Review

New antiviral approaches for respiratory syncytial virus and other mononegaviruses: Inhibiting the RNA polymerase

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ABSTRACT

Worldwide, respiratory syncytial virus (RSV) causes severe disease in infants, the elderly, and immunocompromised people. No vaccine or effective antiviral treatment is available. RSV is a member of the non-segmented, negative-strand (NNS) group of RNA viruses and relies on its RNA-dependent RNA polymerase to transcribe and replicate its genome. Because of its essential nature and unique properties, the RSV polymerase has proven to be a good target for antiviral drugs, with one compound, ALS-8176, having already achieved clinical proof-of-concept efficacy in a human challenge study. In this article, we first provide an overview of the role of the RSV polymerase in viral mRNA transcription and genome replication. We then review past and current approaches to inhibiting the RSV polymerase, including use of nucleoside analogs and non-nucleoside inhibitors. Finally, we consider polymerase inhibitors that hold promise for treating infections with other NNS RNA viruses, including measles and Ebola.

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1. Introduction

1.1. The burden of RSV infection: an unmet medical need

Respiratory syncytial virus (RSV) causes seasonal infections that usually last 1—2 weeks and result in mild cold-like symptoms in the majority of otherwise healthy adults. The RSV season occurs during winter months in regions with temperate climates in the Northern and Southern Hemispheres (Yusuf et al., 2007). However, RSV infection can lead to severe lower respiratory infection in vulnerable populations such as infants, elderly and immunocompromised persons. In 2005, an estimated 33.8 million episodes of RSV occurred worldwide in infants younger than 5 years old. Of these, at least 3.4 million severe cases of lower respiratory infection required hospitalization, and an estimated 66,000 to 199,000 deaths occurred, mostly in the developing world (Nair et al., 2010). Severe RSV infections have also been documented among elderly patients. RSV infection in the elderly results in approximately 177,000 hospital admissions and approximately 10,000—14,000 deaths per annum in the United States. Costs associated with hospitalization are estimated to exceed $1 billion annually (Falsey et al., 2005; Falsey and Walsh, 2005; Thompson et al., 2003). No vaccines are approved for the prevention of RSV infection. Palivizumab, a monoclonal antibody directed against RSV, is approved only for prophylaxis to prevent serious lower respiratory tract disease caused by RSV in high-risk infants, but therapeutic efficacy has not been established (Homaira et al., 2014). Treatment of infants suffering severe RSV bronchiolitis is thus supportive, consisting of oxygen therapy, nutrition, and fluids. Ribavirin was approved in 1986 for aerosol treatment of serious RSV infections in hospitalized children. However, its use is marginal due to the cumbersome route of administration, concern for potential toxicity for exposed medical personnel, and lack of clear efficacy data (Broughton and Greenough, 2004). Therefore, novel therapeutics are needed for use both in the outpatient setting, to reduce the severity of infection and prevent hospital admissions, and in the hospital setting, to ameliorate the severity of symptoms and duration of time spent in the hospital. Since only a few agents for the treatment of RSV infection are in early stage of clinical development, it is important to continue to identify and characterize possible targets for intervention with antiviral drugs.

1.2. The RSV replication cycle

RSV is a member of the family *Pneumoviridae* (previously a subfamily within the Paramyxoviridae (Afonso et al., 2016)) in the order *Mononegavirales,* the non-segmented negative-strand (NNS) RNA viruses. As such, RSV is a relatively simple virus, with a single-stranded RNA genome of just over 15 kilobases. RSV consists of two antigenic subtypes, A and B, subtype A being often associated with the more severe symptom manifestations. The infection cycle begins when RSV interacts with a receptor at the cell surface and fuses its envelope with the plasma membrane (Collins et al., 2013) (Fig. 1). This process delivers the viral nucleocapsid into the cytoplasm of the cell. The nucleocapsid consists of the viral genome RNA, encapsidated along its length with the nucleoprotein (N) to form a helical structure (Bakker et al., 2013; Maclellan et al., 2007; Tawar et al., 2009), and associated with the viral RNA-dependent polymerase, a complex comprising the viral large polymerase subunit (L), phosphoprotein (P), and a transcription factor, M2-1 (Garcia et al., 1993). Once the nucleocapsid has entered the cytoplasm, the polymerase transcribes the viral mRNAs and replicates the genome by generating a positive-sense RNA intermediate, the antigenome, which acts as a template for further genome RNA synthesis (Collins et al., 1984; Collins and Wertz, 1983). Newly
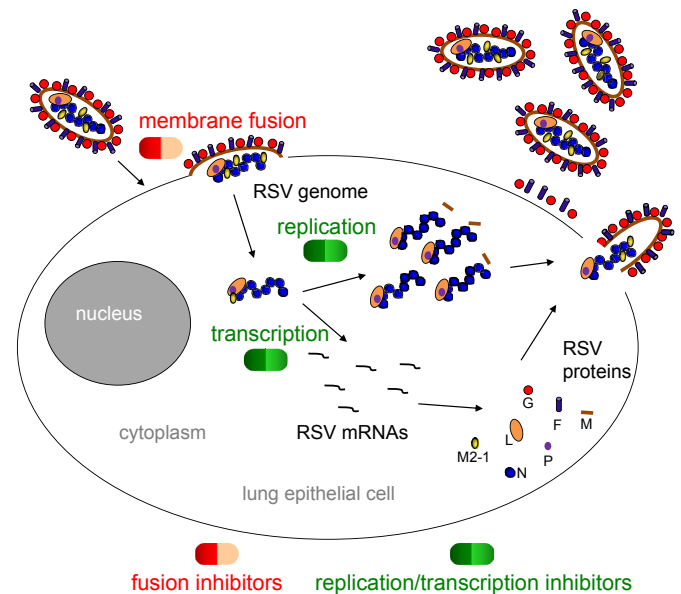

Fig. 1. **RSV replication cycle**. Schematic illustrating the RSV replication cycle. The attachment glycoprotein (G protein, red circle) binds to the chemokine receptor, CX3CR1, on the apical surface of ciliated epithelial cells (Johnson et al., 2015), and the fusion (F) protein (purple cylinder) mediates entry of the nucleocapsid into the cytoplasm (Melero and Mas, 2015). The N-RNA complex (chain of blue circles) serves as a template for the RSV polymerase complex, consisting of the L and P proteins (orange and purple ovals, respectively), to synthesize mRNAs and progeny genomes. Transcription also requires the M2-1 protein (yellow circle). The surface glycoproteins are synthesized, post-translationally modified, and transported through the endoplasmic reticulum and exocytic pathway (Collins and Mottet, 1991; Collins and Mottet, 1992; Olmsted and Collins, 1989). The matrix (M) protein (brown rod) associates with replication complexes in the cytoplasm and interacts with the cytoplasmic tail of the F protein to elicit formation of viral filaments (Baviskar et al., 2013; Ghildyal et al., 2002). This allows nucleocapsids to be packaged into filamentous virus particles, which are released by budding from the cell plasma membrane (Mitra et al., 2012). In addition to the proteins described above and shown in the figure, RSV expresses two non-structural proteins, NS1 and NS2, which act to hinder the innate immune response to infection (Teng, 2012). RSV also expresses M2-2, which is a negative regulator of transcription and, by analogy with the closely related human metapneumovirus, might serve to increase polymerase fidelity (Bermingham and Collins, 1999; Schickli et al., 2008). The SH protein is a viroporin that alters membrane permeability (Carter et al., 2010; Gan et al., 2008; Gan et al., 2012). The steps of the RSV life cycle that are targeted by fusion and replication/transcription inhibitors are highlighted.

synthesized genomes (and antigenomes) become encapsidated with N protein as they are synthesized and associate with the polymerase proteins to form nucleocapsids. Nucleocapsids are transported to the plasma membrane where they associate with other viral structural proteins, and virions are released by budding.

