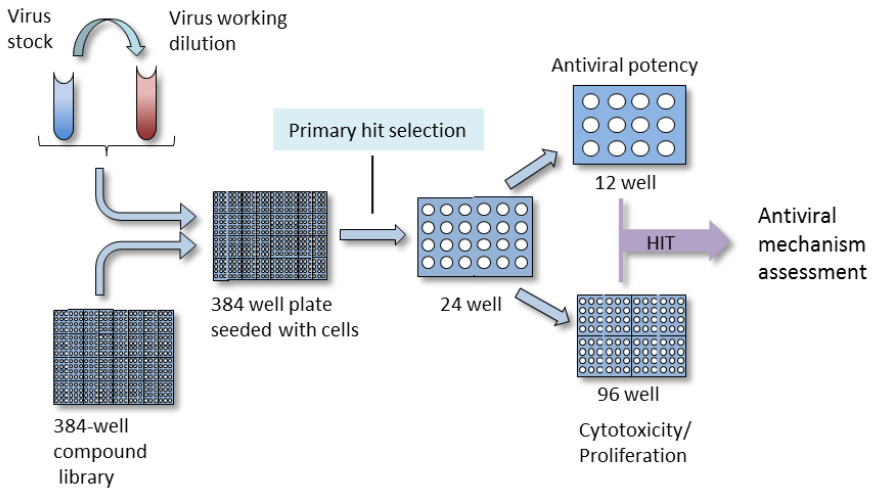


Figure 14. *Schematic overview of the compound collection screening. The main screening was carried out in a 384-well cell culture system seeded with the virus specific susceptible cells. Primary hits were subsequently assessed in two steps using 24- and 12-well formats, complemented by evaluation of the hit effect on cell viability and proliferation. Promising hits were subjected to further assessment of their antiviral mechanism.*

Following the primary screening round, candidate hit compounds were selected for a second screening round in the larger 24-well cell culture format at a four-level concentration span. Promising hits were subjected to additional assessment of antiviral activity at fivefold dilutions in 12-well format along with cytotoxic/cytostatic and proliferation analysis. The antiviral potency of the compounds were established by determining the concentration inhibiting the appearance of virus induced CPE by 50%, IC_{50}. More specifically, 100-200 PFU of RSV or CoV strains and fivefold compound dilutions were added to cells and incubated for 3h at 37°C. For antiviral activity assessment of the GAG-mimetics in paper II the compounds were pre-incubated with the virus for 10 min at room temperature before addition to HEp-2 cells. This step was followed by removal of the inoculum and an additional 2-3 days of incubation under a methyl cellulose overlay supplemented with corresponding concentrations of hit compound. The overlay restricts the virus infection to the cell-to-cell spreading resulting in formation of viral plaques which are subsequently visualized by crystal violet staining.

Antiviral potency of hits against other CoVs was assessed in their respective susceptible cells (see subsection 3:2) using the virus yield reduction assay. Hit compounds at twofold dilutions were added to susceptible cells at 4 h prior to the 1 h period of their inoculation with the virus. This step was

followed by removal of the inoculum, rinsing and addition of assay medium containing corresponding concentrations of compound. The infectious culture medium was harvested and the effect of hit compounds on the propagation of CoVs was monitored either by quantification of amount of viral genomic RNA (SARS- and MERS-CoV), by measuring luciferase expression (MHV-A59-Ren, FCoV-Ren and 229E-Ren), or by $TCID_{50}$ determination in chicken embryo kidney cells (IBV-CoV).

3.4 Cell toxicity and proliferation assays

The potential negative impact of the antiviral candidates on cells was assessed by tetrazolium-based (MTS) cytotoxicity assay and the proliferation assays. Toxicity of the tested candidates was expressed as the concentration of compound reducing cell viability or cell proliferation by 50%, CC_{50}. HEp-2 or MRC-5 cell monolayers in 96-well plates were supplemented with test compounds at fivefold concentrations at a range of 0-500 µM or µg/mL for 48-72h at 37°C. The compound cytotoxicity was estimated by the addition of the MTS reagent for 1-2 h. MTS is metabolized into a colored formazan product by viable cells and was detected by spectrophotometric measurement at an absorbance of 490 nm against a background of 650 nm. Experimental procedure for the cell proliferation assay was similar to the cytotoxicity with incubation of serially diluted compounds for 48-72h at 37°C. Cells were subsequently dissociated with EDTA/trypsin and counted. This allows for assessment of possible cytostatic activity of test compounds. Cells incubated with corresponding amounts of DMSO solvent or water was included as controls.

Estimation of compound cytotoxicity and impact on cell viability on Vero, L929, FCFW and HAE cultures was assessed using the luminescence assay kits CytoTox-Glo™ and CellTiter-Glo® (Promega) based on the live cell membrane permeability and the ATP quantification respectively.

3.5 Elucidation of compound mode-of-action

Following primary and secondary screening rounds identified anti-RSV and anti-CoV hits were subjected to elucidation of their mechanism of antiviral activity (mode-of-action). All methods described in Figure 15 were used as a step by step elucidation of putative mechanisms of antiviral activity. Each

assay provided information aiding identification of the antiviral target. For a more detailed description see **papers III and IV**.

3.5.1 Time-of- addition/removal, and virucidal assays

Firstly, the time-of-addition/removal assay provided initial guidance of whether the hit compound targets the cell, the viral particle, or the virus replication in cells (Figure 15). The time of addition/removal assay evaluates the extent of inhibition of virus infection when the hit compound is added to MRC5 or HEp-2 cells at different time points relative to the virus inoculation. In particular, hit compound was added to and incubated with cells for periods of 2 h occurring either before, during or after a 2 h inoculation of cells with the virus. Following each period of incubation of cells with hit compound and/or the virus, the medium was removed, the cells rinsed and overlaid with methylcellulose, and incubated for 3 days at 37°C. The viral plaques were visualized by staining with crystal violet. Potent activity of a hit whose presence on cells was restricted to a short period before inoculation with the virus strongly suggests that this compound targets cellular receptor sites for the virus. In contrast, the lack of antiviral activity at this period and substantial inhibition during co-incubation of the hit-virus mixture with cells indicate that hit compound targets the viral particle and inactivate its infectivity with or without affecting activities of the virus attachment/entry proteins. To discriminate between these possibilities the activity of hit compound is challenged in virucidal, attachment and fusion assays. A hit showing the most potent activity when present after the virus inoculation of cells usually targets the post-entry events of infection.

