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Nucleosides for the treatment of respiratory RNA virus infections

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Abstract

Influenza virus, respiratory syncytial virus, human metapneumovirus, parainfluenza virus, coronaviruses, and rhinoviruses are among the most common viruses causing mild seasonal colds. These RNA viruses can also cause lower respiratory tract infections leading to bronchiolitis and pneumonia. Young children, the elderly, and patients with compromised cardiac, pulmonary, or immune systems are at greatest risk for serious disease associated with these RNA virus respiratory infections. In addition, swine and avian influenza viruses, together with severe acute respiratory syndrome-associated and Middle Eastern respiratory syndrome coronaviruses, represent significant pandemic threats to the general population. In this review, we describe the current medical need resulting from respiratory infections caused by RNA viruses, which justifies drug discovery efforts to identify new therapeutic agents. The RNA polymerase of respiratory viruses represents an attractive target for nucleoside and nucleotide analogs acting as inhibitors of RNA chain synthesis. Here, we present the molecular, biochemical, and structural fundamentals of the polymerase of the four major families of RNA respiratory viruses: *Orthomyxoviridae*, *Pneumoviridae*/*Paramyxoviridae*, *Coronaviridae*, and *Picornaviridae*. We summarize past and current efforts to develop nucleoside and nucleotide analogs as antiviral agents against respiratory virus infections. This includes molecules with very broad antiviral spectrum such as ribavirin and T-705 (favipiravir), and others targeting more specifically one or a few virus families. Recent advances in our understanding of the structure(s) and function(s) of respiratory virus polymerases will likely support the discovery and development of novel nucleoside analogs.

Keywords

Respiratory syncytial virus, coronavirus, picornavirus, RNA-dependent RNA polymerase, nucleoside analog, rhinovirus, influenza, antiviral

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Introduction to respiratory infections caused by RNA viruses

Respiratory viral infections are a global health concern caused by dozens of different types of viruses. The respiratory diseases resulting from these viral infections represent one of the main causes of death in developing countries.[1] A more thorough understanding of respiratory viruses, their epidemiology, as well as medical impact on the communities they affect will delineate the path toward eventual treatments and future abatement of the illnesses. While symptoms of many respiratory viruses are similar, the viruses themselves are characteristically unique. Categorically, viruses are grouped based on similarities such as the nature of their nucleic acid genome, envelope presence, overall size, and even capsid uniformity.[2] This review focuses on the following families of RNA viruses: *Orthomyxoviridae*, *Paramyxoviridae* and *Pneumoviridae*, *Picornaviridae*, and *Coronaviridae*. *Orthomyxoviridae* comprise negative (–) sense single-stranded (ss) RNA viruses that are segmented, enveloped, and includes the influenza viruses (see Table 1). *Paramyxoviridae* and *Pneumoviridae* are also (–)ssRNA

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Table 1. Viral characteristics: Family, category, and infectivity.

Demonstrative virus	Family	Genome content	Age group affected	Seasonality
Influenza A, B, and C	*Orthomyxoviridae*	(–)ssRNA	All ages	Fall to early Spring
PIV	*Paramyxoviridae*	(–)ssRNA	Young children	Fall, Spring, and Summer
RSV	*Pneumoviridae*	(–)ssRNA	Infants and young children	Fall to early Spring
HMPV	*Pneumoviridae*	(–)ssRNA	Young children	Year-round
Rhinovirus A, B, and C	*Picornoviridae*	(+)ssRNA	All ages	Year-round
Enteroviruses	*Picornoviridae*	(+)ssRNA	All ages	Summer to Fall
HCoV	*Coronaviridae*	(+)ssRNA	All ages	Fall to early Spring
SARS	*Coronaviridae*	(+)ssRNA	Adults	Fall to early Spring
MERS	*Coronaviridae*	(+)ssRNA	Adults	Fall to early Spring

PIV: parainfluenza virus; RSV: human respiratory syncytial virus; HMPV: human metapneumovirus; HCoV: human coronavirus; SARS: severe acute respiratory syndrome; MERS: Middle Eastern respiratory syndrome; ssRNA: single-stranded ribonucleic acid.

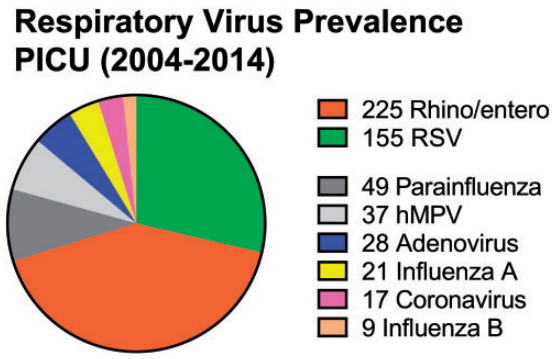

Figure 1. Prevalence of respiratory viruses in a PICU. Prevalence was defined as number of cases per 1000 admissions with respiratory distress.[4] PICU: pediatric intensive care unit.

viruses, but are non-segmented and enveloped, and include parainfluenza virus (PIV), human respiratory syncytial virus (RSV), and human metapneumovirus (HMPV). The *Picornaviridae* family, which contains positive (+)ssRNA viruses are non-enveloped; the key members include the rhinoviruses and enteroviruses. Lastly, the *Coronaviridae* are (+)ssRNA enveloped viruses, which include, chiefly, human coronavirus (HCoV), and severe acute respiratory syndrome (SARS)-associated and Middle Eastern respiratory syndrome (MERS) CoV.[3] Young children, the elderly, and patients with compromised cardiac, pulmonary, or immune systems are at greatest risk for serious disease associated with these RNA virus respiratory infections. In a 10-year study, over 85% of acute respiratory viral infections in critically ill children admitted to a pediatric intensive care unit were caused by either a picornavirus, RSV, PIV, or HMPV (see Figure 1).[4] Other DNA viruses such as adenovirus can be the source of respiratory infections but will not be discussed here.

In addition to their wide variation in viral characteristics, respiratory RNA viruses are also remarkable in their epidemiological variety. They differ in (1) their outbreak calendar, where some are seasonal and others are year-round, (2) their patient profile, whether infant, geriatric, or otherwise healthy adults, and (3) the morbidity and/or mortality associated with infection.

Influenza virus (*Orthomyxoviridae* family)

Influenza virus is a (–)ssRNA virus and a member of the *Orthomyxoviridae* family.[5] There are four influenza genera within this family, called A, B, C, or D. Influenza A and B contain hemagglutinin and neuraminidase envelope glycoproteins. Influenza C and D have a single surface glycoprotein called the hemagglutinin-esterase fusion protein.[6,7] Antigenic variation in these glycoproteins results in limited vaccine protection. Influenza, or the flu, presents with symptoms such as headache, cough, fever, sore throat, malaise, and chills.[8] Generally, the flu lasts from 5 days to 2 weeks and the severity of infection is determined by the host. The highest incidence of influenza infection occurs in younger patients (<25 years old) where a shorter infection is typical, while those at risk for longer and more severe illness and complications associated with infection are the pediatric (<2 years old) and geriatric populations (>65 years old), pregnant women, and immunocompromised individuals.[9,10] It is estimated that 3–5 million cases of the flu occur annually around the globe, with a quarter to half million deaths resulting from these illnesses.[11]

PIV (*Paramyxoviridae* family), RSV, and HMPV (*Pneumoviridae* family)

Until recently, PIV, HMPV, and RSV were all categorized in the *Paramyxoviridae* family due to their phylogenetic proximity in the order Mononegavirales, the non-segmented negative-strand RNA viruses. More recently, RSV and HMPV have been assigned as members of the newly formed *Pneumoviridae* family.[12] While influenza outbreaks are most prevalent in the winter, some viruses such as PIV persist year-round.