1.3. Current targets for new anti-RSV treatment candidates

Although every single step of the RSV replication cycle may represent a potential target for therapeutics, the two most common points of intervention are membrane fusion and RNA synthesis (Fig. 1, for review: (Roymans and Koul, 2010)). Small molecules that block RSV entry by inhibiting virus-cell fusion and cell-cell syncytium formation have been identified using in vitro RSV replication assays (Andries et al., 2003; Bonfanti et al., 2008; Cianci et al., 2004; Douglas et al., 2003). These compounds bind to a three-fold-symmetric pocket within the central cavity of the pre-fusion conformation of RSV F, blocking an important conformational change needed for membrane fusion (Battles et al., 2016; Samuel et al., 2015). Although many fusion inhibitors have impressive antiviral potency in the low-to sub-nanomolar range, most of them could not be advanced into clinical development due to suboptimal

pharmacokinetic properties or safety liabilities (Roymans and Koul, 2010). To date only one, GS-5806, has shown promise in recent early-phase clinical trials (DeVincenzo et al., 2014; Mackman et al., 2015) (for more detailed review on GS-5806: (De Clercq, 2015)). The efficacy and safety of GS-5806 is currently evaluated in hospitalized adults, including lung transplant and hematopoietic cell recipients (ClinicalTrials.gov Identifiers: NCT02135614, NCT02534350, NCT02254408, and NCT02254421).

Multiple approaches to block viral RNA synthesis during RSV replication have been described, including gene silencing by siRNAs (DeVincenzo, 2012) and targeting the nucleoprotein responsible for encapsidating the RNA (Challa et al., 2015; Chapman et al., 2007). One of the most amenable molecular targets for blocking RNA synthesis is the viral RNA polymerase. As described in detail below, the RSV polymerase is responsible for transcribing and replicating the viral genome, by catalyzing at least three enzymatic reactions: (1) RNA polymerization, for synthesis of the RNAs, (2) a poly-ribonucleotidyltransferase (or capping) activity, to add a guanosine cap at the 5′ end of mRNAs, and (3) a methyltransferase activity to methylate the cap. These activities are tightly coordinated to allow appropriate synthesis of mRNAs and full-length replicative RNAs. This coordination is essential not only to produce functional RNA species, but also to avoid generation of abortive RNAs, either uncapped or double-stranded, that could act as stimulators of the host innate immune response. Thus, the polymerase is an attractive prospect for antiviral drug development because of its essential nature, its multiple activities and target sites, and because a failure of the polymerase function may stimulate the innate immune response, potentially augmenting the antiviral effect.

2. Functions and structure of the RSV polymerase complex

2.1. Transcription of mRNAs

Each of the RSV genes is transcribed into a capped and poly-adenylated mRNA. Transcription of RSV mRNAs involves a complex interplay between the polymerase and conserved sequence elements at the boundaries of each of the genes. Fig. 2 shows the arrangement of *cis*-acting elements on the RSV genome, and the products that are generated during transcription. At the beginning of each gene is a 9- or 10-nucleotide *gene start* (*gs*) signal, and at the end is a 12- or 13-nucleotide *gene end* (*ge*) signal (Collins et al., 1986; Kuo et al., 1997). Between each gene is a short non-transcribed intergenic region, of varying length and sequence (Collins et al., 1986). At the 3′ and 5′ ends of the genome are extragenic regions, called the *leader* (*le*) and *trailer* (*tr*), respectively, which are important for initiation of RNA synthesis (Collins et al., 1991; Fearns et al., 2002; Mink et al., 1991; Peeples and Collins, 2000). The genome remains encapsidated throughout the replication cycle, and so the template for the polymerase is a ribonucleoprotein complex, consisting of the RNA wrapped around a chain of interlocking N molecules (Bakker et al., 2013; Tawar et al., 2009). Because of the intimate association of RNA with N protein, the N chain must be transiently displaced to allow the RNA to enter the polymerization active site of the polymerase. Probably for this reason, the polymerase can only initiate RNA synthesis from the 3′ end of the genome, and not at the beginning of individual genes.

The *le* region is 44 nucleotides in length and contains a sequence almost identical to a *gs* signal at positions 3—12 relative to its 3′ end, which is essential for all RNA synthesis (Fearns et al., 2002; Tremaglio et al., 2013). To transcribe the genome, the polymerase begins at position 3, opposite to the *gs*-like template sequence, and first transcribes a short uncapped RNA transcript, which is variable in length, but approximately 25 nucleotides (Tremaglio et al., 2013) (Fig. 2). Having released this RNA, the polymerase remains attached

to the template and can scan to the *gs* signal for the first gene, *NS1*, where it reinitiates RNA synthesis (Kuo et al., 1996). Shortly after reinitiating RNA synthesis, the polymerase adds a guanosine cap and then elongates the RNA until it reaches a *ge* signal (Kuo et al., 1996; Barik, 1993; Liuzzi et al., 2005). The *ge* signal causes the polymerase to reiteratively stutter on the U-tract that it contains and this enables the polymerase to polyadenylate the 3′ end of the mRNA (Harmon et al., 2001). The polymerase then releases the mRNA and scans the genome until it recognizes another *gs* signal, where it reinitiates RNA synthesis (Kuo et al., 1997; Fearns and Collins, 1999a). By continuing to respond to the *gs* and *ge* signals as it moves from the 3′ to the 5′ end of the genome, the polymerase generates a series of capped and polyadenylated mRNAs, corresponding to each of the viral genes. Because the polymerase always begins transcription at the 3′ end of the genome, and because it has a tendency to dissociate from the genome at the gene junctions, the genes at the 3′ end of the genome are expressed at a somewhat higher level than those at the 5′ end, leading to differential gene expression (Aljabr et al., 2016; Barik, 1992; Dickens et al., 1984).

2.2. Capping of mRNAs

Most of the research on NNS RNA virus capping has been performed with vesicular stomatitis virus (VSV), a member of the family *Rhabdoviridae*, but the mechanisms involved are thought to be conserved with RSV and other NNS RNA viruses. Messenger RNAs of NNS RNA viruses require a methylguanosine cap to be recognized by the cellular translation machinery (Lodish and Rose, 1977; Muthukrishnan et al., 1975) and to remain concealed from cellular pattern recognition receptors that signal innate immune responses (Hyde and Diamond, 2015). Furthermore, cap addition occurs co-transcriptionally and is necessary for the polymerase to elongate the RNA and respond appropriately to downstream *ge* and *gs* signals (Li et al., 2008; Neubauer et al., 2016; Ogino, 2014; Galloway and Wertz, 2008; Li et al., 2009; Rose et al., 1977). VSV capping occurs in two stages: (1) addition of guanosine to the 5′ end of the RNA by a 5′-5′ triphosphate linkage to form GpppRNA and (2) addition of two methyl groups, one at the ribose 2′O position of the first nucleoside of mRNA and one on the N7 group of the guanosine cap (Li et al., 2006) (Fig. 3A). Cap addition and methylation are dependent on interaction between the polymerase and the conserved sequence at the 5′ end of each mRNA (the complement of the *gs* signal) (Ogino et al., 2005; Rahmeh et al., 2009; Stillman and Whitt, 1999; Wang et al., 2007) and the biochemistry underlying these reactions is distinct from those of cellular enzymes. Cellular mRNAs are capped by a guanylyltransferase activity, in which the capping enzyme binds covalently to the guanosine monophosphate moiety of GTP and transfers GMP to an RNA containing a 5′-diphosphate end. In contrast, VSV mRNAs are capped by an RNA:GDP polyribonucleotidyltransferase (PRNTase) activity in which the polymerase binds covalently to a 5′-monophosphorylated RNA and transfers it to the GDP moiety of GTP (Ogino and Banerjee, 2007, 2010) (Fig. 3B). This is also likely the case for RSV for which it was also shown that GDP is transferred on the 5′ end of the mRNA (Barik, 1993). Whereas cellular mRNAs are methylated at the guanine-N-7 position before methylation at the 2′-O ribose by two distinct methylases, it has been shown that VSV polymerase contains a single binding site for the methyl donor, S-adenosylmethionine, and guanine-N-7 methylation occurs subsequent to, and is facilitated by, 2′-O methylation (Li et al., 2006; Rahmeh et al., 2009). Similarly, in studies with human metapneumovirus (a close relative of RSV) 2′-O methylation occurred prior to guanine-N-7 methylation, and in this case, can even occur prior to addition of the guanosine cap in an in vitro reaction (Paesen et al., 2015). Thus, while the caps at the 5′ ends of the viral mRNAs
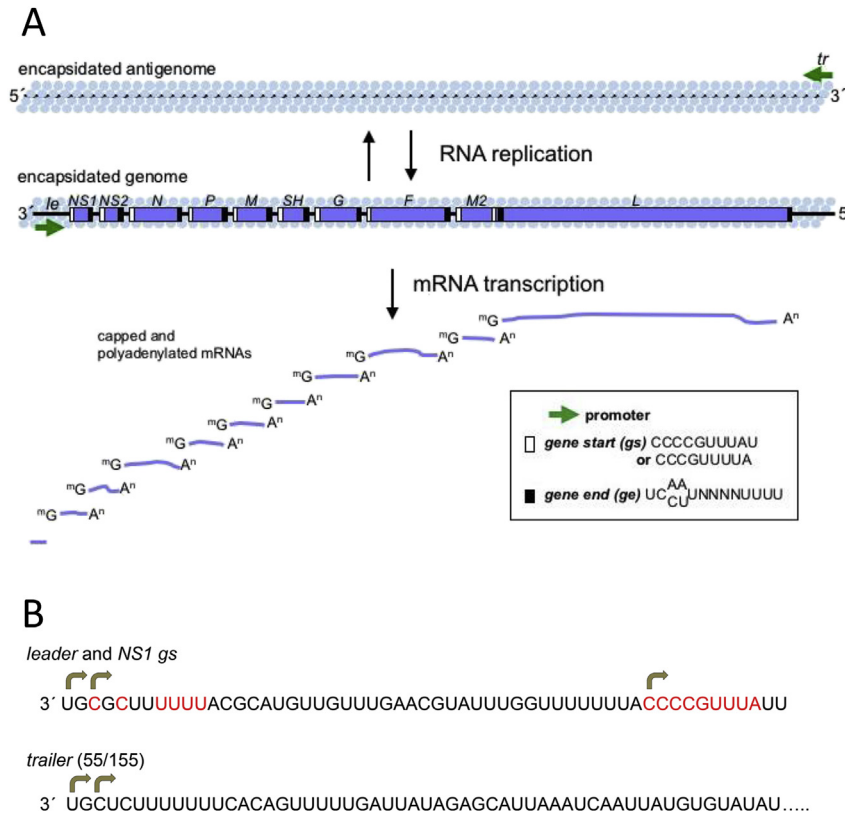
Fig. 2. Genome structure of RSV. A, Schematic of the RSV genome and its RNA products. The ten viral genes are shown as purple boxes and each is flanked with a conserved *gene start* signal (white box) and *gene end* signal (black box). At the 3′ end of the genome is the *leader* promoter region (*le*, green arrow). Transcription results in synthesis of eleven capped and polyadenylated mRNAs, corresponding to each gene, with the exception of the M2.1 and M2.2 proteins that are both transcribed from the same M2 gene. RNA replication results in production of an encapsidated antigenome. The *trailer* (*tr*) promoter at the 3′ end of the antigenome directs initiation of genome RNA synthesis. Both the genome and antigenome RNAs are encapsidated as they are synthesized and remain associated with N protein (blue circles) throughout the replication cycle. The *gene start* and *gene end* sequences are shown. B, Sequences of the leader and trailer promoter regions. The first 55 nucleotides of the genome and antigenome RNAs, containing the *leader* and *trailer* promoter regions, are shown (note that the entire *trailer* promoter region is 155 nucleotides). The core promoter element in the *leader* region, required for both transcription and replication, and the *gene start* signal for the *NS1* gene are shown in red type. The initiation sites in these sequences are indicated with gray arrows.