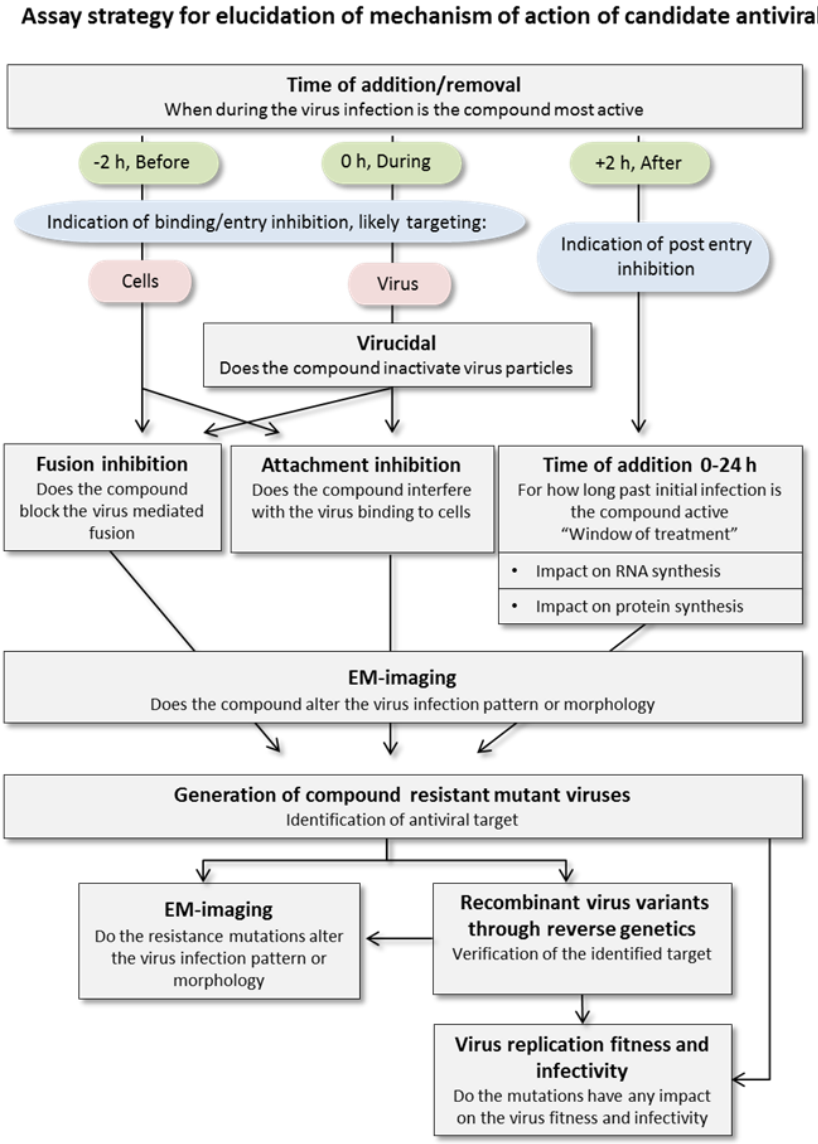


Figure 15*. Assay strategy for the elucidation of the antiviral mechanism of identified hit candidates. Initial time of addition/removal assays resulted in an indication of when during the virus life cycle the compounds had their most potent inhibitory activity and whether it mainly targeted the virus particle or cell components. Analysis of the compound antiviral mechanism by this step-by-step assay strategy generated information on specific viral components involved and their roles in the viral life cycle. The preparation of the drug resistant viral variants and their subsequent analysis revealed more information on the virus escape mechanisms and potential alterations in the virus morphology or infection pattern.*

Virucidal, or the virus-neutralization, assay helps to clarify whether hit compounds irreversibly inactivates infectivity of RSV or 229E-CoV particles. This assay relies on mixing of virus at a high PFU of 10^5/0.5 mL with the hit at three different concentrations, 100, 10 and 1 µM or µg/mL, or with a diluent medium to serve as control. The virus-hit mixture was then incubated in a 37°C water bath for 15 min followed by the immediate serial tenfold dilutions of the mixture to a non-inhibitory concentration of hit and their addition to MRC-5 or HEp-2 cells for plaque titration of the virus. Decreased viral infectivity as related to the control sample indicates virucidal/virus-neutralizing activities of the hit. As mentioned previously apart from disruption of viral particles (virucidal effect) their neutralization may rely on affecting the activities of viral attachment or fusion proteins in their native or active intermediate forms.

3.5.2 Attachment and fusion inhibition assays

Compounds identified as potentially targeting the viral particle by the time-of-addition/removal assay were subjected to elucidation of their specific impact on viral attachment and fusion activities. These assays were utilized for the identified anti-RSV hits in **paper I** and **II**. All steps of the attachment assay were carried out in cold room conditions (~4°C). RSV particles to be used in the assay were metabolically radiolabelled with the ^{35}S-methionine/cysteine and then purified by centrifugation on a three-step discontinuous sucrose gradient. Serial fivefold dilutions of hit compounds in cold medium were mixed with the radiolabelled virus and incubated for 5 min prior to the addition of the mixture to HEp-2 cells monolayers. After a 2 h inoculation period at 4°C the cells were extensively rinsed, lysed, and transferred to scintillation vials for quantification of radioactivity. Substantial decrease of bound viral radioactivity levels compared to the non-treated control indicates that the hit interferes with the attachment of viral particles to cells.

The candidate impact on virus mediated fusion was studied through the use of a firefly luciferase reporter assay. One set of BHK-21 cells was infected with RSV A2 strain and 24 h later transfected with a plasmid expressing firefly luciferase under the control of a T7 promoter while another set of BHK-21 cells were transfected with a plasmid expressing a T7 RNA polymerase [305, 306]. A principle of this assay is that, the RSV-mediated fusion of these two sets of cells results in expression of the firefly luciferase and thus the development of luminescence. Hit compounds at serial fivefold dilutions were added to the cells infected with RSV and transfected with the T7 promoter controlled plasmid and incubated for 15 min at 37°C. Cells

expressing the T7 RNA polymerase were dissociated with EDTA/trypsin and added to the hit treated cells. This step was followed by co-incubation of cells for 6 h 37°C, after which the cells were lysed and the levels of luminescence measured. Decreased level of luminescence as related to the mock-treated sample indicates that the hit compound is a fusion inhibitor.

3.5.3 Classical time-of-addition assay/impact on viral RNA synthesis

Hits that in the time-of-addition/removal assays were identified as post-viral entry inhibitors were further assessed in the classical time-of-addition assay to identify the time-span or the latest addition time relative to inoculation of cells at which the hit still retains antiviral activity. Using this assay the impact of anti-CoV hit K22 on viral RNA synthesis was studied in **paper IV**. More specifically, 229E-CoV was adsorbed to precooled MRC-5 cells for 45 min at 4°C after which the inoculum was removed and cells rinsed. Hit K22 (10 μM) was added at 2 h intervals from 0-12 h relative to the end of the virus adsorption period and all samples were harvested at 24 h p.i. followed by the quantification of viral RNA by reverse transcriptase PCR (RT-PCR). Estimation of the time-span for the most potent hit activity may also provide a hint for its possible post-entry target that can be assessed by analysis of viral RNA or protein production, or replication kinetics.

3.5.4 Generation of drug resistant viral variants

Following assessment of the step of the viral life cycle targeted by hit compounds additional assays were used for more specific identification of the antiviral target. Through the preparation and analysis of drug resistant viral variants specific gene or protein targets could be identified. Resistant virus variants were obtained through the 10-13 consecutive passages of RSV in HEp-2 or 229E-CoV in MRC-5 cells in the presence of fixed concentrations of hits P13 and C15 in **paper I** or increasing concentrations of hits PG545 in **paper II** and K22 in **paper IV**. Similar passages of the virus in the absence of hits were also performed to serve as controls for comparative analysis of the drug resistant viruses. Viral variants that resisted the selective pressure from the hits were plaque purified followed by estimation of their respective resistance compared to original and mock passaged virus by the plaque reduction assay as described in subsection 3.3. Resistant variants were then subjected to nucleotide sequence analysis through RNA purification of virus material, cDNA preparation by RT-PCR and subsequent sequencing with amplification of DNA fragments of the coding regions of the RSV F- and G-proteins and the 229E-CoV complete genome. Sequences were analyzed by

Sequencher 4.9 software. Presence of a specific aa alteration(s) in at least several plaque purified drug resistant virus variants suggests the involvement of the mutated viral component in virus resistance to the hit compound.