Human PIV has four types (1 to 4) and was known historically to induce respiratory complications mainly in children and the immunocompromised; however, more recently, it has been identified as a concern in the adult population as well.[13] Symptoms of PIV include upper and lower respiratory tract infection, middle ear inflammation, bronchitis, pneumonia, and croup, the last of which results in the most hospitalizations in the pediatric patients infected by this virus.[14,15] Up to one-third of the nearly 5 million annual cases of lower respiratory tract infection in children is at least partially due to the presence of PIVs.[16]

RSV and PIV infections are among the most common reason for hospitalization of young children.[17,18] The two strains of RSV, A and B, are distinguished by genetic variations in the G surface glycoprotein.[19] Dissimilar to PIV, RSV occurs mostly in the winter months in its target pediatric population. Symptoms include runny nose, nasal inflammation, cough, sore throat, low-grade fever, wheezing, bronchiolitis, and pneumonia.[20] Current estimates in developing and industrialized countries suggest as many as 33 million cases of RSV worldwide in the pediatric population less than 5 years old, 10% of which require hospitalization, and 2% to 18% of hospitalized cases result in mortality. This amounts to between 66,000 and 600,000 deaths in young children annually.[18,21]

HMPV, like RSV and influenza, tends to have greatest prevalence in the winter and studies have shown that by the age of 5 years, nearly all children have been infected with this virus.[22] The clinical manifestations of infection with this virus are upper and lower respiratory tract infections, bronchiolitis, middle ear inflammation, fever, chills, pneumonitis, and wheezing.[23] Of note, HMPV tends to occur in populations with seasonal inconsistency as studies done on Italian populations shortly after its discovery from 2000–2002, showed a range of infection from 7% to 40% depending on the year. Patterns of seasonal irregularity like this have been noted with other respiratory viruses, particularly RSV and influenza.[24]

Rhinoviruses and enteroviruses (*Picornaviridae* family)

Rhinoviruses are thought to be the cause of up to two-thirds of what is termed the common cold, worldwide. Children tend to experience up to 12 of these infections, or colds, per year, while this incidence drops in adults to just 2–3 per year.[25] There are three distinct species of rhinovirus, RV-A, RV-B, and RV-C, each of which infects humans at different periods throughout the year.[26] Symptoms include cough, fever, sneezing, nasal congestion, sore throat, fever, and headache and usually last 7–10 days after an initial 48-h viral incubation.[27]

In addition to the three rhinoviruses, four enterovirus species result in disease in humans, EV-A, EV-B, EV-C, and EV-D, while EV-E through EV-H, and EV-J affect non-human hosts.[28,29] Enteroviruses differ from rhinoviruses in that while rhinoviruses are limited to the respiratory airways, enteroviruses infect a wide range of cell types. They result in a large array of complications associated with the respiratory, gastrointestinal, and central nervous systems. Manifestations of enterovirus infection range from a febrile cold to encephalitis, pneumonia, viral meningitis, and even death.[30] Although EV-C and EV-D are the principal enteroviruses that cause respiratory illness, EV-A also includes EV71. EV71 causes hand-foot-and-mouth disease, a highly contagious pathogen in children that mainly results in a maculopapular rash, blisters on the limbs, and ulcers in the mouth.[31] EV71 is most prevalent in the summer months and tends to be more ubiquitous in tropical zones of the globe. In rare cases of severe EV71 infection, respiratory illness can lead to pulmonary edema, hemorrhage, and lung failure.[32]

Coronaviruses (CoV; Coronaviridae *family)*

Presently, six HCoV are recognized: HCoV-229E, HCoV-NL63, HCoV-OC43, HCoV-HKU1, and the well-known SARS and MERS CoV.[33] These CoV can be further characterized based on genera of Alpha, Beta, Gamma, or Delta; the Alpha and Beta CoV comprise the six viruses mentioned above, and are those that infect humans. CoV 229E and OC43 are both pathogens associated with the common cold, but can cause pneumonia as well.[34] HCoV-NL63 and HCoV-HKU1 infection show similar clinical features to those in patients with 229E and OC43, but clinicians have also reported bronchiolitis, croup, and pneumonia in infected individuals.[35,36] The first CoV recognized as pandemic threat is SARS-CoV. SARS was discovered in 2002–2003 after a perplexing epidemic of pneumonia among hospital workers in China.[37] By the end of its global epidemic, SARS disseminated to 29 countries, infecting over 8000 individuals, and killing roughly 10% of those infected.[38] Roughly a decade later, a similar pattern occurred with MERS, which began in 2012 in Jordan with an outbreak of a respiratory illness among hospital workers, one of whom died of the infection. Later that year, a man with pneumonia and multiple organ failure in Saudi Arabia was found to have the MERS pathogen.[39] Adults are the target population for both SARS and MERS with a median age range of 39–50 years; MERS occurs predominantly in men whereas SARS does not. The clinical features

of both SARS and MERS range from mild to severe respiratory illness, fever, chills, cough, shortness of breath, vomiting, and diarrhea, with the latter displaying a more lethal pneumonia and renal failure.[40,41] Even worse than SARS mortality, retrospective analysis has shown that of the 2040 confirmed cases of MERS, 35% were fatal.[42]

Viral polymerase: An important molecular target for antiviral therapy

Nucleoside analogs represent one of the dominant classes of antiviral agents due to their widespread use against the common chronic infections caused by human immunodeficiency virus (HIV), hepatitis B virus, and herpesviruses. In the past 15 years, multiple nucleoside and nucleotide analogs have been developed as direct-acting agents against RNA virus infections such as hepatitis C virus (HCV), but have not yet been successfully applied to acute infections caused by respiratory viruses. Only a handful of non-nucleoside drugs have been developed for the treatment and prevention of these viruses. Such drugs include the FDA approved oseltamivir, zanamivir, and peramivir for influenza virus infection, palivizumab for RSV prevention, as well as the two discontinued clinical candidates targeting rhinovirus, pleconaril and rupintrivir. These molecules possess limitations preventing their widespread use, such as short therapeutic window and risk of resistance selection for the neuraminidase inhibitors, and only partial protection associated with prophylactic use for palivizumab. This has provided the impetus for the approval of new drugs with a broader therapeutic use.

The recognized advantages of direct-acting agent nucleosides over other classes of antiviral agents are (1) their propensity to cover a broad-spectrum of virus strains and sometimes species and (2) their high barrier to antiviral resistance. Both properties are best explained by the mechanism of action common to most antiviral nucleosides: targeting viral polymerases.[43] After being metabolized by host kinases to their triphosphate form, antiviral nucleotides compete with natural nucleoside triphosphates (NTPs) to bind to the active site of viral polymerases and alter DNA or RNA synthesis. The nucleotide binding site of these proteins is usually well conserved among virus families and any changes in neighboring amino acids often comes at a cost for the enzyme and the virus. The functional and structural features of RNA polymerases of respiratory viruses targeted by antiviral nucleosides are described in the following paragraphs.