are biochemically indistinguishable from cellular caps, the capping machinery of the virus is distinct from that of the host, making it a potentially attractive target for antiviral drugs.

2.3. Genome replication

Genome replication differs from mRNA transcription in a number of ways. First, during replication the polymerase initiates opposite the first nucleotide of the *le* promoter rather than opposite the third nucleotide; second, the polymerase disregards the *gs* and *ge* signals as it moves along the genome; and third, the replicative RNA becomes encased with N protein as it is synthesized. These factors are probably interlinked resulting in replication products that are uncapped, unpolyadenylated, and encapsidated (Fig. 2). In a similar manner to transcription, replication initiation also depends on the *gs*-like sequence within the *le* region, but the polymerase initiates opposite the first nucleotide of the template, possibly by becoming pre-loaded with the initiating nucleoside triphosphates (NTPs) (Fearns et al., 2002; Noton et al., 2010; Noton and Fearns, 2011). Whereas during transcription, the polymerase is highly unprocessive in the *le* region, and can only elongate RNAs efficiently following cap addition, during RNA replication, the polymerase is super-processive and elongates to the end of the template to generate the antigenome (Fearns et al., 2002; Noton and Fearns, 2011; Cowton and Fearns, 2005; McGivern et al., 2005). The antigenome contains a promoter at its 3′ end that is very similar to that of the *le* promoter, and signals genome synthesis initiation by a

similar mechanism (Mink et al., 1991; Peeples and Collins, 2000; Noton et al., 2010). The antigenome and genome RNAs are encapsidated as they are synthesized, and the association of the nascent RNA with N protein is likely what causes the replicating polymerase to be processive (McGivern et al., 2005). Encapsidation depends on a supply of soluble N protein and so RNA replication does not occur until later in infection when N protein has reached a sufficiently high level. N protein is maintained in a soluble RNA-free form by association with P protein to ensure RNA binding specificity (Fearns et al., 1997; Murphy et al., 2003). Encapsidation depends on sequences throughout the first 35 nucleotides of the *le* region including 5′pppApC at the 5′ end of the RNA (Noton and Fearns, 2011; McGivern et al., 2005). Replicative RNA initiated at position 1 of the *le* and *tr* promoters contains 5′pppApC, but RNA initiated at position 3 during transcription initiation does not; one plausible mechanism for encapsidation initiation is that the RNA sequence at the 5′ ends of the antigenome and genome, including 5′pppApC, and other sequences encoded by the promoters, specifically interact with the N-P complex. This initial interaction could function as a nucleation event, with additional N protein being added by N-N oligomerization and nucleoprotein-RNA interactions. Alternatively, the polymerase may play a role in recruiting and delivering N protein to the nascent RNA (Qanungo et al., 2004).

2.4. Polymerase structure and assignment of functional domains

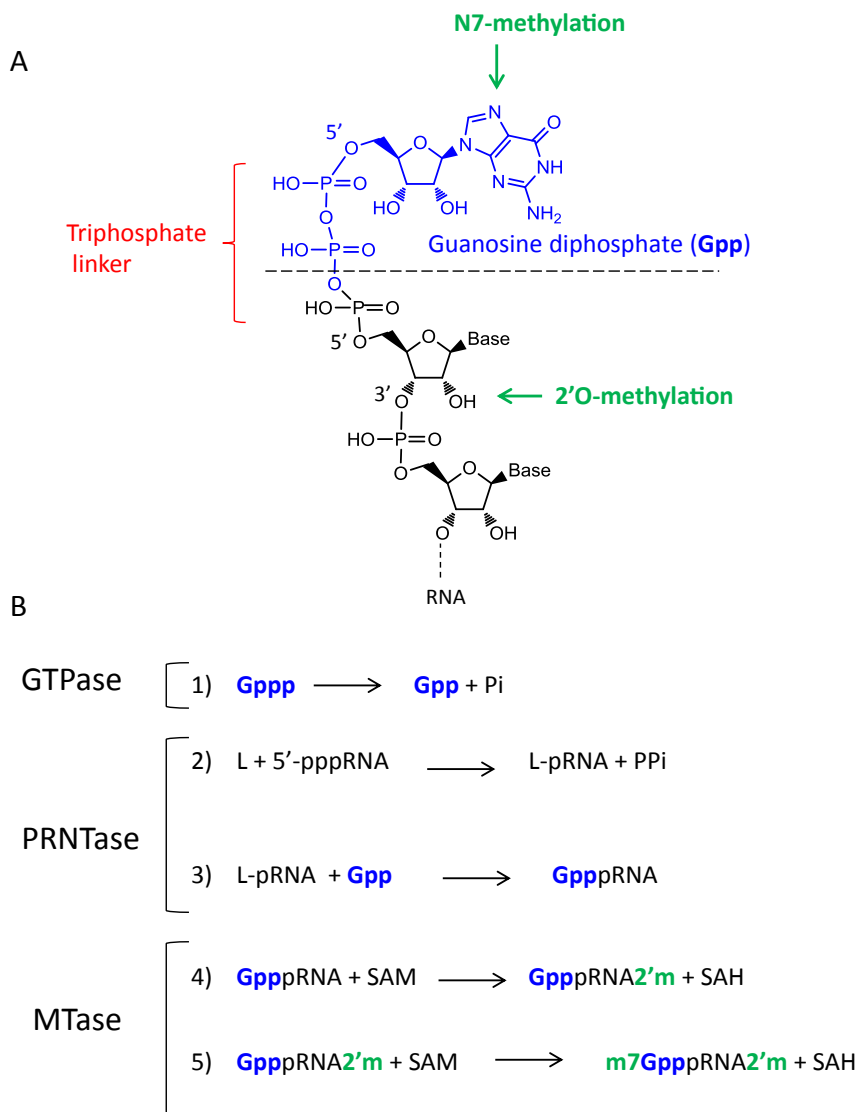The core RSV polymerase consists of L and P proteins of 250 and