3.5.5 Generation of recombinant viral variants and their replication fitness

A question as to whether the identified aa alteration is indeed responsible for the drug resistance of the virus needs to be confirmed by preparation of recombinant viruses harboring this specific aa mutation. This was performed to confirm resistance of 229E-CoV variants to the K22 hit in **paper IV**. Three different variants of the gene fragment comprising resistance specific mutations were cloned into a TOPO-TA plasmid vector and subsequently used for recombination into a vaccinia virus system expressing 229E-CoV [307]. This reverse genetics system produced fully viable recombinant 229E-CoV with alterations in the specific target protein. Confirmation of resistance of the recombinant variants in comparison to wild type virus was conducted by the plaque reduction assay.

Replication fitness of the drug resistant recombinant viruses was assessed in the replication kinetics and EM assays. Concentrated preparations of wild type 229E-CoV and recombinant viruses to be used in the replication kinetics studies were prepared by centrifugation of infectious supernatant medium through the layer of 20% sucrose. To study the replication fitness, 229E-CoV was adsorbed to precooled monolayers of MRC-5 cells for 1 h at 4°C, followed by removal of inoculum, cell rinsing and addition of fresh warm medium. Infected cells and supernatant fluid were harvested at different time points of their incubation at 37°C (0-72 h) relative to the end of virus inoculation. The amount of viral RNA produced in supernatant fluid and in cells was quantified by RT-PCR. Virus infectivity was quantified in samples of infectious culture medium by the viral plaque assay.

3.5.6 Electron microscopy

Electron microscopy (EM) imaging of cells infected with the drug resistant viral variants may help to analyze replication fitness of these viruses. For this analysis, MRC-5 cells seeded on melinex polyester film were infected with wild type or recombinant K22 resistant 229E-CoVs with or without the presence of K22 hit for 18 h at 37°C. The cells were subsequently fixed with glutaraldehyde and processed for EM as described by Widehn and Kindblom [308]. By studying potential changes in the infection pattern of the recombinant virus compared to the wild type infection, the expression of

genes with compound specific mutations could be related to phenotypic alterations in the infected cells.

4 RESULTS AND DISCUSSION

4.1 Paper I and III

In **paper I** we identified two novel anti-RSV hits targeting the viral fusion/entry process. These hits were identified by screening of the ChemBioNet collection of compounds using a 384-well HEp2-cell culture based screening method, a comprehensive protocol of which is described in **paper III**. Preliminary and secondary screening for compound prevention of the virus induced cytopathic effect resulted in the selection of 221 and subsequently 13 hits respectively. The two hits that displayed the most promising inhibitory qualities, namely the N-(2-hydroxyethyl)-4-methoxy-N-methyl-3-(6-methyl[1,2,4]triazolo[3,4-a]phthalazin-3-yl)benzenesulfonamide P13 and the 1,4-bis(3-methyl-4-pyridinyl)-1,4-diazepane C15 (Figure 16) were selected for further assessment of the antiviral potency and cytotoxicity as well as elucidation of their antiviral mechanisms.

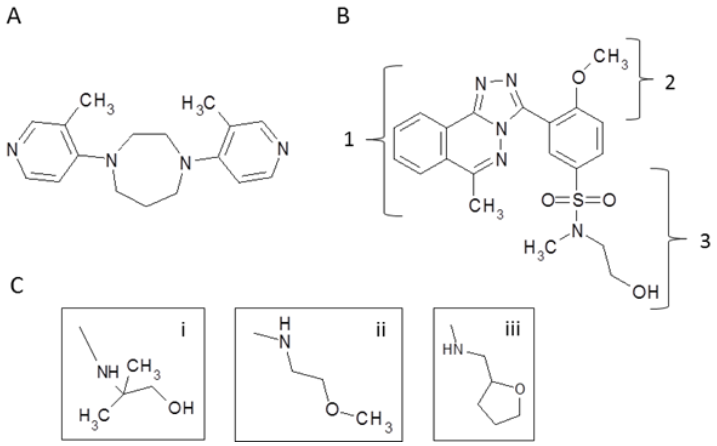


Figure 16. *Structure of the diazepane-based C15 (A) and the benzenesulfonamide-based P13 (B) with indications of the different groups of the P13 molecule subjected to structure-activity assessment by analysis of analogs. Substituents at group 3 that improved the anti-RSV activity of P13 are depicted (C).*

Both compounds exhibited anti-RSV capability in the submicromolar range with IC_{50} values, determined by the RSV plaque reduction assay in HEp-2 cells, at concentrations of 0.11 and 0.13 µM for P13 and C15 respectively. In

combination with cytotoxicity assessment the selective index values were 2818 and 577 for P13 and C15 respectively.

P13, displaying the better inhibitory profile of the two hits, was selected for additional analysis of its structure activity relationship. Results based on the screening of fifteen commercially available structurally similar compounds (analogs) identified the specific structures of the molecule important for its antiviral activity (Figure 16 B and C). Removal of group 1 resulted in complete loss of activity. Similar results were observed for group 2 since changing its position on the benzene ring, replacement by a methyl group or complete removal resulted in a marked reduction or abolished anti-RSV activity. Substitution of group 3 demonstrated a way of improving the compound activity since replacing it with any of the three groups shown in figure 16 C resulted in a ~2.5 fold improvement in antiviral activity.

An initial elucidation of the antiviral mechanism of the hits aimed at defining a stage of the RSV life cycle where these compounds were most active. This was assessed by a time-of-addition/removal assay where P13 and C15 were added to the cells at different time points relative to the virus inoculation. Both compounds most potently inhibited RSV infection when present during the virus inoculation of cells indicating their interference with viral components during the RSV attachment to and entry into the cells (Figure 17 A-B).

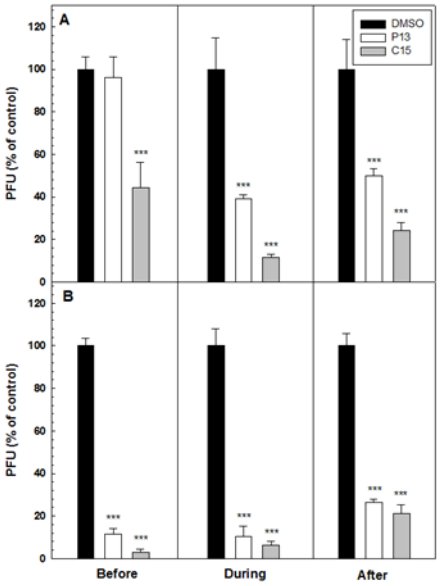


Figure 17. *Hits P13 and C15 exhibited the most pronounced anti-RSV activity when present during initial RSV infection of cells as evaluated by the time of addition/removal assay. P13, C15, or DMSO solvent was added to HEp-2 cells at different time points, before/during/after, relative to the virus inoculation. Presence of hits on cells was limited to a 2h period (A) or extended until the development of viral plaques (B).*

Since P13 and C15 interfered with early events of the RSV life cycle, we wanted to discriminate whether these hits showed direct inactivating (virucidal) properties against viral particles or interfered with the virus attachment to or entry into cells. Co-incubation of these hits with RSV particles did not affect their infectivity thus excluding the potential virus inactivating activity of P13 and C15. Furthermore, evaluation of the compound impact on the RSV capability to attach to cells showed little or no interference with this step indicating that neither of the compounds targeted RSV binding to cell surface receptors. These results further supported an assumption that P13 and C15 affect the stage of RSV life cycle occurring after attachment of viral particles to cells.