Structure and function of the polymerases of respiratory RNA viruses

The polymerase of influenza virus

Influenza virus is a (–)ssRNA virus and a member of the *Orthomyxoviridae* family.[5] The viral genome has eight segments in influenza A and B and seven segments in influenza C and D. In influenza A, these

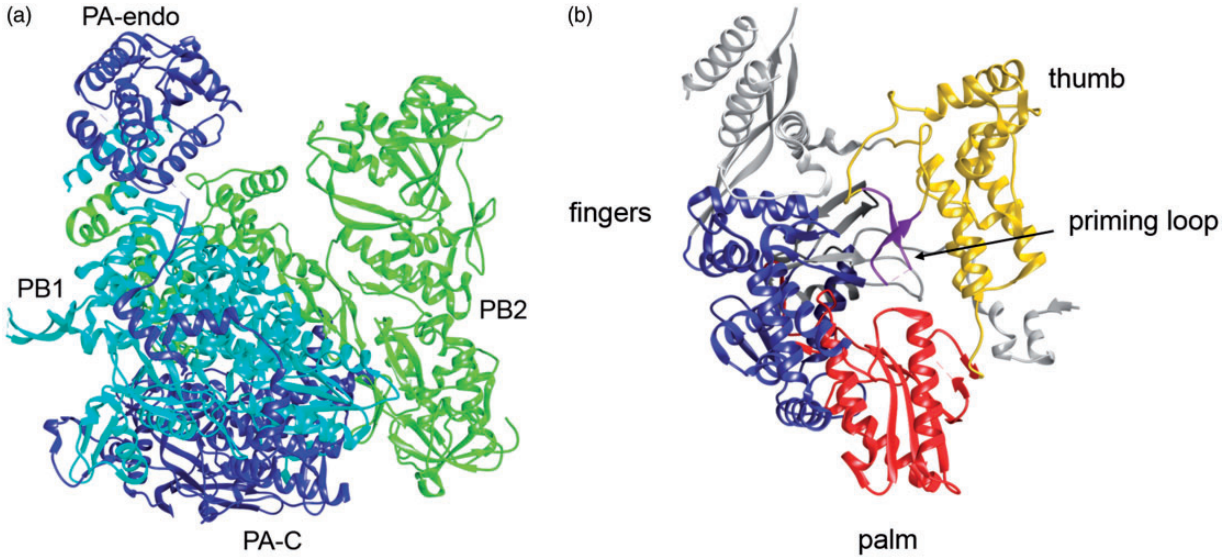

Figure 2. (a) The ribbon model of the influenza A polymerase complex, which is composed of three subunits that assemble a 270 kDa trimer. The trimer consists of the (PA) protein (blue), PB1 (teal), and PB2 (green). (b) Within the PB1 domain is the RdRp, which adopts a right-handed arrangement with the fingers (blue), palm (red), thumb, (yellow), and a priming loop (purple).[46] PA: polymerase acidic; PB1: polymerase basic protein 1; PB2: polymerase basic protein 2; RdRp: RNA-dependent RNA polymerase.

encode for 11 or 12 proteins. These are non-structural protein 1 (NS1), non-structural protein 2 (NS2), matrix protein M1 and ion channel protein M2, polymerase acidic (PA) protein, polymerase basic protein (PB1), polymerase basic protein 1-F2, polymerase basic protein 2 (PB2), nucleoprotein (NP), hemagglutinin (HA), and neuraminidase (NA).[44] Some viruses express the PB1-N40 protein. All four species of influenza adopt similar arrangements with the viral genomic segments forming viral ribonucleoprotein complexes associated with one heterotrimeric polymerase. Influenza A polymerase is composed of three subunits that yield a 270 kDa heterotrimeric complex. The longest viral RNA segments encode for the PA protein, PB1, and PB2, which assemble to form the influenza polymerase complex (see Figure 2).[45] The three subunits interact non-covalently to exert their polymerase activity. The polymerase transcribes viral RNA into messenger RNA (mRNA) and then replicates it using a complementary RNA intermediate.[5] The process of transcription includes cap snatching, where short-capped cellular RNA are bound by the PB2 subunit, cleaved from the PA endonuclease domain, and then utilized for priming mRNA synthesis by the PB1 domain.[46–51]

The PB2 subunit has an N-terminal domain (PB2-N) from residues 1 to 247 and a C-terminal domain (PB2-C) from residues 248 to 760.[52] PB2-N, including a lid domain, interacts with the C-terminal extension and thumb of PB1.[52] The PB2-C includes several notable structural features and subdomains such as the C-terminal nuclear localization signal, the PB2 627-domain, the PB2 cap-627 linker, the mid domain, and a cap binding domain.[46] Based on structural biology, the PB2 domain has a key exterior, positively charged residue at the 627 position within a flexible loop that partially wraps around an alpha helix to form what is known as a phi-loop.[53] Importantly, this residue is in the middle of a set of highly conserved, basic residues forming a net positive charge. A signature structural element is the conserved P[F/P]AAAPP motif on the N-terminal side of the 627 residue that is part of the alpha helix previously described.[53] Mutation of the P620 or F621 residue significantly decreased the ability of the virus to replicate, presumably by causing a slight kink in the alpha helix that alters the polymerase function. The exact role of the PB2 627 domain remains unclear, but recent evidence suggests it is not necessary for in vitro binding and transcription of viral RNA; this has not been proven true in a cell-based format.[54]

The PB1 domain is at the center of the polymerase complex and within its center is a classic right-handed shape with the signature fingers, palm, and thumb subdomains (see Figure 2).[5] These subdomains are described as conserved RNA-dependent RNA polymerase (RdRp) motifs pre-A/F and A-E.[52] The pre-A/F motif describes the fingertips and a small loop, which spreads over to the thumb domain; the tip of this loop is stabilized by an alpha helix within the PA domain. Residues within the pre-A/F may guide and bind NTPs and the incoming template. In addition to these subdomains, N-terminal and C-terminal extensions interact with PA and PB2 domains. The fingers and palm are covered by a linker connecting the two subdomains of PA. The PB1 possesses a β-hairpin loop within motif E from residues 641 to 657 in the thumb subdomain of influenza A.[46] In de novo initiation, it has been shown with other related polymerases that this priming loop may serve as a platform for the initial NTP on the 3′ end of the template and ensure against double-stranded RNA. Biochemical investigations have shown that the proline found within the loop tip motif of 648-Ala-His-Gly-Pro is necessary for in vitro and cell-based RNA synthesis. This loop may also be necessary during replication mode for terminal de novo initiation but unnecessary for internal initiation and transcription.[55]

The PA endonuclease domain or the PA subunit, as it will be named here, has little homology to other proteins and its exact enzymatic function was discovered only recently. The subunit was expressed in insect cells to reveal an N-terminal third (PA-Nter) and a C-terminal two-thirds (PA-Cter) subdomains. They have molecular weights of 25 and 55 kDa, respectively. These two subdomains are connected by a flexible linker. The endonuclease activity was originally thought to occur through the PB1 or PB2 domains but the structure of the PA-Nter was solved by two groups to reveal that the catalytic residues for endonuclease activity reside in the PA domain, not in the PB1 subunit as originally thought.[50,51] The PA endonuclease domain contains a signature (P)DXN(D/E)XK motif that is conserved among influenza viruses and coordinate divalent cations such as magnesium or manganese.[56] Although the exact quantity and identity of ions present in the native influenza enzyme are unclear, the endonuclease activity is strongly activated by metal ion binding through hydrolysis of ssDNA and ssRNA substrates.

The polymerase of RSV, HMPV, and PIVs

Human metapneumovirus and human RSV are non-segmented, negative strand RNA viruses from the Pneumoviridae family of the order *Mononegavirales*. The polymerases from this class of viruses are multifaceted with multiple enzymatic functions contained within a single protein. It exists as part of a ribonucleoprotein complex, or replicase, composed of L, N, P, and M2-1 proteins in complex with RNA. These

include RNA synthesis activities carried out by an RdRp domain but also include capping and methylation functions.

The RdRp carries out transcription and replication of its genome in response to cis-acting elements within the genome.[57] The genome of RSV is approximately 15 kilobases long and is transcribed into capped and poly-adenylated mRNAs.[58] The HMPV genome is 13 kilobases long. Current understanding of HMPV transcription is based on knowledge gained from the more extensive characterization of RSV transcription, which is the focus here.