Fig. 3. Capping of mRNA by NNS RNA viruses. A, Structure of the cap at the 5′-end of the viral mRNA. B, Proposed stepwise mechanism of RNA capping by RSV polymerase, based on vesicular stomatitis virus and other related viruses. Step 1: a molecule of GTP is hydrolyzed to GDP (blue). The transfer of a guanosine at the 5′-end of the nascent viral RNA is provided by the polyribonucleotidyltransferase (PRNTase) activity. Step 2: the L protein forms a covalent intermediate with the 5′-end of the nascent RNA after departure of pyrophosphate (PPi). Step 3: the GDP molecule attacks the phosphodiester bond between L and the RNA, forming the GpppRNA cap product. Step 4: the RNA is methylated (green) by the methyltransferase (MTase) domain of L, first at the 2′-position on the ribose of the first nucleotide, and then at the N7 of the first guanine in the cap structure. S-adenosyl methionine (SAM, or AdoMet) serves as a co-substrate of the MTase to transfer two methyl groups, leading to the formation of two molecules of S-adenosyl-L-homocysteine (SAH). For review: (Decroly et al., 2012).

27 kDa, respectively. L and P together with the N-RNA template, are sufficient for the polymerase to synthesize RNA and respond appropriately to *gs* and *ge* signals (Collins et al., 1995, 1996; Grosfeld et al., 1995; Mazumder and Barik, 1994; Noton et al., 2012; Yu et al., 1995). However, the L-P complex requires M2-1 as a transcription elongation factor to be fully processive (Collins et al., 1996), particularly for mRNAs longer than ~500 nucleotides (Fearns and Collins, 1999b; Mason et al., 2003). M2-1 is not required for RNA replication, but in this case elongation is facilitated by concurrent encapsidation, as described above. Thus, the viral components required for RSV transcription and replication are the N-RNA template, the core polymerase L-P, and M2-1 or N-P, respectively. Currently, high-resolution structures are available for the RSV N-RNA complex, M2-1, and a fragment of N bound to a peptide of P (Bakker et al., 2013; Tawar et al., 2009; Ouizougun-Oubari et al., 2015; Tanner et al., 2014). In addition, a structure for the complete

N-P complex of human metapneumovirus has been elucidated (Renner et al., 2016). As yet, the structure of the L-P complex remains unresolved. This has been difficult due to the large size of the L protein and the challenges of isolating sufficient quantities of highly purified protein. However, the structure of the VSV L protein in complex with a fragment of P protein has been resolved at high resolution (Liang et al., 2015) (Fig. 4A). In addition, a crystal structure of a C-terminal fragment of the human metapneumovirus L protein has been resolved, showing an overall conserved fold when compared with the MTase domain of VSV (Paesen et al., 2015) (Fig. 4B). These breakthroughs in structural studies, combined with sequence homology and mutational analyses, mean it is now possible to identify functional domains within the polymerase.

When the amino acid sequences of the L proteins of the NNS RNA viruses were first determined, sequence alignment identified six highly conserved regions, I to VI (Poch et al., 1990). In the three-
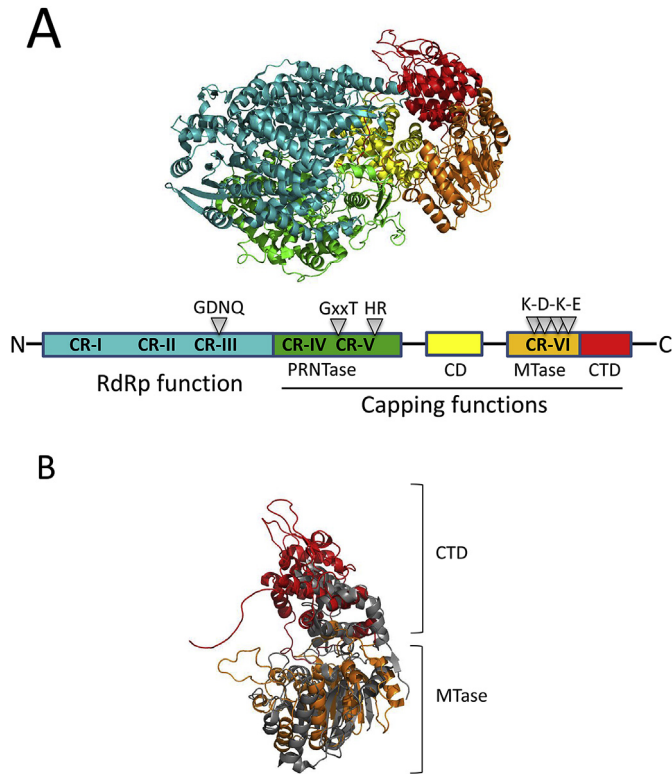
A

B

Fig. 4. Structure of RNA polymerases from viruses related to RSV. A, Structure of VSV RNA polymerase. The L protein of VSV carries the RdRp function in its N-terminal region (cyan), followed by the capping functions composed of the PRNTase (green) and the MTase (orange) activities. B, superimposition between the MTase (orange) and CTD (red) domains of HMPV and HMPV (gray). VSV protein structure is taken from PDB = 5a22 (Liang et al., 2015); HMPV protein structure is taken from PDB = 4ucl (Paesen et al., 2015).

dimensional structure of VSV L protein, conserved regions I-III comprise a "doughnut" structure, with the remainder of the L protein appearing as four globular appendages (Fig. 4A). Conserved regions IV and V form a single appendage that is folded over the hole of the doughnut. Conserved region VI and flanking sequence are a separate appendage. The two remaining appendages are referred to as a connector domain and a C-terminal domain, which comprise a poorly conserved sequence proposed to be involved in organizing the enzymatically active domains (Liang et al., 2015). The doughnut has a structure of a right hand, with fingers, palm and thumb domains, which is characteristic of RNA and DNA polymerases and contains catalytic motifs shared amongst RdRps (Noton et al., 2012; Poch et al., 1989; Morin et al., 2012). Conserved regions IV and V contain the residues required for addition of the guanosine cap. These include a GxxT motif, which is thought to be important for NTP binding, and an HR motif, in which the histidine becomes covalently bound to RNA (Fig. 4A) (Li et al., 2008; Neubauer et al., 2016; Ogino and Banerjee, 2007, 2010). Conserved region VI contains a motif similar to that of a ribose 2′O methyltransferase (Bujnicki and Rychlewski, 2002; Galloway et al., 2008), and the function of this appendage in methylation by VSV L protein has also been confirmed with biochemical assays (Fig. 4B) (Rahmeh et al., 2009; Paesen et al., 2015; Li et al., 2005). The similarity in structure between RSV and VSV polymerase is unknown. In any case, both proteins almost certainly undergo significant conformational rearrangement(s) following initiation of RNA synthesis to release the nascent RNA from the active site, and to facilitate functional interaction between the capping and methyltransferase domains and the RNA polymerization domain.

3. Inhibitors of RSV polymerase

Recombinant enzymes represent in general a major and well-defined class of protein targets for drug discovery campaigns. Although recombinant RSV polymerase can be purified in functional form (Noton et al., 2012), the use of purified RSV polymerase for high-throughput screening has not yet been reported. Nonetheless, several RSV inhibitors have been identified by screening compounds using infectious RSV, cell-based replicon assays, and an in vitro assay utilizing nucleocapsids isolated from RSV-infected cells. Follow-up studies on these compounds have identified a number of polymerase inhibitors, including both nucleoside and non-nucleoside inhibitors. The most extensively characterized nucleoside and non-nucleoside inhibitors are described below.