To identify a specific viral component targeted by P13 and C15, the hit resistant viral variants were generated. After 10 consecutive RSV passages in HEp-2 cells in the presence of continuous selective pressure generated by 10 μM of respective hit, RSV variants that were at least 1000 times less sensitive to P13 and C15 than the wild type RSV were selected. Such an extended drop in hit sensitivity of selected RSV variants suggests a high target specificity of these hits. Comparative nucleotide sequence analysis of a number of plaque purified resistant RSV variants, original virus, and a mock passaged virus revealed mutations in the RSV fusion component, the F-protein. Variants resistant to P13 displayed mutations in the cysteine-rich (T400I) and the HR1 (N197T) regions while C15-resistant variants all carried mutations at position D489G in the HR2 domain (Figure 18). The fusion process of RSV is a critical step in the viral life cycle where the F-protein through a series of conformational events mediates the fusion of viral and cellular membranes to deliver the genetic material to the cytoplasm. The critical importance of fusogenic activity of the F-protein during RSV infection of cells has made it an attractive target for antiviral intervention [309]. Note that alterations in the F-protein conferring resistance to P13 and C15 were located within the same mutation-rich domains found in RSV variants resistant to other fusion inhibitors. (Figure 18, Table 1). It should be emphasized that these domains mediate key functions of the F-protein, and most mutations were located in or near the HR2 region or in the FP. This confirms that these regions are important for the F-protein fusion activity, and also reveal the most frequent mutational “escape routes” in the RSV attempt to evade an inhibitor. Since P13 and C15 did not show direct virucidal activity it is unlikely that these inhibitors target the native/prefusion form of the F-protein. Instead, an intermediate form of this protein, where the two HR regions are transiently exposed to mediate the virus-cell fusion, are more likely to be targeted [103]. C15 and P13 may bind to HR1/HR2 and inhibit viral fusion by preventing the required affinity interaction between these two regions, hindering the

apposition of the two membranes, or stabilizing the intermediate in an extended form of the F-protein which would then be unable to proceed into the collapsed hairpin structure of the post-fusion form (Figure 13).

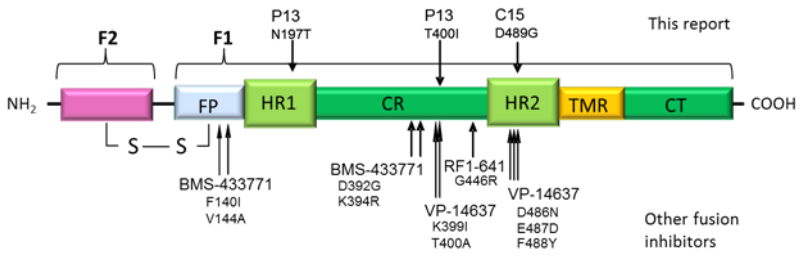


Figure 18. *Schematic illustration of the disulfide linked subunits F1 and F2 of the RSV F-protein and the sequential order of the essential regions including the fusion peptide (FP), heptad repeat 1 (HR1), cysteine-rich region (CR), HR2, transmembrane region (TMR), and cytoplasmic tail (CT). Indicated are the mutations identified in P13 and C15 resistant RSV variants, and in RSV variants resistant to fusion inhibitors reported by others (see text above).*

To further validate the F-protein as a target of P13 and C15, these hits were evaluated for their capability to prevent the F-protein mediated fusion by using a luciferase reporter activation assay in BHK-21 cells. P13 and C15 displayed a dose dependent inhibition of the RSV induced cell-to-cell fusion activity confirming their role as potent fusion inhibitors (Figure 19).

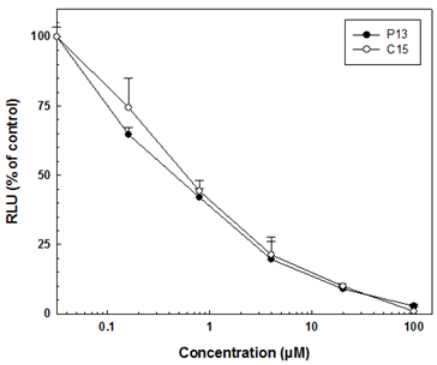


Figure 19. *P13 and C15 prevent the RSV mediated cell-to-cell fusion of BHK-21 cells. The luciferase activity, induced by the RSV mediated fusion of cells, is expressed as a percentage of the number of relative light units (RLU) detected in the presence of hit relative to the mock-treated controls.*

Together, potent anti-RSV activity and low cytotoxicity of P13 and C15 fusion inhibitors warrant further investigation of these hits as potential drugs for treatment of RSV disease.

4.2 Paper II and III

Antiviral strategies targeting the attachment and entry events early in virus infectious cycle have the advantage of the generally low abundance of virus particles which are affected by an inhibitor prior to the attachment to and entry into host cells thus preventing virus multiplication and reducing the risk of host specific adverse events.

It has been shown that RSV interaction with GAG chains on host cell surfaces is important to facilitate the virus binding and entry events which provides an opportunity to use GAG-mimetics such as sulfated oligo- and polysaccharides for antiviral intervention. The main mechanism through which negatively charged GAG mimetics inhibit RSV attachment to cells is by targeting the positively charged GAG-binding regions of the virus attachment G-protein, an event that shields the viral protein thus preventing its interaction with GAG receptors (Figure 20).

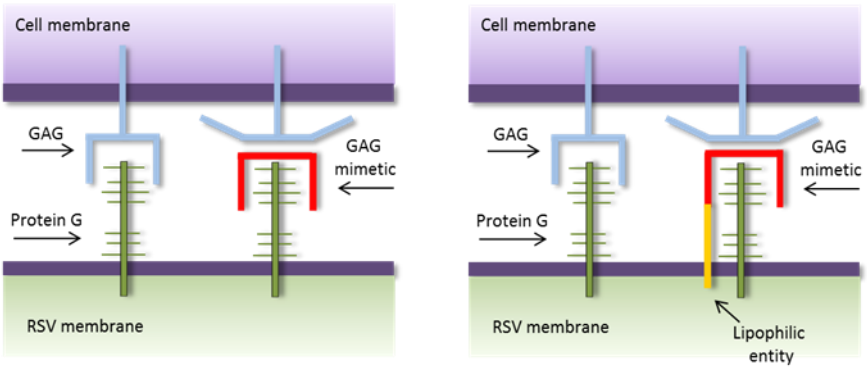


Figure 20. *Antiviral mechanism of the GAG-mimetics. Indicated in the right panel is a plausible mechanism behind the improved anti-RSV activity of GAG-mimetics modified with a lipophilic entity which may be inserted into the viral envelope.*

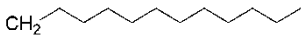
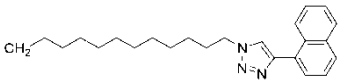
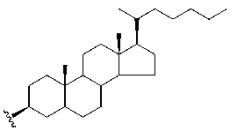
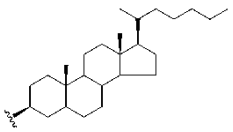
These sulfated polyanionic compounds have been shown to have a broad spectrum of inhibition among various GAG-binding viruses (HIV, HSV, and RSV) due to presence of clusters of positively charged aa residues in the GAG-binding domains of the viral attachment proteins in all these viruses [248, 310, 311]. The main inhibitory mechanism of GAG-mimetics relies on electrostatic interactions with the virus attachment proteins. However, these interactions tend to be weak and reversible [312] resulting in a non-persistent inhibition that is also sensitive to "dilution effects". Hence, the compound needs to be present continuously at high concentrations during the virus binding stage, and even then it is difficult to reach a complete inhibition of

the virus infection [313, 314]. Therefore, interactions of GAG mimetics with the virus attachment proteins need to be altered to achieve a sustainable inhibition and irreversible inactivation of viral particles.