At the beginning and end of each viral gene lies a conserved region (CR) of 9–10 nucleotides and 12–13 nucleotides, respectively. These are termed the *gene start (gs)* and *gene end (ge)* signals with an intergenic, non-transcribed region between these genes. At the 3′ side of the genome, prior to the first gene, is the *leader (le)* extragenic region and at the 5′ end is the *trailer (tr)* extragenic region. The lengths of these extragenic regions can vary based on the virus but in RSV the *le* region is 44 nucleotides long. To transcribe its RNA, the polymerase initiates at the third nucleotide to transcribe a short uncapped transcript of about 25 nucleotides.[59] This RNA is released but the polymerase remains affixed to the template where it proceeds along until it encounters the *gs* signal for the first gene and subsequently begins RNA synthesis. These mRNAs are modified with a 5′ methyl cap and when the *ge* signal is reached, a 3′ polyadenylated tail is added, and the mRNA is released. The polymerase then scans for the next *gs* region. The genome is replicated starting at the leader promoter in a processive manner to yield a positive sense antigenome RNA. The 3′ and 5′ ends of the antigenome contain the trailer and leader complement. The trailer complement ultimately directs genome RNA synthesis.

The core RSV polymerase consists of a 250 kDa large (L) protein of approximately 2000 amino acids and a 27 kDa phosphoprotein (P) that synthesizes an RNA product upon the addition of an RNA template.[59–62] The P protein is thought to act as a chaperone to aid in the stability and expression of the polymerase. During RNA synthesis, the P protein anchors the L protein to the N protein and also binds to the M2-1 transcription antitermination factor.[63–66] This matrix protein, M2-1, serves as an elongation factor and is necessary for the polymerase to be fully processive in producing long mRNA products.[67]

No structure is available for any L protein from the paramyxoviruses and pneumoviruses, largely due to the size, solubility, and complexities with yielding enough highly pure protein. However, the cryo-electron microscopic (EM) structure of the L-P complex from a highly similar virus, vesicular stomatitis virus (VSV), has recently been solved.[68] VSV is a non-segmented, negative strand virus from the *Rhabdoviridae* family and given the high sequence conservation of the L protein, the structure of VSV L has provided important

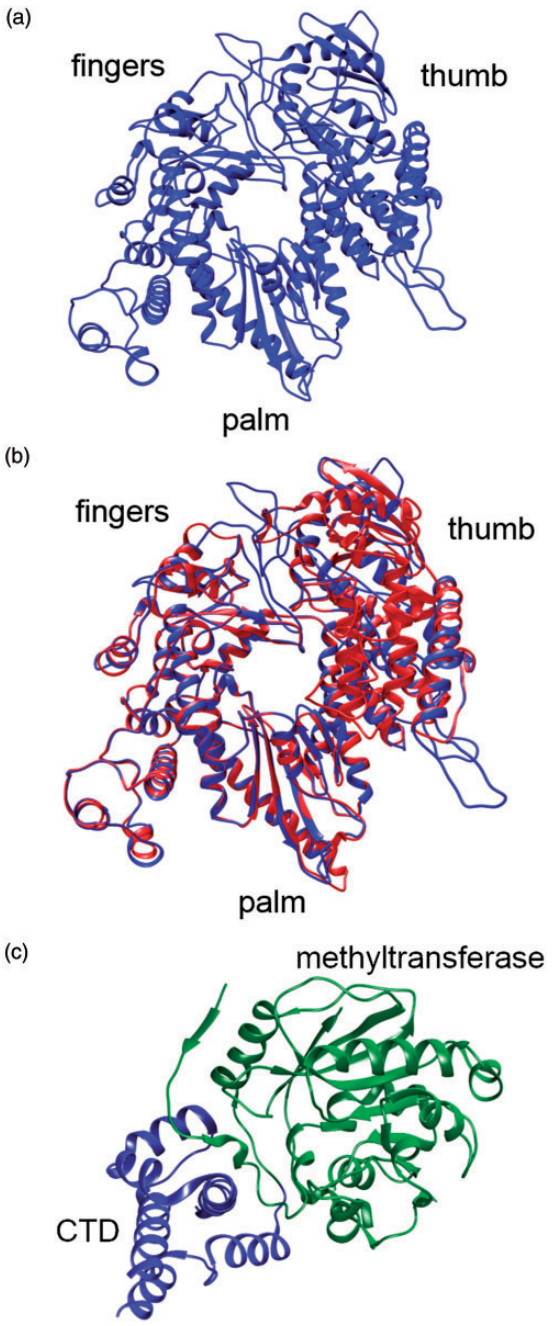

Figure 3. (a) A homology-model of the RdRp domain of the RSV L protein shows a right-handed fold with structural similarities to VSV (b) when the two structures are overlayed (RSV RdRp is shown in blue, VSV RdRp is shown in red).[68,71] (c) The three-dimensional structure of the methyltransferase (green) and C-terminal (blue) domains of the HMPV L protein.[72] RdRp: RNA-dependent RNA polymerase; RSV: respiratory syncytial virus; VSV: vesicular stomatitis virus; HMPV: human metapneumovirus.

structural insights. The L proteins of RSV, VSV, and other related negative sense RNA polymerases can be divided into six CR.[69] The first three regions (CRI-III) of VSV generate a doughnut-like structure in negative stain EM and cryo-EM analysis.[68,70] The remaining CRs appear as globular appendages on this doughnut. The doughnut structure adopts a classic right-handed configuration in the cryo-EM reconstruction composed of fingers, palm, and thumb domains, like other polymerases (see Figure 3). The CRIV and CRV contain conserved residues and catalytic motifs necessary for enzymatic function.

Among these is the GxxT[n]HR motif, a highly conserved set of residues necessary for NTP binding and the HR motif, which forms covalent histidine RNA intermediate.[73] Within CRVI there is a motif with sequence similarity to the 2′-*O*-ribose methyltransferase (MTase) domain, which has been characterized in the VSV L system.[74] While it is unknown exactly how similar the capping mechanism of VSV is to RSV and HMPV, the detailed mechanism of the VSV capping biochemistry provides insights. The capping of VSV is unique in that it is accomplished through an unconventional RNA:GDP polyribonucleotidyltransferase (PRNTase) rather than a guanyltransferase.[75–77] The VSV capping occurs in two parts starting with conversion of GTP to GDP through a GTPase followed by the covalent attachment of a histidine to pRNA to form a phosphamide bond. This GDP then serves as a nucleophile to attack the pRNA and results in the release of the GpppRNA.[58] The second part of the reaction consists of the MTase reaction, which uses S-adenosylmethionine to methylate nitrogen 7 and the 2′-oxygen of the cap.

A recent crystal structure of the MTase domain from HMPV has provided additional clues into this reaction (see Figure 3).[72] A 406-residue fragment was expressed, consisting of the CR-IV containing the putative MTase with an additional C-terminal K-K-G motif (termed CR-VI+). With the exception of the K-K-G motif, the fold of the HMPV MTase domain indicates a conserved fold compared to VSV.[72] While the CR-VI+ was active, the reaction rate was very slow and structural and biochemical results did not clearly identify active site residues. This suggests that MTase requires other co-factors or additional parts of the L protein to be catalytic.