3.1. Nucleoside analogs

ALS-8112 − In an effort to discover new chemical scaffolds for RSV inhibitors, a series of sugar-modified nucleoside analogs was screened against the subgroup A serotype of RSV in a cell-culture virus replication assay (Wang et al., 2015). In parallel, the 5′-triphosphate counterparts were tested against crude isolates of RSV nucleocapsids containing the active viral RNA polymerase. The original scaffold identified from both assays was 2′difluoro-4′azido-cytidine, an analog of gemcitabine also known to inhibit hepatitis C virus (HCV) polymerase (Smith et al., 2009) (Fig. 5A). Further modifications at the 2′- and 4′- positions aimed to improve anti-RSV potency and selectivity. This work led to the identification of a series of novel 2′- and 4′-modified nucleosides, including ALS-8112 (Fig. 5A). ALS-8112 did not display any anti-cancer nor anti-HCV properties, which is different from gemcitabine and 2′difluoro-4′azido-cytidine. Instead, ALS-8112 inhibited a broad panel of RSV A and B subtypes, as well as related paramyxo- and rhabdoviruses (Deval et al., 2015). The polymerization function of the L protein was identified as the molecular target of ALS-8112 by selecting and characterizing drug resistance-associated mutations located in the L gene. In enzymatic assays, using purified recombinant polymerase, 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, since ALS-8112-TP did not inhibit polymerases from host or viruses unrelated to RSV such as HCV. To explain the unfavorable binding of ALS-8112-TP to the active site of HCV polymerase, it was proposed that the side chain of Asn291 may be responsible for the discrimination of the 4′ClCH$_2$ group of the nucleotide analog (Deval et al., 2015).

ALS-8176 - Because of the low oral bioavailability of ALS-8112, a series of 2′, 3′-diester prodrugs were evaluated for improved pharmacokinetic properties (Wang et al., 2015). One prodrug, ALS-8176 (Fig. 5B), formed high levels of monophosphate and triphosphate forms 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 (Deval et al., 2015). This represents a difference in RSV RNA titers of >4 log$_{10}$ copies/mL compared to vehicle-treated animals. Subsequently, a randomized, double-blind, clinical trial evaluated ALS-8176 given for 5 days to healthy adults inoculated with RSV (DeVincenzo et al., 2015). 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 1.3−2.3 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.
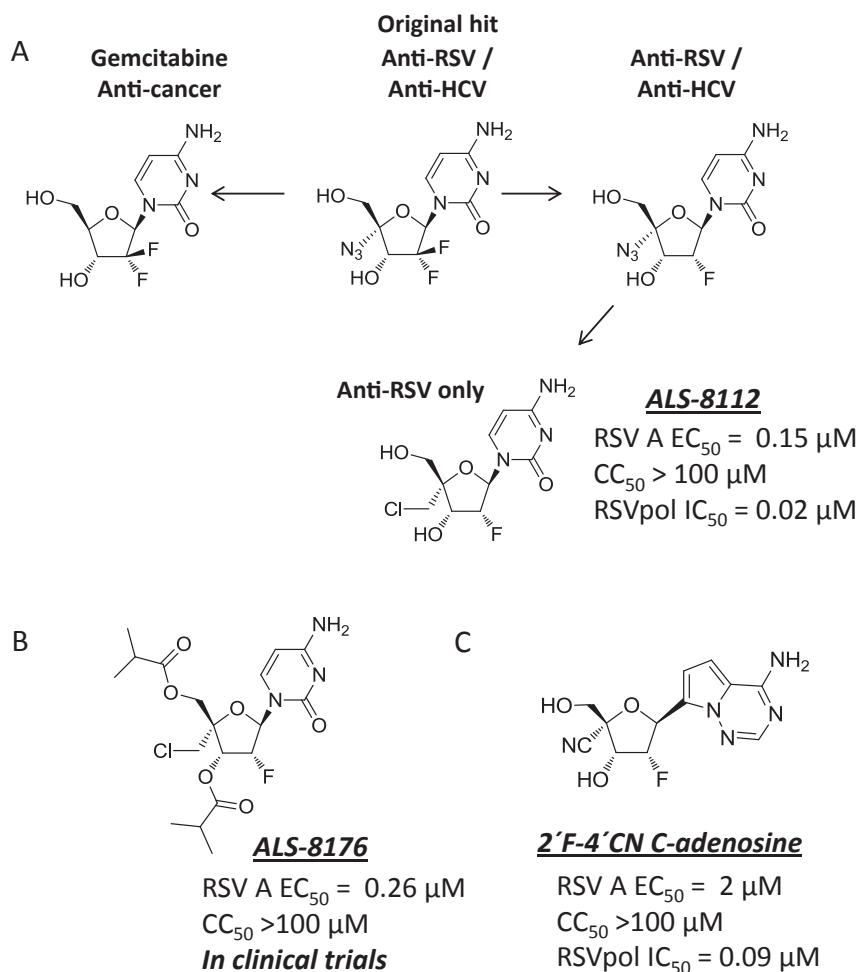
Fig. 5. 2′-fluoro nucleoside analogs inhibiting RSV polymerase. A, Identification of 2′difluoro-4′azido-cytidine as a dual inhibitor of HCV and RSV, leading to the discovery of ALS-8112 as a specific inhibitor of RSV (Smith et al., 2009). B, Structure and in vitro antiviral activity of ALS-8176. C, Structure and in vitro antiviral activity of the anti-RSV nucleoside analog 2′F-4′CN C-adenosine (Clarke et al., 2015).

This is an important result that represents the first proof-of-concept validation that an RSV polymerase inhibitor can be efficacious in the clinic.

Resistance to ALS-8112 and ALS-8176 - After 32 in vitro passages of RSV in the presence of ALS-8112, four mutations were selected within the region of the L gene of RSV encoding the polymerization function (Deval et al., 2015). These four amino acid mutations (M628L, A789V, L795I, and I796V) were associated with resistance to ALS-8112 and ALS-8176. In biochemical assays, the presence of the substitutions in recombinant L-P enzyme caused an increase in discrimination of ALS-8112 triphosphate relative to natural CTP. The resistance phenotype provided by the mutations was conferred by the $4'ClCH_2$ moiety of ALS-8112 triphosphate, with other 4′-halomethyl derivatives also being affected (Deval et al., 2016). This resistance pattern is consistent with the fact that three of the four amino acid changes are positioned in the vicinity of conserved region III, which contains the conserved residues $_{810}$-GDNQ-$_{813}$ responsible for nucleotide incorporation by NNS RNA virus polymerases (Fig. 6) (Malur et al., 2002; Sleat and Banerjee, 1993). Although the resistance mutations were identified and characterized from in vitro selection experiments, no resistant viruses have been identified in any nasal wash samples from RSV-infected individuals treated with ALS-8176 as part of the human challenge study, indicating that ALS-8176 exhibits a high barrier to viral resistance.

Fig. 6. Putative position of ALS-8112-resistance mutations in RSV polymerase. A homology-based three-dimensional structure of the RdRp domain of the RSV L protein (blue) was generated using the VSV L protein (cyan, PDB = 5a22). The proposed positioning of residues 628, 789, 795, and 796 from the RSV polymerase model is shown in red, and the active site residues GDNQ are indicated with an orange asterisk.

Other chemical series with a 2′fluoro group — Following up on the previously identified 2′fluoro nucleosides, a new series of C-linked nucleosides have been reported (Clarke et al., 2015). One of

these molecules, (2'deoxy-)2'fluoro-4'-cyano-5-aza-7,9-dideaza adenosine (2'F-4'CN C-adenosine, Fig. 5C) inhibited RSV replication in tissue cultu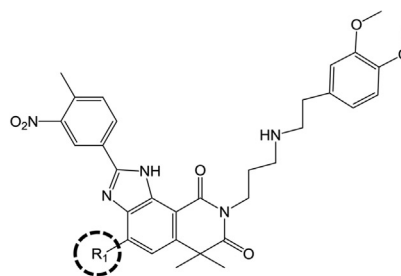re experiments, and its triphosphate form also inhibited the RNA polymerization activity of RSV polymerase. This molecule displayed high selectivity against incorporation by mitochondrial DNA and RNA polymerases, while other analogs were less selective. The potential for these compounds to be further developed and tested for in vivo efficacy will depend on their capacity to form high levels of NTPs in lung tissues upon oral dosing in animals and humans, which remains unknown. Additionally, escape mutations associated with resistance to 2'F-4'CN C-adenosine from in vitro RSV passaging experiments have not yet been reported.