In **paper II** we found that sulfated oligosaccharide muparfostat formerly known as PI-88 exhibited potent anti-RSV activity. Muparfostat was originally identified as a potent anti-cancer drug [315] but have since then also been reported to have inhibitory activity against a number of GAG-binding viruses such as HSV, HIV, dengue and flavivirus [246, 247, 251] as well as being an antimalarial inhibitor [316]. However the inhibitory effect on RSV infectivity appeared to be reversible and non-virucidal. Since previous studies from our laboratory [250] indicated that addition of a lipophilic group to muparfostat greatly improved its anti-HSV properties, in **paper II** we studied the possibility of improving the anti-RSV potency of muparfostat and other specific sulfated oligosaccharides by their coupling to various lipophilic structures. Muparfostat, acting as the scaffold for most of the modifications, consists of a mixture of mainly tetra- and pentasaccharides which were modified by the conjugation of different lipophilic groups at their reducing end.

A set of fifteen modified structures were evaluated in a mini-screen at 100 µg/ml for their ability to inhibit RSV infection and compared to unmodified muparfostat. This initial screen identified four compounds that exhibited improved antiviral potency manifested as near-complete or complete inhibition of RSV infectivity and greater virucidal properties (Table 2). Compounds 14 and PG545, both coupled with cholestanol, exhibited similar antiviral potency but since PG545 displayed a stronger virucidal activity, coinciding with the project aim, it was selected for further assessment of antiviral activity and cytotoxicity in addition to the elucidation of its antiviral mechanism.

Table 2. *Anti-RSV activity of selected oligosaccharide glycosides*

Compound	Structure of glycoside		Residual infectivity (%) [b]	Virucidal activity[c] (% of residual infectivity)
	Oligo-saccharide component[a] (No. of residues)	Aglycone component		
Muparfostat	Mainly penta- and tetra-saccharide	None	13.5 ± 5.1	94.0
3	Penta-saccharide		0.9 ± 1.5	47.3
5	Penta-saccharide		0.0	57.3
14	Tetra-saccharide		0.0	13.3
PG545	Tetra-saccharide[d]		0.0	9.9

[a] Manα(1→3)/Manα(1→2)

[b] Percentage of the number of viral plaques found with drug treated virus (100 μg/ml) relative to mock treated controls

[c] Residual RSV infectivity after incubation for 15 min at 37°C of ~10^5 PFU of the virus with 100 μg/ml of a test compound.

[d] Composed of α(1→4)-linked glucose residues (maltotetraose).

Coupling of cholestanol to sulfated tetrasaccharide (PG545) improved the anti-RSV activity by ~5 times as related to unmodified sulfated oligosascharide muparfostat. In addition, PG545 reached complete inhibition of RSV infectivity at 20 µg/ml, a quality that muparfostat did not display even at 500 µg/ml (Figure 21). Furthermore, while muparfostat completely lacked virucidal activity at the highest concentration tested, PG545 displayed strong dose-dependent virus inactivating (virucidal) properties with only marginal residual infectivity left at 100 µg/ml.

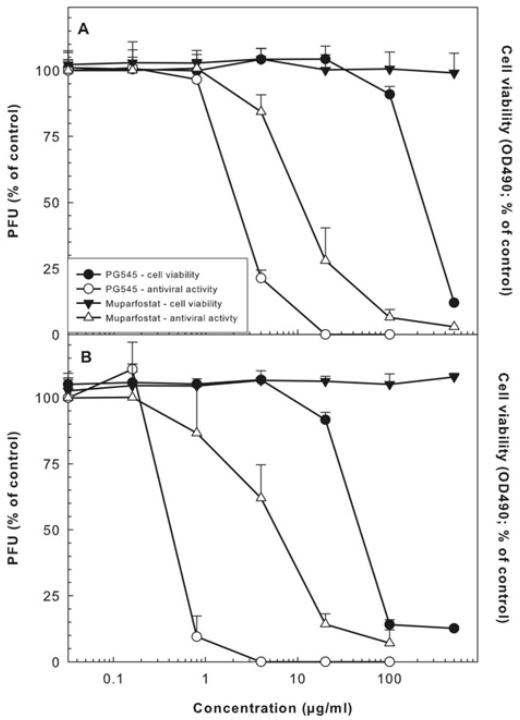


Figure 21. *Effect of PG545 or muparfostat on RSV infectivity and HEp-2 cell viability. Antiviral and cytotoxicity assays were conducted with (A) and without (B) the presence of serum in culture media. Results are expressed as a percentage of mock-treated controls.*

Another interesting feature of PG545 was the potent inhibition of vesicular stomatitis virus, a *Rhabdoviridae* family member with documented sensitivity to GAG-mimetics [317], and lack of inhibitory activity against influenza A virus that uses sialic acid for its attachment to cells. This further supported the interpretation that PG545 selectively targets the GAG-binding viruses.

Since the lipophilic cholestanol group of PG545 may potentially interact with apolipoproteins in serum, the antiviral and virucidal activity as well as the

cytotoxicity was also evaluated in the absence of serum in the culture medium. Both the antiviral activity and cytotoxicity was decreased by approximately five times in the presence of serum. Because of the intended use of PG545 in the mucus-covered respiratory epithelium, the activity was also evaluated in the presence of human nasal secretions. This body fluid decreased anti-RSV activity of PG545, an effect that could be attributed to the presence of a cholestanol moiety which due to its lipophilic nature is prone to interaction with other lipophilic structures present in mucus such as surfactant proteins or free lipids. These unwanted interactions of PG545 that decreased its anti-RSV activity could be overcome by increasing the initial PG545 concentration.

Initial evaluation of the PG545 mechanism of anti-RSV action was made using the time-of-addition/removal assay as previously mentioned for paper I, and the detailed procedure of this assay is presented in **paper III**. As expected PG545 displayed the most potent inhibition when present at the cell surface at the time of virus inoculation suggesting that this compound mainly targets early events of RSV infection of cells, but may also interact with cellular components to a lesser extent.

Furthermore, PG545 prevented the attachment of radiolabeled RSV particles to cells by ~50%. This partial reduction of RSV binding suggested that the virucidal activity of this compound could be attributed both to the partial inhibition of the virus binding to cells and to its interaction with other components of viral particles such as possible insertion of the cholestanol moiety of PG545 into viral envelope lipids (Figure 20). However this interpretation would require further studies.

To identify specific viral components targeted by PG545 and muparfostat, we attempted to select for the drug resistant RSV variants generated under selective pressure of these compounds. However, selection for resistant variants proved to be a challenge and in spite of 13 passages in the presence of increasing concentrations of PG545 the isolated virus was only 3-4 times less sensitive to the drug than original virus. In contrast, RSV variants resistant to muparfostat displayed a 7-9 times reduction in sensitivity after 10 passages. In spite of the relatively low resistance of PG545 selected variants, nucleotide sequence analysis revealed mutations in the attachment G-protein in all plaque purified variants examined (Figure 22). These aa alterations were located in the highly conserved domain in or near the central cysteine noose of the G-protein suggested to have a role in receptor specificity [82]. Similar analysis for muparfostat resistant variants also revealed a mutation in the G-protein at aa N191T, slightly downstream of the alterations identified

in the PG545 variants. This mutation was likely to confer resistance to muparfostat (Figure 22) and was located in the GAG-binding region of this protein. The anti-RSV compound NMSO3 (MBX-300) [274] which exhibits similar structural features to PG545, i.e., it comprises sulfated sialic acid coupled to two lipophilic acyl chains, targeted the G-protein and the drug resistant variants comprised a set of mutations in the G-protein including the F168S aa alteration, which was also detected by us in the PG545 resistant mutant viruses. Interestingly, coupling of cholesterol to the inhibitory peptides derived from the fusion proteins of HIV, and Nipah and Hendra paramyxoviruses has greatly improved their antiviral activities [318, 319].