The polymerase of rhinoviruses

Human rhinovirus (HRV), enterovirus 71 (EV71), and poliovirus (PV) are nonenveloped, positive strand RNA viruses and are all members of the *Picornaviridae* family. Picornaviruses replicate their genome using an RdRp, called $3D^{pol}$.[78] Replication

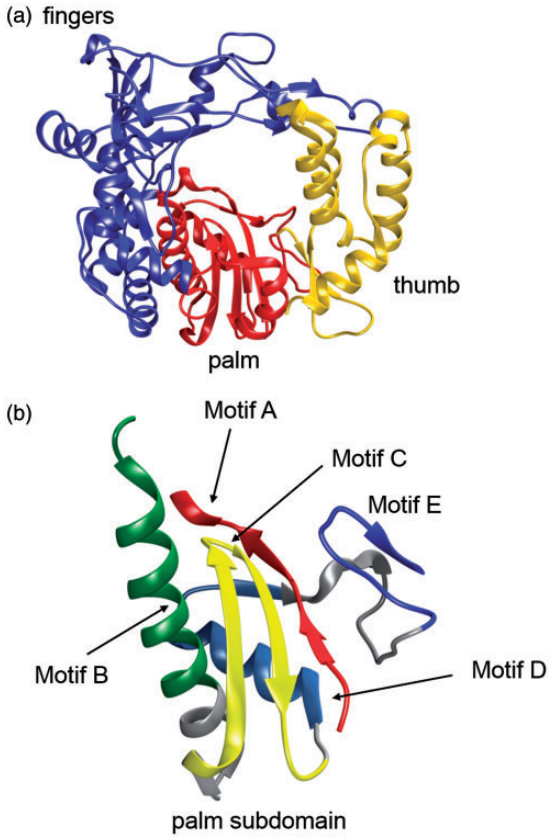

Figure 4. (a) The HRV16 $3D^{pol}$ in a ribbon representation showing the classic right-handed model with the fingers (blue), palm (red), and thumb (yellow) subdomains.[86] (b) The palm subdomain showing motifs A–E. HRV: human rhinovirus.

takes place in one of two forms: primer-dependent format or de novo RNA synthesis.[79] De novo RNA synthesis, which uses a single initiation nucleotide, gives the 3′-hydroxyl group for adding the sequential nucleotide whereas a primer-dependent format uses a protein-based primer or an oligonucleotide for the hydroxyl group donor.[80] The polymerases from the picornavirus family only use a protein-primed mechanism of initiation.[80,81] The $3D^{pol}$ uses a small viral peptide (VPg) to initiate both plus and minus RNA synthesis in vivo, making *Picornaviridae* unique in their initiation mechanism.[78] RNA synthesis is initiated using a highly conserved tyrosine residue within VPg using cis-acting replication element as a template whose position varies depending on the genus.[78,82] The $3D^{pol}$ aids in the binding of two UMP molecules to the tyrosine hydroxyl group of VPg.[83–85] The product of this reaction, VPg-pU is then extended by an additional uridine to form VPg-pUpU.[86]

The $3D^{pol}$ is located at the C-terminal end of a longer viral polyprotein of approximately 250 kDa and the structures of $3D^{pol}$ have been solved for EV71, HRV16, and PV, among many others.[86–88]

The structures of these polymerases are largely similar and have sequence domains A–G indicating this conservation.[89] The 460-residue polymerase domain adopts a right-handed configuration with fingers, palm, and thumb subdomains providing an optimal arrangement for substrate and metal cation access during the catalytic cycle. The palm, fingers, and thumb subdomains contain these sequence motifs (motifs A–E in the palm subdomain shown in Figure 4).[90,91] These motifs have specialized roles in catalysis including nucleotide binding and overall structural integrity of the active site.[80] The palm contains the active site of all the RdRps and its structure consists of an antiparallel beta-sheet surrounded by three alpha helices.[90] Additional substructures within the fingers domain are referred to as the index, middle, ring, and pinky fingers.[82,92] All but the pinky fingers build an extended beta-sheet that seems to be conserved. The index finger within the fingers subdomain makes an important contact with the thumb subdomain and pushes the ring finger down to trap it. This conformation results in the ring finger being the roof for NTP entry and making important interactions with the triphosphate. An additional structural feature of motif F is a positively charged tunnel that modulates the interactions of NTP.[93] This tunnel aides in the diffusion of nucleotides and is conserved among this family of viruses.

Proteins and enzymes rarely exist in a monomeric state in nature but are energetically driven to higher order, oligomeric states through polar and hydrophobic interactions and disulfide bond formation. The development of increasingly sophisticated structural biology techniques, including high-resolution X-ray crystal structures and cryo-EM, has provided a snapshot into how polymerases may adopt such oligomeric states. The understanding and characterization of the oligomeric states place these multifunctional enzymes in a greater biological context. An example of such oligomeric states and how this impacts the catalytic function is the polymerase from PV. PV RdRp is described as having macromolecular contacts at two polymerase interfaces (Interface I and Interface II).[94] Interface I is defined by the interaction of the thumb domain from one polymerase with the palm of another polymerase. Interface II describes interactions of the N-terminus of one polymerase with the thumb of a neighboring polymerase within the crystal lattice. Additionally, PV can form tube-like structures suggesting it is a highly dynamic structure able to undergo and adopt multiple conformational arrangements.[95] The oligomeric state of PV polymerase is required for membrane-associated RNA replication in infected cells, as demonstrated by mutating residues involved in protein–protein interactions.[96,97]

The polymerase of CoV

The CoV are part of the larger nidovirus family and have exceptionally large genomes of up to 32 kilobases in length.[98] CoV are positive sense RNA viruses, with a notable example being SARS as one of the most pathogenic member of this viral family.[99] The CoV genome has a 5′-cap, is polyadenylated on the 3′end, and generates a total of 16 non-structural proteins (nsp1 to nsp16). The 5′ two-thirds of its genome encodes for non-structural proteins that combine to form a replication and transcription complex that completes viral RNA synthesis.[100]

The Nsp12 protein is the RdRp and is typically composed of a N-terminal domain composed of a nidovirus-specific RdRp-associated nucleotidyltransferase (NiRAN) and a C-terminal containing the RdRp domain, which contains a set of six conserved motifs (motifs A-F) responsible for recognizing substrates and template.[98,101] The NiRAN domain has only been identified in nidoviruses and is approximately 300 residues long in CoV.[101] In SARS-CoV, a reverse genetic system identified this motif as a requirement for replication of its viral genome. While NiRAN activity has not been directly observed outside of a reverse genetic system for CoV, based on the nucleotidylation activity of EAV nsp9, the NiRAN domain is hypothesized to play a role in protein priming, capping, or as a potential universal ligation mechanism.[98]

The active site of the polymerase is located within motif C and is composed of conserved (within the Nidovirus family) ser-asp-asp residues. Importantly, conserved aspartates found in motif A of SARS-CoV, which combined with those found in motif C, contribute to the polymerase and RNA synthesis activities. This is different from other positive strand RNA viruses which contain a GDD active site. Motif A along with motif C aid in coordinating the metal ions necessary for catalysis. The SARS-CoV harbors a signature sequence in motif G necessary for primer-dependent RNA synthesis.[102,103]

Due to difficulties in obtaining large enough amounts of highly pure protein, the structures of CoV nsp12 have not been solved either by X-ray crystallography or cryo-EM. Therefore, the structural information currently available is based solely on structural models obtained via sequence alignment and homology modeling techniques. These models indicate a right-handed fold composed of fingers, palm, and thumb subdomains with clearly defined entry and exit channels, consistent with RdRp domains for other structurally characterized positive sense RNA polymerases (for example, foot-and-mouth disease virus) but clearly distinct from the known molecular topology of negative-stranded RdRps. No structural models

predict the presence of a priming loop. These data combined with biochemical data indicating no de novo initiation of RNA synthesis may account for the functionality of motif G.