3.2. Non-nucleoside inhibitors

BI-compound D - A few years ago, the ingenious adaptation of an enzyme assay designed to capture transcripts in a microplate format uncovered a series of RSV polymerase complex inhibitors (Mason et al., 2004). The principle of the assay is simple: a crude isolate of RSV nucleocapsids is incubated in a transcription buffer containing radiolabeled NTP. The radiolabeled polyadenylated mRNA products of the RSV polymerase (Long strain) are captured by strand complementation using an oligo(dT) probe coupled to biotin. Using this approach, a weak inhibitor of RSV transcription, designated BI-compound A, was identified. Several analogs, such as BI-compound D (BI-D), were synthesized to optimize the inhibition potency by about 50-fold compared to BI-compound A (Fig. 7A) (Liuzzi et al., 2005). Some of the analogs in the imidazoquinolines family not only prevented replication of infectious particles in cell culture, but also inhibited the RSV polymerase-dependent transcription of a minigenome. However, the in vivo antiviral effect in a murine model was very modest. Three amino acid residues, originally selected in vitro with BI-compound C, confer resistance to this series of inhibitors. They are located within conserved region V of the L protein, the domain responsible for cap addition (Fig. 7B) (Liuzzi et al., 2005). Experiments studying the mechanism of inhibition by BI-D showed that it inhibited full-length mRNA synthesis and instead caused the production of aborted ~40–50 nucleotide length transcripts containing a 5' triphosphate moiety (Liuzzi et al., 2005), consistent with a capping defect. In addition, BI-D has been shown to affect polymerase processivity within the promoter region (McCutcheon et al., 2015), indicating it has multiple effects on polymerase function. Interestingly, RSV-infected cells treated with BI-D secreted higher levels of interferon and expressed higher levels of interferon-stimulated genes than infected untreated cells, despite the fact that untreated cells contained considerably more RSV RNA (McCutcheon et al., 2015). This suggests that the abortive RSV transcripts produced in the presence of BI-D potentiated the interferon response, probably through the RIG-I pathway. Thus, inhibitors that target the capping activity of the RSV polymerase have the potential to activate the innate immune response in addition to inhibiting the polymerase, which could increase their potency. However, further evaluation is necessary to determine if such inhibitors cause increased immunopathology.

YM-53403 - In cases where it is not possible to implement high-throughput enzymatic assays, cell-based assays remain a viable option for screening of large number of compounds. Resistance selection and target validation by reverse genetics are used later on for validating the mechanism of action of newly discovered inhibitors. A good example of this approach is YM-53403 (Fig. 8A), an inhibitor of RSV from a benzazepine series discovered by measuring the cytopathic effect associated with virus replication (Sudo et al., 2005). In this cell-based high-throughput screening campaign,

BI-compound D: $R_1 = NH_2$
RSV A EC_{50} = 0.021 / 0.32 μM
RSV B EC_{50} = 0.25 μM
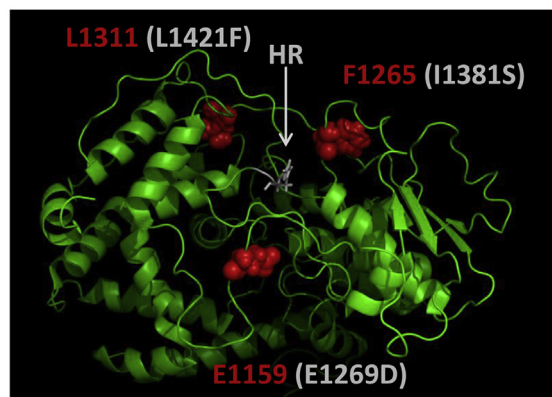CC_{50} = 9.3 μM
BI-compound C: $R_1 = H$

Fig. 7. Non-nucleoside inhibitors of RSV polymerase (Part 1). A, Structure and in vitro antiviral activity of the anti-RSV non-nucleoside inhibitors BI-compound D (Liuzzi et al., 2005) and (Tiong-Yip et al., 2014). B, Proposed position of amino acids responsible for the resistance to BI compounds in the capping domain of RSV polymerase. Resistance to BI-compound C has been associated with mutation E1269D, I1381S, and L1421F in RSV L, corresponding respectively to residues E1159, F1265, and L1311 in VSV L capping domain (side chains shown in red) (Liuzzi et al., 2005). The side chain of the catalytic histidine from the HR motif in VSV L capping domain is shown in gray.

some compounds identified in the primary screen were up to 100-fold more potent against RSV than ribavirin. YM-53403 emerged as one of the most promising molecules from this series. Although it was originally reported to be active against RSV subtype B (Sudo et al., 2005), this result could not be confirmed in a subsequent study (Tiong-Yip et al., 2014). When cells were repeatedly infected in the presence of YM-53403, the selection of a single mutation Y1631H within the L gene made the mutated virus completely insensitive to the inhibitory effect of YM-53403. Although no detailed mechanistic studies have been reported, in an in vitro assay using recombinant RSV polymerase, YM-53403 inhibited RNA synthesis from a promoter template (McCutcheon et al., 2015), indicating that it has a similar mechanism of inhibition as one of its derivatives, AZ-27, described below.

AZ-27 - Other derivatives of YM-53403 were synthesized to increase the inhibitory potency against RSV (Xiong et al., 2013). In particular, the compound AZ-27 (Fig. 8A) had an EC_{50} = 0.01 μM against RSV subtype A and an EC_{50} = 1.3 μM against RSV subtype B (Tiong-Yip et al., 2014). The in vitro antiviral potency of AZ-27 was specific to RSV, with no inhibition observed against metapneumovirus, influenza A virus, human rhinovirus, or
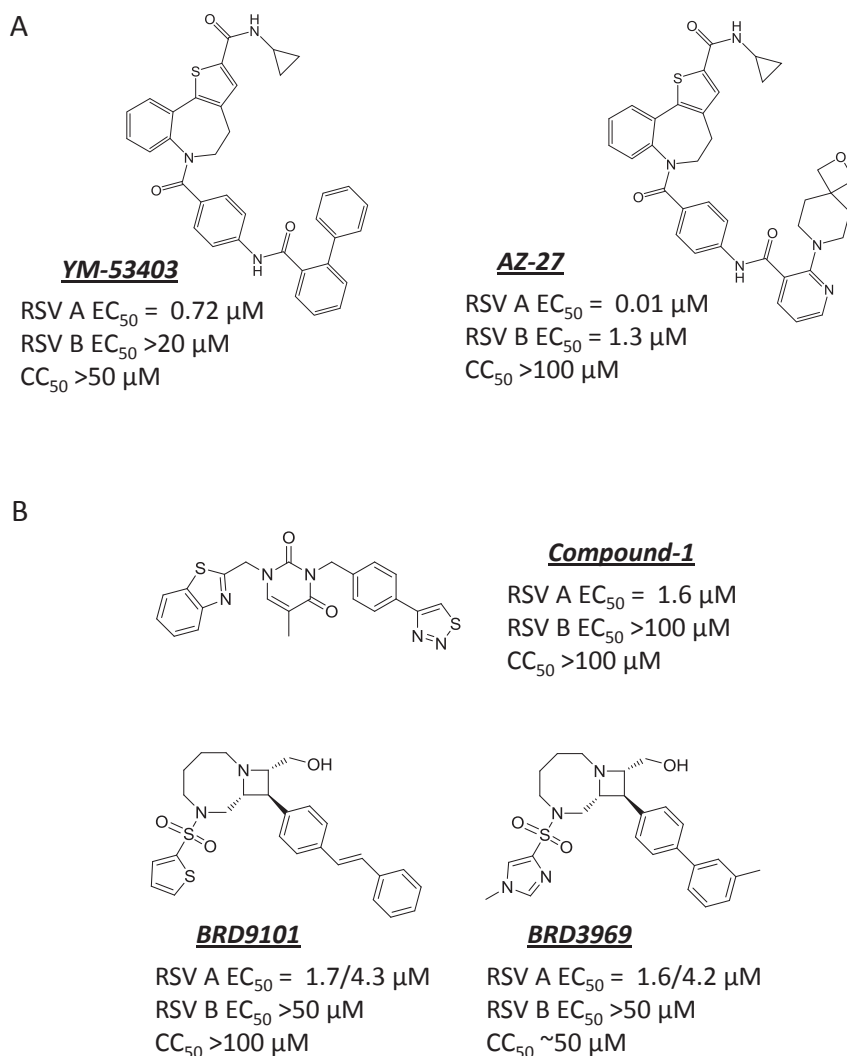
A