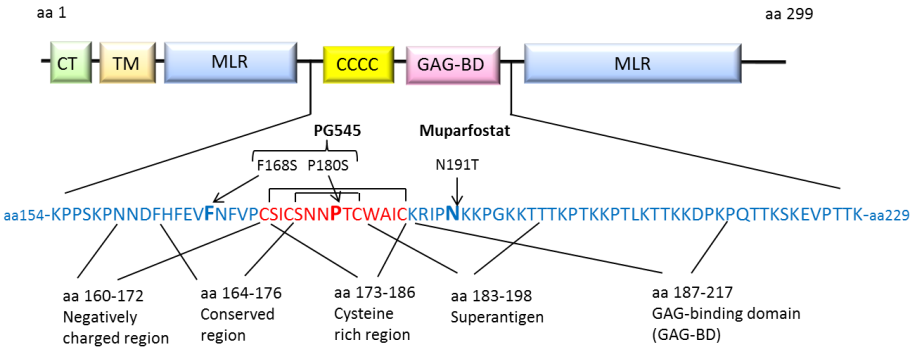


Figure 22. *Schematic representation of the RSV G-protein showing aa alterations identified in the PG545 and the muparfostat resistant RSV variants.*

Together, coupling of a lipophilic entity to polysulfated oligosaccharides significantly improved their anti-RSV activity. This observation together with previous reports of this strategy [250, 251] confirms its effectiveness in improving the inhibitory potential of GAG-mimetics. The cholestanol component of PG545 not only improved its anti-RSV but also resulted in emergence of the virus inactivating (virucidal) activity, a feature absent in muparfostat and other sulfated polysaccharides

4.3 Paper IV

Antiviral targeting of the post-entry events of virus life cycle, often enzymatic activities of viral "replicase" proteins, has proven to be an efficacious way for treatment of viral diseases.

In the post entry stage of the viral life cycle many viruses establish a site for their replication inside the cells, usually in the form of an organelle-like structure. This feature is a classic example of how the viruses in many

different and remarkable ways can utilize the host resources to their own advantage. In particular, CoVs recruit intracellular membranes from ER and modify them to form a complex membrane network with the subsequent formation of CM, large clusters of DMVs, VPs and other structures described in section 1.3.3 (Figure 8) [150, 151]. These membrane formations serve as the virus replication factories providing necessary stability and shelter for this process.

In **paper IV** we sought to identify new anti-CoV hit compounds through screening of the ChemBioNet library using experimental procedures similar to those described in **paper I** and **III**. The 229E-CoV strain was used for this assay system and the screening strategy resulted in the identification of a benzamide-based hit, K22, (Figure 23) that in a standard plaque assay in MRC-5 cells reduced the virus infectivity with IC_{50} at a concentration of 0.7 μM and a selective index of 157.

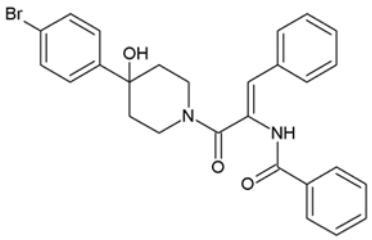


***Figure 23**. Structure of K22, the (Z)-N-(3-(4-(4-bromophenyl)-4-hydroxypiperidin-1-yl)-3-oxo-1-phenylprop-1-en-2-yl)benzamide.*

The mechanism of action studies, as for those described in **paper I** and **II,** were initiated with a time-of-addition/removal assay. The results of this assay revealed the most potent activity when K22 was added to cells after their inoculation with 229E-CoV (Figure 24 A). This suggested that K22 targeted the post entry stage of CoV cycle, an interpretation further supported by the lack of the virus-inactivating (virucidal) activity of K22 on viral particles. To further elucidate the suggested post-entry mode of action, the impact of K22 on viral RNA synthesis was studied. A standard time-of-addition experiment with addition of compound at 2 h intervals during the first 24 h of 229E-CoV infection of MRC-5 cells, revealed that the viral RNA and infectivity levels reached near complete inhibition upon K22 addition up to 6 h p.i. (Figure 24 B, C) thus supporting the early post-entry inhibition mechanism.

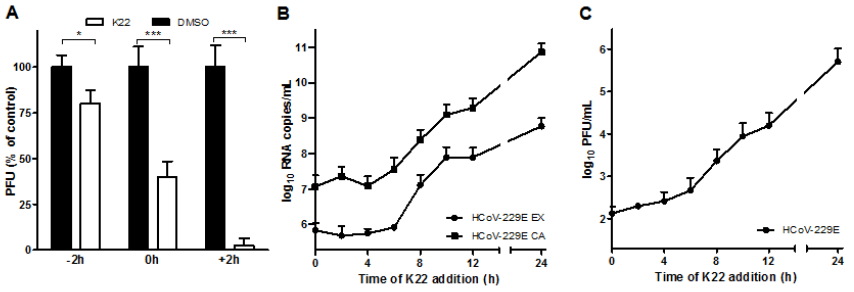


Figure 24. *Elucidation of the K22 mechanism of anti-HCoV-229E activity. The time of addition/removal assessment indicated that K22 affected post-entry steps (+2) of the HCoV-229E life cycle (A). Classical time of addition (0-24 h) evaluation showing that K22 most potently reduced production of viral RNA (B) and infectious virus (C) when added up to 6 h p.i.*

In addition, EM studies were conducted to investigate the effect of K22 on characteristic structural alterations of MRC-5 cells induced by 229E-CoV. As shown in Figure 26 A (left panel), the virus induced clusters of DMVs were clearly seen in the perinuclear area, and clusters of virus particles were found within ER compartments of MRC-5 cells infected with 229E-CoV in the absence of K22. In contrast, none of these virus-induced membrane rearrangements could be observed in cells treated with K22 thus supporting the idea of inhibition of the CoV life cycle at a post entry step preceding the assembly of new virions, and likely aimed at the membrane bound replication process.

For a more precise identification of the K22 target, compound resistant viral variants were generated during 10-13 consecutive passages of 229E-CoV in MRC-5 cells under the selective pressure of increasing concentrations of K22 (2-16 µM) in two separate experiments. Subsequent nucleotide sequence analysis revealed that plaque purified viral variants exhibiting a strong compound resistance all displayed mutations in the 1ab gene fragment coding for nsp6. This protein possesses 6-7 hydrophobic TM regions out of which at least 6 span the lipid bilayer [320]. The H121L and M159V aa changes, each identified in separate selection experiments, were predicted to be located in or near the TM regions 4 and 5 of nsp6 (Figure 25).