A complete characterization of the in vitro nsp12 RdRp activity has demonstrated overall weak activity. Initial evidence suggested that a previously uncharacterized N-terminal domain may have been required for polymerase activity.[104] However, the addition of this domain using a C-terminally tagged protein still yielded protein with poor activity and processivity. Based on these results and to increase the in vitro activity of the nsp12, two other non-structural proteins, nsp7 and nsp8, were added to the nsp12 protein in a primer extension mode.[100] The addition of nsp7 and nsp8 to nsp12 appear to activate and increase the processivity of the polymerase allowing it to produce an RNA synthesis product of 340 nucleotides in the presence of Mg^{2+}. Linking the nsp7 and nsp8 subunits together also increased the polymerase reaction efficiency suggesting that nsp7–nsp8 complex formation may influence the reaction rate. Importantly, nsp14 can interact with an nsp7–nsp8–nsp12 complex without influencing RNA synthesis activities.[100] Nsp14 contains an exoribonuclease domain that has been shown to decrease nucleotide mismatch, in many ways similar to the proofreading exoribonuclease activities correlated with a polymerase.[105]

Nucleoside analogs against respiratory RNA viruses

In this section, we aim to answer the following questions that are important to medicinal chemists, biologists, and drug developers working in the field of Virology. Are there clinically relevant nucleoside analogs that are potent against one or multiple respiratory viruses? How were these molecules first identified, and which ones have been approved for commercial use? Why aren't molecules like ribavirin and its analogs more widely used to treat respiratory viral infections? What are the current approaches to develop new nucleoside analogs against respiratory RNA viruses?

Ribavirin as a broad antiviral against respiratory viruses

The broad-spectrum antiviral effect of ribavirin, a guanosine analog, was first reported in the 1970s.[106,107] It was found at the time that ribavirin inhibits 16 DNA and RNA viruses, including herpesviruses, vaccinia, VSV, as well as respiratory infections caused by influenza A and B viruses and parainfluenza 1 virus. Ribavirin is currently approved for the treatment of chronic HCV infection in combination therapy and against severe RSV infection in monotherapy. In the case of RSV infection, ribavirin is administered as a small-particle aerosol that requires use of a mask and a tent. Ribavirin was originally developed against influenza based on its efficacy in a mouse model of influenza,[108,109] but its effect in human clinical trials was less clear, so it was not approved for the treatment of influenza.[110] Its clinical use for the treatment of RSV infection via aerosol delivery remains limited due the inconvenient route of administration, lack of clear evidence for efficacy, and safety concerns associated with anemia and risk of teratogenicity.

Studies evaluating the mechanism of action of ribavirin have produced contradictory results. It is usually acknowledged that ribavirin exerts its main effect through its monophosphate metabolite by inhibiting the host inosine monophosphate dehydrogenase (IMPDH) enzyme, leading to depletion of intracellular GTP pools, which results in indirect inhibition of RNA synthesis during viral replication (for review[111]). The nucleoside form of ribavirin is also believed to enhance T-cell-mediated immune response through increased expression of interferon-gamma and tumor necrosis factor-alpha.[111] In addition, it has been proposed that prolonged replication of PV in the presence of ribavirin increases the viral mutation frequency and decreases infectivity.[112] One hypothesis is that the mutagenic effect of ribavirin is caused by its triphosphate form that is recognized by the viral RNA polymerase.[112] Once incorporated into the viral genome, ribavirin monophosphate could equally base pair with cytidine and uridine, therefore causing random mutations throughout the viral genome.

7DMA and NITD008 for human rhinoviruses

Ribo-cytidine and adenosine analogs containing a methyl group at the 2′-position on the ribose are known inhibitors of HCV and other related members of the *Flaviviridae* family.[113–119] One of the most potent molecules of this series, 7-deaza-2′-C-methyladenosine (7DMA, MK-0608), was once a development candidate for the treatment of HCV infection (Figure 5).[117,120] The adenosine analog 7DMA also inhibits HRV type A infection in vitro, with EC_{50} values ranging from 2 to 12 μM.[121] A subgenomic replicon assay was used in transient transfection experiments to demonstrate that 7DMA is equipotent against multiple strains of HRV type C. This important proof-of-concept experiment demonstrated that 2′-methyl nucleosides prevent picornavirus replication, most likely by inhibiting the viral RNA polymerase function. This class effect was

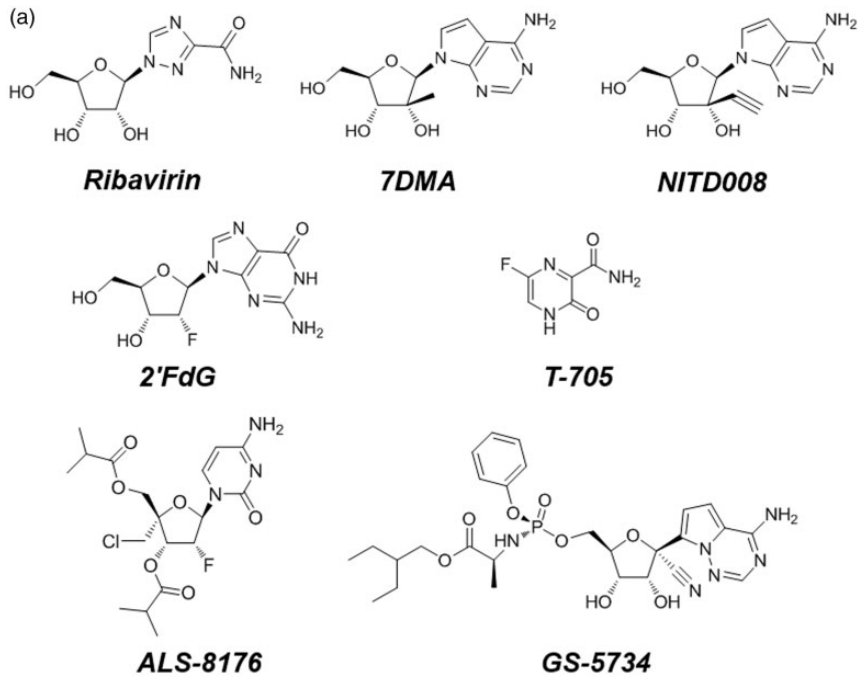

Figure 5. The chemical structures of a range of antiviral compounds. These structures describe the broad-spectrum ribavirin approved for HCV and severe RSV, the inhibitors NITD008 and 7DMA for rhinoviruses, the influenza virus inhibitors 2′FdG and T-705, ALS-8176 for RSV virus infection, and GS-5734, an Ebola virus inhibitor. HCV: hepatitis C virus; RSV: respiratory syncytial virus; 7DMA: 7-deaza-2′-C-methyladenosine; 2′FdG: 2′-deoxy-2′-fluoro guanosine.

confirmed with NITD008, another adenosine analog containing a 2′-C-ethynyl on its 2′-ribose (Figure 5). Just like 7DMA, NITD008 was previously known to inhibit Flaviviruses, and was once a development candidate for the treatment of dengue infection.[122] NITD008 blocks the replication of dengue virus in cell culture and in mice by inhibiting the RdRp activity of the NS5 protein.[122,123] Therefore, the possibility that NITD008 would inhibit other (+)ssRNA viruses is not unexpected. Indeed, NITD008 blocks the replication of EV71, another enterovirus-related to rhinovirus.[124] This in vitro antiviral effect was confirmed in a separate study that also demonstrated in vivo efficacy.[125] Investigators in the latter study infected 2-week-old AG129 immunocompromised mice with EV71 by intraperitoneal inoculation. Treatment of the infected animals with NITD008 given orally at 5 mg/kg twice a day for 4 days resulted in 100% survival at the end of the study, compared to 0% survival for the vehicle control group. NITD008 cannot be developed in the clinic due to severe toxicity seen in 14-day studies in rats and dogs.[122] However, the results summarized here indicate that nucleoside analogs targeting the viral RNA polymerase of rhinovirus, EV71, and other enteroviruses have the potential to be efficacious in preclinical animal models, providing a rationale to conduct human studies with safer molecules sharing the same mode of action.