YM-53403

RSV A EC$_{50}$ = 0.72 μM
RSV B EC$_{50}$ >20 μM
CC$_{50}$ >50 μM

AZ-27

RSV A EC$_{50}$ = 0.01 μM
RSV B EC$_{50}$ = 1.3 μM
CC$_{50}$ >100 μM

B

Compound-1

RSV A EC$_{50}$ = 1.6 μM
RSV B EC$_{50}$ >100 μM
CC$_{50}$ >100 μM

BRD9101

RSV A EC$_{50}$ = 1.7/4.3 μM
RSV B EC$_{50}$ >50 μM
CC$_{50}$ >100 μM

BRD3969

RSV A EC$_{50}$ = 1.6/4.2 μM
RSV B EC$_{50}$ >50 μM
CC$_{50}$ ~50 μM

Fig. 8. Non-nucleoside inhibitors of RSV polymerase (Part 2). A, Structure and in vitro antiviral activity of the anti-RSV non-nucleoside inhibitors YM-53403 (Sudo et al., 2005), and AZ-27 (Tiong-Yip et al., 2014). All EC$_{50}$ and CC$_{50}$ values are reported from (Tiong-Yip et al., 2014). B, Structure and in vitro antiviral activity of the anti-RSV non-nucleoside inhibitors Compound-1 (Laganas et al., 2014), BRD9101 and BRD3969 (Duvall et al., 2016). For BRD9101 and BRD3969, the first EC$_{50}$ value is from a CPE reduction infectious assay, and the second from a sub-genomic replicon assay.

cytomegalovirus. Experiments performed with the RSV mini-genome and recombinant polymerase complex showed that AZ-27 inhibited the initiation of *de novo* RNA synthesis at the promoter, resulting in inhibition of both mRNA transcription and genome replication to the same extent (Noton et al., 2015). The Y1631H mutation that had previously been identified with YM-53403 conferred cross-resistance to AZ-27 (Tiong-Yip et al., 2014), but not against nucleotide analogs (Deval et al., 2016). Instead, it was found that, when combined, the nucleoside analog ALS-8112 and AZ-27 acted in synergy to inhibit RSV replication. Y1631 lies in a poorly conserved linker region between the connector domain and the methyltransferase domain in conserved region VI. It is not present within the C-terminal crystal structure of HMPV L protein and this region was not traced on the VSV L structure because it is in a poorly ordered linker. Therefore, the mechanism by which AZ-27 inhibits RNA synthesis initiation remains elusive. While Y1631 is proximal to the RNA capping and methylation domains of RSV polymerase, it is conceivable that AZ-27 could interfere with one of these functions, although the data obtained so far clearly show that

the dominant inhibition effect is on RNA synthesis initiation (Noton et al., 2015).

Other molecules that induce the selection of the Y1631H mutation - Interestingly, the same Y1631H substitution was observed in an independent study where novel candidates were found by screening a sub-genomic RSV replicon against a library of about one million compounds (Laganas et al., 2014). One of the most potent inhibitors identified in this study was a thymine derivative, referred to as Compound-1 (Fig. 8B). Compound-1 is in a distinct structural class from YM-53403 and AZ-27 and induced the double selection of Y1631H and I1413T in the L protein. In this case, the Y1631H substitution was shown to be the most important for conferring resistance. As with YM-53403 and AZ-27, Compound-1 also showed greater potency against RSV subgroup A than against subgroup B viruses. Similar results were also obtained with related compounds from a separate series, BRD9101 and BRD3969 (Duvall et al., 2016) (Fig. 8B). The mechanisms of inhibition by Compound 1, BRD9101 and BRD3969 have not yet been determined, but the mechanism of inhibition of BRD3969 appears to be distinct from

that of YM-53403 and AZ-27 (Duvall et al., 2016). The fact that the same resistance mutation can be selected by multiple compound scaffolds in independent screening campaigns suggests that tyrosine 1631 may be in a "hot spot" for inhibition by small molecules, but that different molecules have different effects on polymerase activity. Alternatively, it is possible that this substitution causes structural alterations in the polymerase that allow it to perform its activities, but that prevent these small molecules from binding other sites.

4. Inhibitors of RNA polymerases of viruses related to RSV

The order *Mononegavirales* includes a number of important human pathogens including measles, mumps, parainfluenza, rabies, Ebola, and Marburg viruses. These viruses differ in terms of pathogenesis, tropism, immune evasion, and mechanisms of viral entry and assembly. However, the overall strategy of mRNA transcription and genome replication, described above for RSV, is likely to be conserved between all NNS RNA viruses. Furthermore, since the polymerase regions directly involved in enzymatic activities are conserved, the structural elements and biochemical mechanisms underlying RNA polymerization, cap addition, and cap methylation are likely to be similar. Studies on measles, Marburg, and Ebola viruses have identified additional inhibitors, some of which are broad spectrum and have activity against RSV as well. The following section reviews molecules either inhibiting a specific NNS RNA virus polymerase other than that of RSV, or that display broad anti-*Mononegavirales* polymerase activity.

4.1. Measles polymerase inhibitors

ERDRP-0519 – Measles virus RNA polymerase inhibitors have been discovered by screening a library of >30,000 molecules (Ndungu et al., 2012; Sun et al., 2008; White et al., 2007). This screening campaign used recombinant viruses containing a reporter gene, which presented several advantages compared to wild type virus, such as the ease of use and the strength of the signal. Again, the absence of biochemical or structural data was compensated by reverse genetics experiments to determine the viral target and inhibitor binding site of structurally related compounds such as AS-136a and ERDRP-0519 (Krumm et al., 2014; Yoon et al., 2009) (Fig. 9A). Prolonged incubation of measles-infected cells in the presence of these small molecules induced the selection of multiple resistance mutations, including a T776A mutation in the L gene of measles. The mutated residue is in close proximity to the GDNQ motif within conserved region III of the L protein that is responsible for RNA polymerization activity. The ERDRP-0519 inhibitor is active in vivo in a ferret model of infection (Krumm et al., 2014). The precise mechanism of inhibition of these molecules remains to be determined. For example, it is not known whether they interact with the polymerase at the time of initiation or elongation of RNA synthesis. In any case, this result is an important step towards demonstrating that it is possible to achieve strong in vivo efficacy using non-nucleoside molecules targeting the RNA polymerase of a paramyxovirus related to RSV and other pneumoviruses.

4.2. Filovirus polymerase inhibitors

T-705 - The nucleoside precursor 6-fluoro-3-hydroxy-2-pyrazinecarboxamide (T-705, favipiravir) was originally developed against influenza virus (for review: (Furuta et al., 2013)). The molecule has also been shown to inhibit a number of other RNA viruses including members of *Orthomyxo-*, *Noro-*, *Bunya-*, *Arena-*, and *Flaviviridae* (Furuta et al., 2013). In vitro, the antiviral potency of T-705 against Ebola replication is very weak, with an EC_{50} value

of 67 μM (Fig. 9B) (Oestereich et al., 2014). Nonetheless, treatment with T-705 given orally resulted in 100% survival in a lethal Ebola-infected mouse model (Oestereich et al., 2014; Smither et al., 2014). It has been proposed that T-705 exerts its activity by targeting viral polymerases, although this has not been demonstrated in the case of Ebola virus. In cells, T-705 is efficiently converted to a ribofuranosyl 5′-triphosphate form (T-705 RTP) by cellular enzymes (Furuta et al., 2009). The RNA polymerases of influenza virus and norovirus recognize T-705 RTP as substrate during RNA synthesis, which results in enzyme inhibition by delayed chain termination (Furuta et al., 2005; Jin et al., 2013, 2015). It has also been suggested that T-705 causes error catastrophe as a consequence of introducing lethal mutations (Baranovich et al., 2013). Sissoko et al. recently reported the experimental use of T-705 in Ebola-infected patients during the peak of the 2014–2015 outbreak in Guinea (Sissoko et al., 2016). Although T-705 was well tolerated, it did not provide any significant antiviral effect. In this context, more work will be needed to understand the precise mechanism of action of T-705 against Ebola virus and its actual potential as an anti-Ebola agent, alone or in combination.

BCX4430 - BCX4430 is an adenosine analog with a C-nucleoside base modification and 1′-imino group on the sugar moiety (Fig. 9B). BCX4430 is also a broad-spectrum agent that shows weak in vitro antiviral activity not only against positive-strand RNA viruses such as rhinovirus ($EC_{50} = 3$ μM) and dengue virus ($EC_{50} = 33$ μM), but also against negative-strand RNA viruses such as influenza ($EC_{50} = 11$ μM), RSV ($EC_{50} = 11$ μM), Ebola ($EC_{50} = 11.8$ μM) and Marburg viruses ($EC_{50} = 4.4–6.7$ μM) (Warren et al., 2014). In a macaque lethal model of Marburg virus disease, treatment with BCX4430 resulted in 100% protection, even when administered 48 h post-infection. Similarly to what was observed with T-705, it is not known why BCX4430 is efficacious in vivo despite its weak in vitro inhibition potency. Based on biochemical experiments performed with recombinant HCV polymerase, it has been proposed that BCX4430 targets viral RNA polymerases and acts as a non-obligate chain terminator. BCX4430 is currently in Phase I clinical trials to evaluate safety, tolerability and pharmacokinetics in healthy volunteers (ClinicalTrials.gov Identifier: NCT02319772).