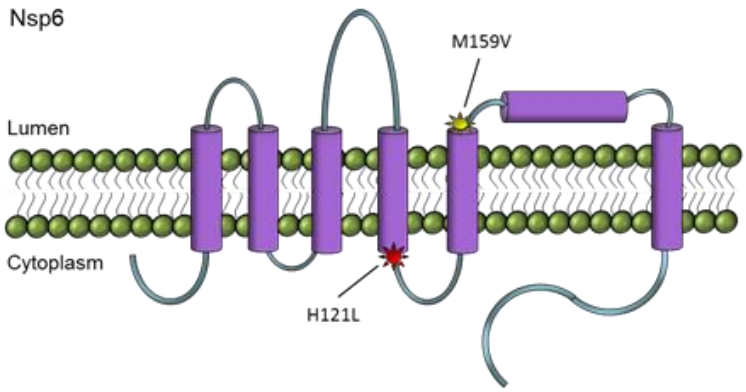


Figure 25. *Cartoon structure of nsp6 showing location of putative TM regions. Mutations conferring 229E-CoV resistance to K22, i.e., the H121L and the M159V aa substitutions are indicated.*

CoV nsp6 as well as nsp3 and nsp4 contain multiple hydrophobic domains, although not all of them are necessarily used as TM regions indicating their important functions in other hydrophobic interactions [320]. Presence of multiple TM regions in these proteins strongly suggest their function as an membrane anchor for other nsps that form the RTC, and their function in recruitment and modification of cytoplasmic membranes. Recently, it was suggested that nsp3 and nsp6 have a membrane proliferating function and that the combined activity of nsp3 and nsp4 could mediate pairing of membranes [147]. In addition, nsp6 was observed to induce vesicle formation, a role that has previously been suggested for this protein in inducing autophagosomes [321].

In addition to 6 TM regions, three luminal loops, and both the N- and C-terminal tail on the cytosolic side was predicted for nsp6 (Figure 25). The C-terminal end has been shown to be highly conserved between different CoVs indicating its important function [322]. A free hydrophobic domain in the C-terminal tail or an intermediately located hydrophobic region that does not span the bilayer, could easily interact with other parts of the RTC, connect apposing membranes into DMV proximity or function in forming the DMVs [320, 323, 324].

To confirm that the mutations identified in nsp6 confer 229E-CoV resistance to K22, three recombinant mutant viruses, carrying the nsp6 mutations individually or combined, were generated by the use of a reverse genetics vaccinia virus system as described by Thiel et al. [307]. Analysis of

sensitivity of these recombinants to K22 confirmed the aa alterations H121L and M159V as being responsible for the drug resistance.

Subsequent studies of the recombinant nsp6 mutant viruses were conducted to assess whether these specific changes in the nsp6 would affect their replication kinetics or infectivity. The recombinants showed similar levels of intra- and extracellular RNA as wild type virus, however the infectivity of the recombinants was reduced by ~ 34-fold. The results indicate that a native nsp6 function may not be essential for RNA synthesis but required for production of a fully infectious virus particle.

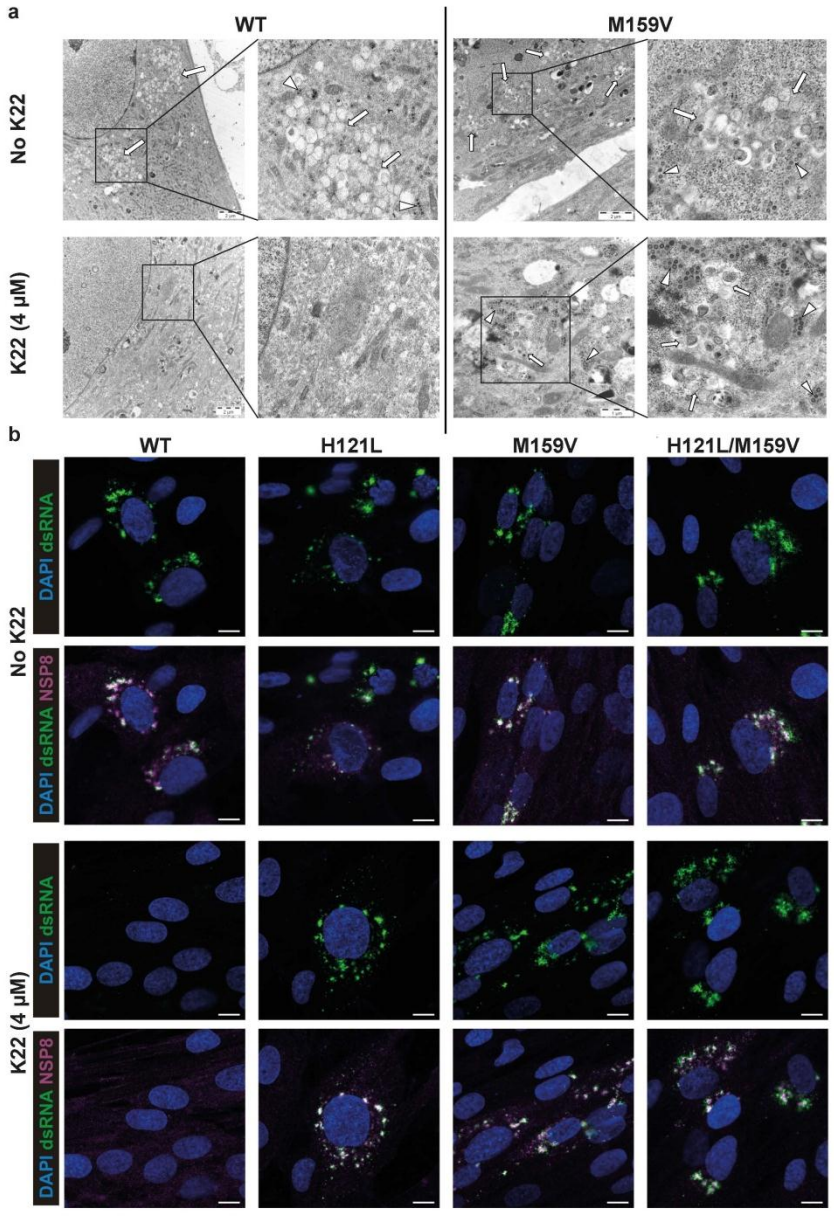


Figure 26. *The EM (A) and IF (B) images of MRC-5 cells infected with wild type (WT) 229E-CoV or recombinant nsp6 mutant viruses H121L, M159V, or H121L/M159V with or without the presence of K22.*

Additional assessment of the nsp6 recombinants was conducted by EM and IF imaging analysis. MRC-5 cells infected with wild type 229E or recombinant viruses with and without the presence of K22 were fixed at 18 h p.i. and processed for image analysis. For the IF assay CoV nsp8 and dsRNA were used as markers for the CoV replication complex and viral RNA synthesis respectively. For wild type 229E-CoV the characteristic staining pattern of nsp8 and dsRNA was observed only in MRC-5 cells that were not treated with K22 (Figure 26B) thus confirming a potent and complete inhibition of CoV-229E replication by K22. Analysis of the three nsp6-recombinants revealed that these mutant viruses displayed similar IF staining in both K22 treated and untreated cells thus confirming their K22 resistance, and also showing that the resistance mutations in nsp6 did not prevent the formation of the replication transcription complex associated with nsp8. Furthermore, EM-imaging analysis of the same experimental set-up revealed a reduced number of virus induced DMVs present in the untreated samples of the M159V mutant as compared to wild type 229E-CoV (Figure 26A). The observed reduction of the DMV numbers in the nsp6-recombinant correspond with the reduction in the mutant specific infectivity and support the idea that the CoV replication and subsequent virion assembly is firmly connected.