2′-Deoxy-2′-fluoro nucleosides for influenza

Fluorinated nucleosides are well known for their antiviral and anticancer properties (for review[126]). In particular, 2′-deoxy-2′-fluoro guanosine (2′FdG) was at one time considered a potential candidate for influenza treatment (Figure 5). In vitro, 2′FdG inhibits influenza A virus replication with an EC_{50} of about 20 μM, without causing apparent cell toxicity.[127] In ferrets, treatment with 2′FdG at 20 mg/kg starting 1 h post-infection significantly reduced H3N2 influenza A virus titers in nasal washes, associated with reduction in fever and inflammation.[128] Although time-of-addition experiments suggested that the molecule inhibits an early step of virus replication, more direct evidence for the mechanism of action came from enzyme inhibition studies.[129] In cell-free transcription experiments, 2′FdG triphosphate inhibited influenza A virus RNA polymerase activity by competing with natural GTP. The inhibition of the enzyme was caused by the incorporation of 2′FdG monophosphate into the viral RNA.[129] More recently, the related nucleoside analog 2′-deoxy-2′-fluoro cytidine (2′FdC) was evaluated against the highly pathogenic H5N1 and the pandemic H1N1 strains.[130] When administered intraperitoneally, 2′FdC significantly enhanced survival of BALB/c mice infected with a lethal dose of either H5N1 or H1N1

viruses. Although these studies show compelling evidence of in vivo efficacy in preclinical species, 2′FdG and 2′FdC are not suitable candidates for clinical development. One of the main limitations of these molecules is their lack of specificity for influenza virus polymerase. The ability of a nucleotide to inhibit distant molecular targets is not detrimental per se. As such, 2′-deoxy-2′-fluoro nucleotides and their derivatives interact with the RNA polymerase of HCV.[131–134] But the substitution of the 2′-hydroxy by a fluoro group also makes the resulting nucleotides broad substrates for viral[135] and human[136] DNA polymerases. In the latter study, the authors have shown that the monophosphate form of both 2′FdC and 2′FdG can be incorporated into DNA by human DNA polymerase alpha and gamma. This might explain the changes in cell cycle distribution and cytostatic effect caused by prolonged in vitro incubation with 2′FdC.[133] Paradoxically, the same molecule was well tolerated when administered intravenously to rats and woodchucks for up to 90 days.[137] One hypothesis for this discrepancy is that a low organ exposure of the phosphorylated metabolite(s) of 2′FdC could limit the toxic effect on dividing cells in these animals.

T-705 (favipiravir) for influenza

The antiviral 6-fluoro-3-hydroxy-2-pyrazinecarboxamide (T-705, favipiravir, AVIGAN) has been approved in Japan for the treatment of influenza infection since 2014. T-705 is a nucleoside precursor inhibiting influenza virus with broad-strain coverage[137,139] (Figure 5). It is often proposed that T-705 exerts its antiviral activity through its NTP form (T-705 RTP) by directly inhibiting the RdRp activity of influenza A virus polymerase,[140] but the exact mode of action and precise molecular interaction between the nucleotide and the viral polymerase has been elusive. In vitro, T-705 is efficiently converted to its ribofuranosyl 5′-triphosphate (T-705 RTP) form by cellular enzymes.[141] Treatment of influenza A virus-infected cells with T-705 results in a significant increase of lethal mutations within the viral genome, a phenomenon also described as error catastrophe.[142] The lethal mutagenesis hypothesis is supported by enzymatic assays showing that T-705 RTP is efficiently recognized by influenza A virus polymerase both as a guanosine and an adenosine analog.[143] In addition, single events of T-705 RMP incorporation into RNA by influenza A virus polymerase delayed but did not block the extension of the RNA primer strand. The antiviral potency of T-705 covers other virus families well beyond orthomyxoviruses. T-705 has been shown to inhibit a number of diverse RNA viruses unrelated to influenza, including representatives of noroviruses, bunyaviruses, arenaviruses, flaviviruses, and filoviruses.[144–152] It is interesting to point out that the mutagenic effect of T-705 has also been documented for HCV.[153] At the biochemical level, we showed that T-705 is recognized as substrate for RNA synthesis not only by viral polymerases, but also by human mitochondrial RNA polymerase.[154,155] This host-based interaction did not result in any measurable in vitro mitochondrial toxicity, but it raised more questions about the mechanism of action of the compound. Recently, the possibility that T-705 exerts its main antiviral effect without converting to its triphosphate form came from the observation that T-705 ribonucleoside is chemically unstable under biological conditions.[156] Even though T-705 does not seem to potently inhibit the human IMPDH enzyme,[157] its very broad antiviral spectrum and its capacity to induce lethal mutagenesis are somewhat reminiscent of ribavirin, another nucleoside that inhibits HCV replication through host-based mechanisms. Therefore, the possibility that T-705 exerts its inhibition through interactions with host proteins cannot be ruled out and remains to be further explored. Considering its similarities with ribavirin in terms of antiviral spectrum and mode of action, it will be interesting to see if T-705 becomes more widely used in patients suffering from respiratory viral infections, or if it will remain limited to stockpiling for potential influenza pandemic in Japan.

ALS-8176 (lumicitabine) for RSV and HMPV

The discovery of ALS-8112, the parent molecule of the prodrug ALS-8176 (lumicitabine), was the result of a screening campaign using a focused library of structurally diverse nucleoside and nucleotide analogs tested against RSV in an in vitro infectious assay.[158] The main scaffold identified from this screen was 2′difluoro-4′azido-cytidine. Further modifications at the 2′- and 4′- positions to improve anti-RSV potency and selectivity, led to the identification of ALS-8112 (2′fluoro-4′chloromethyl-cytidine) (Figure 5). In vitro, ALS-8112 inhibits a broad panel of RSV A and B subtypes, as well as related pneumo-, paramyxo-, and rhabdoviruses.[159] In particular, we recently reported that ALS-8112 inhibits RSV and HMPV with similar in vitro potency.[160] The molecular target of ALS-8112 was determined by two independent methods. The polymerization function of the RSV L protein was identified as the target of ALS-8112 inhibition, first, by selecting and characterizing drug resistance-associated mutations located in the *L* gene. When introduced into a wild-type RSV genome, four amino acid mutations (M628L, A789V, L795I, and I796V)

were phenotypically associated with resistance to ALS-8112.[159] Enzymatic assays using purified recombinant RSV polymerase were critical to validate the mode of action of ALS-8112. In these assays, the 5′-triphosphate form of ALS-8112 (ALS-8112-TP) caused immediate chain termination of RNA synthesis and inhibition of the viral polymerization activity. This inhibitory effect was specific to RSV polymerase, since ALS-8112-TP did not inhibit polymerases from host or viruses unrelated to RSV such as HCV. The lack of inhibition against HCV was rationalized by molecular modeling, predicting steric clashing of ALS-8112-TP inside the active site of HCV polymerase. Because of the low oral bioavailability of ALS-8112, a series of 2′,3′-diester prodrugs was evaluated for improved pharmacokinetic properties. One prodrug, ALS-8176, formed high levels of monophosphate and triphosphate in the lungs when administered orally to nonhuman primates. Because of its high oral bioavailability, ALS-8176 was evaluated for in vivo efficacy in African green monkeys infected with RSV. At the end of treatment, RSV RNA was undetectable in bronchoalveolar lavage samples from all four ALS-8176-treated animals.[159] Subsequently, a randomized, double-blind, clinical trial evaluated ALS-8176 given for 5 days to healthy adults inoculated with RSV.[161] The reduction in viral load in nasal washes associated with ALS-8176 treatment varied from 73% to 88% depending on the dose regimen. RSV RNA was undetectable 1.3 to 2.3 days after the start of ALS-8176 treatment compared with 7.2 days for placebo. Assessment of symptom scores and quantity of mucus produced also showed a clear effect on RSV-induced disease. This important result represents the first proof-of-concept validation that an RSV replication inhibitor can be efficacious in humans. ALS-8176 is currently in clinical development for the treatment of RSV infection in hospitalized infants and adults (ClinicalTrials.gov identifier: NCT02202356, NCT02935673).