GS-5734 - Other C-linked nucleoside analogs have been reported recently as anti-Ebola agents (Warren, 2015). Adding a 1′-cyano group to a C-linked adenosine provided potency against Ebola virus polymerase and selectivity for viral polymerases (Fig. 9C). In tissue culture experiments, the parent nucleoside GS-441524 was moderately active against Ebola replication with EC_{50} values around 1.5 μM. Its weak antiviral activity may be attributed to inefficient intracellular phosphorylation, which was improved by adding a monophosphate prodrug to the parent nucleoside (Fig. 9C). The resulting compound, GS-5734, is a broad-spectrum inhibitor with efficacy against a variety of viruses, including RSV. It 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 (Warren et al., 2016). 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. Following Phase I safety testing in healthy human volunteers, GS-5724 was first given for compassionate use in October 2015 to an Ebola-infected patient when a nurse who had survived the disease developed a recurrence in the central nervous system (Jacobs et al., 2016). In conclusion, GS-5734 represents the first Ebola polymerase inhibitor to demonstrate robust therapeutic efficacy in a non-human primate model and reach evaluation of safety and efficacy in human clinical trials.
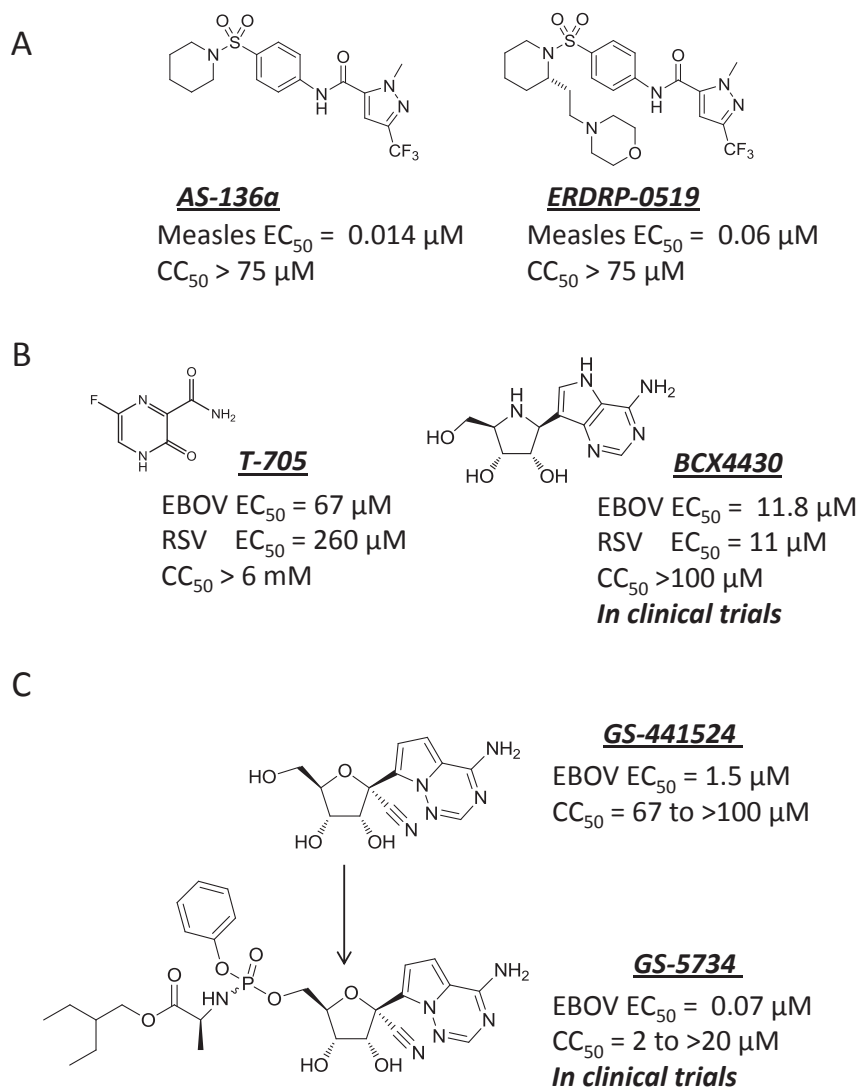
Fig. 9. Inhibitors of measles and Ebola virus polymerases. A, Structure and in vitro antiviral activity of the measles virus inhibitors AS-136a and ERDRP-00519 (Ndungu et al., 2012; Sun et al., 2008; White et al., 2007). B, Structure and in vitro antiviral activity of the Ebola virus inhibitors T-705 (Oestereich et al., 2014), and BCX4430 (Warren et al., 2014). C, Structure and in vitro antiviral activity of the Ebola virus inhibitors GS-441524 and GS-5734 (Warren et al., 2016).

5. Conclusion and future directions

In this review, we have summarized the efforts taken to develop inhibitors of NNS RNA virus polymerases, focusing mainly on RSV for which most molecules have been identified. We have discussed inhibitors of RSV RNA polymerase that are either nucleoside analogs targeting the active site of the polymerization domain, or non-nucleoside inhibitors binding to other regions of the polymerase. Currently, the most clinically advanced drug candidate for RSV infection is the nucleoside analog ALS-8176, which acts as a chain terminator of RNA synthesis. In general, the mechanism of action of non-nucleoside inhibitors of RSV polymerase is less well understood. Given that the polymerase likely undergoes multiple conformational changes to initiate RNA synthesis, elongate, cap, and methylate the RNA, non-nucleoside inhibitors might have allosteric effects that impact multiple activities. The latest Ebola virus crisis has served as a reminder that it is extremely challenging to conduct antiviral drug discovery in response to a rapidly expending epidemic and has highlighted the need for continued research on the polymerases of a variety of human pathogens. The recent Ebola drug discovery efforts also highlighted that its viral RNA polymerase represents a viable target for small molecule inhibitors. The fact that inhibitors such as GS-5734 are active against Ebola virus and RSV opens up the possibility that in future, inhibitors approved for use against a highly prevalent virus, such as RSV, might have the potential for being deployed against rare emerging threats, such as Ebola, Marburg and Nipah viruses.

Key future research directions to advance polymerase inhibitors as antiviral treatments include elucidation of the structures of the polymerase of RSV and other related human pathogens, including structures of the polymerase in association with ligands and at different stages of RNA synthesis. This is necessary to provide a better mechanistic understanding of modes of inhibition, and improve molecule design. In addition, the possibility of combining inhibitors for synergistic effects should be explored; for example, the combination of ALS-8112 with AZ-27 had a synergistic effect on inhibition of RSV replication in vitro (Deval et al., 2016). This combination approach might raise the barrier to resistance and increase antiviral efficacy. As more polymerase inhibitors progress into clinical trials, there will be greater opportunity to evaluate

synergistic effects between drugs and determine the likelihood of broad cross-resistance.

Acknowledgments

List of abbreviations

Term	Definition
AdoMet	S-adenosyl methionine
CC_{50}	half-maximal cytotoxic concentration
EC_{50}	concentration resulting in 50% of the maximum effective response
F	fusion protein
G	glycoprotein
GDP	guanosine diphosphate
ge	gene end
gs	gene start
GTP	guanosine triphosphate
HCV	hepatitis C virus
HMPV	human metapneumovirus
IC_{50}	concentration resulting in 50% of the maximum inhibition
L	large polymerase subunit
le	*leader* promoter
M	matrix protein
MTase	methyltransferase
N	nucleoprotein
NNS	non-segmented negative sense
NTP	nucleoside triphosphate
P	phosphoprotein
PPi	pyrophosphate
PRNTase	polyribonucleotidyltransferase
RdRp	RNA-dependent RNA polymerase
RSV	respiratory syncytial virus
RT	reverse transcriptase
RTP	ribofuranosyl 5′-triphosphate
SAH	S-adenosyl-L-homocysteine
SAM	S-adenosyl methionine
TP	triphosphate
tr	*trailer* promoter
VSV	vesicular stomatitis virus

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