The potential activity of K22 against other CoVs from the alpha, beta and gamma serogroups was also assessed (Figure 27). K22 displayed inhibitory activity of varying potency against all CoVs tested with the most potent inhibition observed for the feline and infectious bronchitis CoVs as well as for the newly identified human strain MERS-CoV (Figure 27b, d, f). In addition to the antiviral activity assessment in monolayer cell cultures, the activity of K22 on 229E-ren, expressing renilla luciferase, and MERS-CoV strains was also evaluated in differentiated cultures of human airway epithelium (HAE). These cultures resemble the structure and function of the native human airways and hence the drug evaluations in these cultures are of greater biological relevance. Both 229E-CoV-ren and MERS-CoV were inhibited by K22 in these cultures as assessed by the inhibition of production of dsRNA. The activity of K22 on MERS-CoV infected HAE cultures was also visualized by IF (Figure 27g). These results further support that K22 is a promising candidate drug for the treatment of CoV infections. Furthermore, assessment of K22 mode of action identified a new druggable target for CoVs, i.e., a membrane-bound viral RNA synthesis.

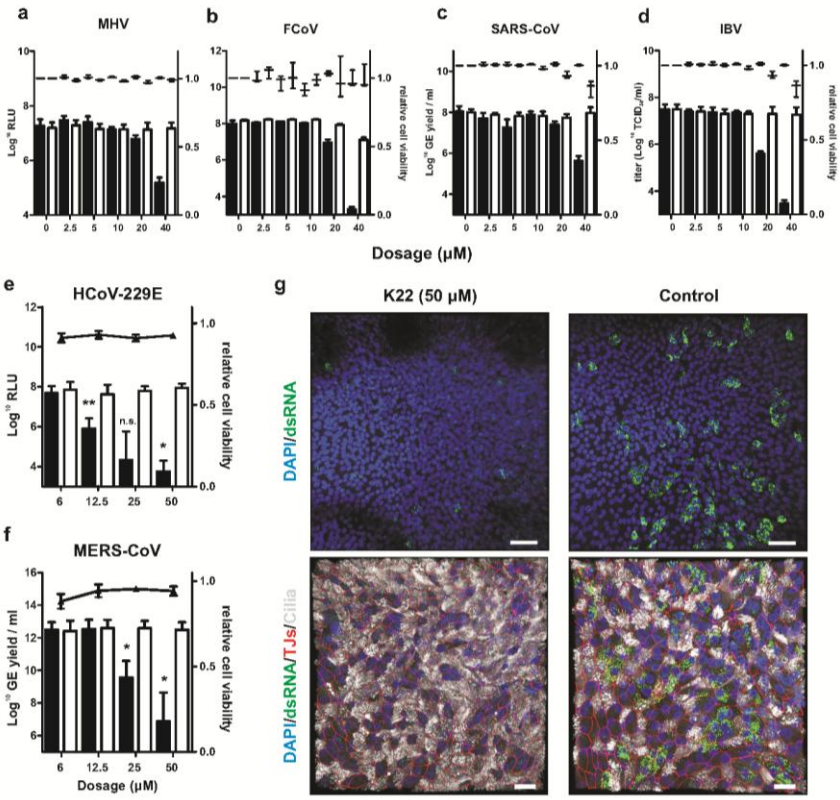


Figure 27. *The effect of K22 on diverse animal and human CoVs (a-d). Antiviral activity (bars) and cell toxicity (data points) of K22 and control (black and white bars respectively) during murine hepatitis (MHV), feline (FCoV), SARS-CoV and chicken infectious bronchitis (IBV) CoV infection. Antiviral activity of K22 on HCoV-229E and MERS-CoV in differentiated human airway epithelial (HAE) cultures (e-f). IF analysis of HAE cultures infected with MERS-CoV with or without the presence of K22 (g) (TJs stands for the tight junctions staining)*

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In this work screening of collections of compounds using a cell based 384-well screening system of whole virus led to identification of three novel anti-RSV and one anti-CoV candidate drugs. By applying a systematic step-by-step approach with a set of assays suitable for the particular virus studied, the antiviral mode-of-action of these candidate drugs was elucidated resulting in (i) identification of novel druggable targets for antiviral intervention and (ii) extending our knowledge about particular structures or activities essential for RSV or CoV life cycle.

In particular, P13 and C15 were identified as potent inhibitors of RSV fusion due to targeting of the two HR domains of the F-protein. These domains are critical for viral fusion because of their intrinsic capability to forcibly overlap each other, an event that reduces the length of the virus and cell interconnecting F-protein thus closely apposing and merging the viral and cellular membranes. The specific targeting of this essential fusion mechanism resulted in potent and highly specific inhibition of RSV infectivity in the submicromolar range without major adverse effects on cells, but unfortunately also in a rapid generation of the drug resistant RSV variants. Mutations conferring RSV resistance to P13 and C15, and to fusion inhibitors reported by others (Table 1), occurred in or near the HR regions of the F-protein. To circumvent this difficulty we plan to modify the P13 structure to extend its activity towards more extensive targeting of the HR domain or according to the multiple target strategy, to supplement P13 treatment with HR1/HR2-derived peptide analogs that could compete with and block interaction of native domains.

PG545 glycoside, a conjugate of synthetic sulfated tetrasaccharide and cholestanol, was found to target the native RSV virions including its attachment component, the G-protein. Although sulfated oligo- and polysaccharides are known to be potent inhibitors of the attachment of RSV to cells and many other GAG-binding viruses, these compounds failed in large clinical trials to protect women against HIV [203, 325]. One reason behind this failure is the irreversible and non-virucidal mode of antiviral activity of these compounds. To circumvent this difficulty we have modified the structure of these inhibitors by coupling of a lipophilic cholestanol group to the reducing end of a sulfated oligosaccharide maltotetraose. The resulting compound, PG545, exhibited enhanced anti-RSV potency including

emergence of the virus inactivating (virucidal) activity, a feature absent in native sulfated oligo- and polysaccharides. The use of GAG-mimetics and their modifications such as PG545 seems to be of special importance in treatment of RSV disease. These compounds may target the putative receptor binding region (conserved and cysteine rich) as well as GAG-binding domain of the G-protein that partly overlaps the "superantigen" sequence believed to induce an allergy-type Th2 cellular response. PG545 may block these regions thus preventing the virus attachment to susceptible cells alleviating the Th2 response and thereby the severity of RSV disease. Since RSV infection of the mucus covered respiratory epithelium is an obvious target for PG545 the observed tendency of the drug to interact with unspecific lipophilic structures needs to be considered in future optimizations.

K22 was identified as a potent inhibitor of the membrane-bound viral RNA synthesis, which represents a new druggable target of the CoV replication process. Apart from 229E-CoV, the compound also affected infectivity of potential pandemics-causing SARS- and MERS-CoVs. Little is known about the specific contributions of the different CoV nsps to the recruitment and modification of host intracytoplasmic membranes and our results support the idea of nsp6 being a crucial contributor to this process. Further studies of the specific interplay between the nsp6 protein and the K22 structure would reveal more information on the inhibitory activity as well as the function of nsp6. Notably, even though the double membrane structures characteristic for CoV infection are not present in cells infected with RSV, this virus also creates specific replication areas in the cytoplasm, inclusion bodies, which might be accessible for similar antiviral intervention. K22 is currently evaluated in an additional structure-activity relationship approach in combination with optimization of the K22 structure for improved physiochemical properties. An optimized activity/safety profile will allow for proceeding with K22 into in vivo studies as a promising candidate for treatment of CoV infection.

Finally, as already assayed for K22, the in vivo potential of the three anti-RSV candidates identified in this work should be assessed in a biologically relevant model of RSV/CoV infection, i.e., in cultures of well-differentiated airway epithelial cells. These cultures resemble the epithelium of human airways by comprising fully differentiated ciliated and mucus-producing secretory cells, and therefore are of obvious importance in validation of candidate drugs against respiratory viruses.

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