GS-5734 for Ebola virus and CoV

The recent Ebola virus outbreak of 2013–2016 in West Africa triggered increased efforts to identify new antivirals targeting filoviruses. As a result, the development of a new series of C-linked nucleoside analogs with anti-Ebola properties was soon reported.[162] In a cell-based infectious assay, the 1′-cyano C-linked adenosine derivative (GS-441524, or compound 4) was moderately active against Ebola replication with EC_{50} values around 1.5 μM, whereas the 1′-methyl and -ethynyl counterparts were completely inactive. GS-441524 is also a broad-spectrum inhibitor of a variety of RNA viruses from four families (*Filoviridae, Flaviviridae, Paramyxoviridae*, and *Pneumoviridae*), including HCV and RSV.[163,164] However, the addition of a 2′-C-methyl group, as in the case of the GS-6620,[165,166] significantly reduces the antiviral spectrum to HCV only. The relatively weak antiviral activity of GS-441524 across all viruses (0.5–50 μM EC_{50}) was attributed to its inefficient intracellular phosphorylation, which could be improved by adding a monophosphate prodrug to the parent nucleoside. The resulting compound, GS-5734 (Figure 5) inhibits the Zaire and Sudan species of Ebola virus and Marburg virus with EC_{50} values ranging from 0.01 to 0.20 μM, and exhibits moderate cytotoxicity ($CC_{50} = 2$ to >20 μM) in multiple human cell types. GS-5734 exhibits the same broad antiviral spectrum as its parent molecule.[163] The triphosphate form of GS-5734 is recognized as substrate by RSV polymerase, but its incorporation into RNA does not lead to immediate chain termination.[162] The favorable in vitro data led to further evaluation of GS-5734 in a macaque lethal model of Ebola virus disease. Complete protection was achieved when GS-5734 was administered at a daily intravenous dose of 10 mg/kg, beginning on Day 3 post-infection.[162] Following Phase I safety testing in healthy human volunteers, GS-5724 was first given as a 14-day course for compassionate use to an Ebola-infected nurse who had survived the disease and developed a recurrence in the central nervous system.[167] Soon after, a neonate who had congenital Ebola virus infection received three different experimental therapies, including a 12-day treatment with GS-5734.[168] In both cases, patients cleared the virus and survived the infection.

The characterization of the broad antiviral spectrum of GS-5734 was further expanded to another (+) ssRNA virus family: *Coronaviridae*. It was shown that GS-5734 inhibits SARS-CoV and MERS-CoV replication in multiple in vitro systems, including primary human airway epithelial cell cultures with sub-micromolar EC_{50} values.[169] GS-5734 was also effective against other human and bat CoV. In a mouse model of SARS-CoV infection, prophylactic and early therapeutic administration of GS-5734 reduced lung viral load and improved clinical signs of disease as well as respiratory function. Although there is limited data to confirm the proposed mechanism of action of GS-5734 against each virus, it is generally assumed that the molecule targets the RdRp function of the viral polymerase. In the case of CoV, this is supported by the identification of two mutations (F476L and V553L) within the predicted fingers subdomain of the RdRp protein nsp12 from murine hepatitis virus.[170] These mutations emerged over 23 passages and confer 4- to 6-fold resistance to GS-5734, combined with overall reduced replication fitness. At this point, the precise mechanism of action of GS-5734 against CoV remains elusive. It is possible

that GS-5734 triphosphate is not excised by the proofreading activity of nsp14 because of lack of immediate chain termination, as observed for RSV polymerase. In this case, could the resistance mutations identified in nsp12 alter the chain termination profile of GS-5734, and make it more susceptible to excision? Such studies are needed, not only to understand how GS-5734 works but also to design new molecules against CoV polymerases.

Conclusion and future directions

In this review, we summarized the exciting advances in discovery and development of novel nucleoside analogs as potential new treatments for respiratory RNA virus infections. The medical need is high because very few drugs have been approved for the treatment of respiratory viral infections despite worldwide health impacts attributed to them. The approved drugs include zanamivir, oseltamivir, peramivir, and favipiravir (Japan only) for influenza virus and palivizumab for RSV, all of which have limitations that prevent their widespread use in a therapeutic setting. Drug candidates intended for use against rhinovirus infections, such as the capsid inhibitor pleconaril and the protease inhibitor rupintrivir, have been tested in the clinic without success.

The first nucleoside analog developed for respiratory viral infection was ribavirin, but despite its approval for use in RSV, its utility for treating severe viral infections remains low. Therefore, the concept of nucleoside analogs against respiratory viruses remains relatively new and needs to be further explored.

What are the molecular determinants of polymerase selectivity against nucleotide analogs? We currently do not understand well how specific changes made in nucleotide analogs alter their recognition as substrates for RNA synthesis, and how substrate selectivity differs among positive and negative strand RNA virus polymerases. For example, many 2′-modified nucleotide analogs are known to inhibit HCV polymerase, often with an antiviral spectrum extended to flaviviruses and picornaviruses. However, there is no clear mechanistic basis to explain why none of these compounds inhibit (–)ssRNA viruses, or even other (+)ssRNA viruses such as CoV. Could the exonuclease/proofreading activity of CoV polymerases excise chain terminators and resume RNA synthesis? Are there specific amino acid within the active site of (–)ssRNA virus polymerases responsible for the discrimination of 2′-C-methyl nucleotides? These hypotheses have not been tested, in part, due to the difficulty to conduct biochemical and structural studies on viral polymerases from respiratory viruses. Until recently, the production of soluble, pure viral protein targets has been limiting, especially in the case of large protein complexes. As mentioned earlier in this review, the development of robust expression systems for influenza polymerase trimer, as an example, have made it possible to use X-ray crystallography and potentially cryo-electron microscopy to provide molecular visualization of binding pockets for small molecule inhibitors, entry and exit channels for substrate(s), and potential new ways to disrupt domain interactions. These structural insights will tremendously aid the development of new drugs as well as to further elucidate the mechanisms of action and binding of existing drugs to their protein targets. Molecular modeling is also a useful approach that we and others have used to rationalize the differences in selectivity of lumicitabine against RSV and HCV polymerase.[159] More studies such as these ones will be needed to rationally design new nucleotide analogs targeting respiratory virus polymerases.

In the past, many nucleoside analogs failed during development for safety/toxicity reasons, especially molecules with suboptimal specificity for their viral polymerase target and those used for chronic treatment of infections such as HIV and HCV. In the context of acute respiratory infections, evaluation of safety must be based on both the intended duration of treatment and the targeted patients, which sometimes include vulnerable populations such as children and the elderly. Other considerations to ensure successful future development of nucleoside analogs directed against respiratory infections will be to optimize delivery to the lung by evaluating different routes of administrations, including aerosol formulations, and developing lung-targeting nucleoside prodrugs. Despite these challenges, the prospect of developing nucleoside analogs directed against respiratory RNA virus infections represents an exciting new avenue in antiviral research.

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