Inhibition of viral RNA-dependent RNA polymerases with clinically relevant nucleotide analogs

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Abstract

The treatment of viral infections remains challenging, in particular in the face of emerging pathogens. Broad-spectrum antiviral drugs could potentially be used as a first line of defense. The RNA-dependent RNA polymerase (RdRp) of RNA viruses serves as a logical target for drug discovery and development efforts. Herein we discuss compounds that target RdRp of poliovirus, hepatitis C virus, influenza viruses, respiratory syncytial virus, and the growing data on coronaviruses. We focus on nucleotide analogs and mechanisms of action and resistance.

1. Introduction

The fight against emerging and existing viral infections is necessarily multifactorial. The economic and personal burden of viral infections is well understood and established, especially in the case of seasonally fluctuating or chronic illnesses [1–10]. Lessons from both recent and ongoing outbreaks of previously uncharacterized viruses highlight the need for development of an

array of countermeasures, both to curb ongoing situations as well as to prepare for future emergences of pathogens [11–16]. Most distressing is that, despite several recent outbreaks, the world was overall underprepared for handling a novel coronavirus. One of the potential tools in handling viral outbreaks are broad spectrum antiviral drugs capable of managing an array of viral infections. However, most of the approved direct acting antivirals are selective, with a mechanism of action tailored to a given protein target. Herein we focus on viral RNA–dependent RNA polymerases (RdRp) as a primary target for nucleotide analog inhibitors using poliovirus (PV), hepatitis C virus (HCV), influenza viruses, respiratory syncytial virus (RSV), and coronaviruses such as SARS-CoV-2 as highlighted examples. We describe important principles of viral RdRp inhibition, defined mechanisms of action (MOA), drug resistance, and discuss the potential for the development of nucleotide analogs with a broader spectrum of activities.

Several barriers exist along the road of antiviral development. If a considerable overlap between a compound's given effects is seen between host and viral proteins, treatment may unsurprisingly cause more harm than good. Additionally, the degree of similarity between viral targets can define if any given compound is broad in its spectrum of activity. However, compounds that are more broad spectrum may also begin to affect similar host proteins [17,18]. Viruses can be classified based upon their genome structure and replication strategy (Fig. 1), with significantly different genes encoded from one family to another. Even among RNA viruses, only a single gene product is shared between all known members, the RdRp, responsible for copying viral genomic information [19,20]. Fundamentally, genome replication of an RNA virus requires a virally encoded RdRp, which is therefore a logical target when designing antiviral compounds. Additionally, the production of RNA from an RNA template is not a catalytic activity found in human polymerases, limiting the potential to affect any native cellular processes when targeting the viral protein [19]. However, the structure of RdRp enzymes or enzyme complexes can differ significantly among RNA viruses.

A defining feature of almost all RNA viruses is their relatively high error rate when compared to DNA viruses, partially due to a "sloppy" RdRp and generally a lack of proofreading capability [21,22]. There are few exceptions, including coronaviruses, that possess a proofreading $3'$ to $5'$ exonuclease activity [23]. A high error rate drives up the number of mutations per round of genome replication, allowing for high degrees of diversity and in turn adaptability. This increase in genetic diversity sets the stage for when antiviral treatment is applied, as resistant variants may emerge [24].
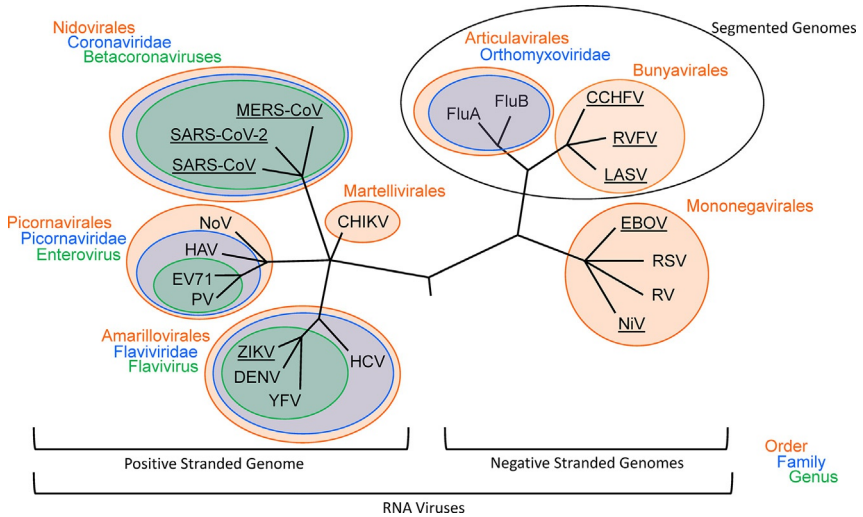
Fig. 1 Phylogenetic tree outlining relatedness between pathogenic RNA viruses. Relations are as outlined by the International Committee on Taxonomy of Viruses (ICTV). Groupings are shown at the levels of Orders, Families, and Genera. Viruses found on the WHO R&D Blueprint priority list are underlined. Abbreviations are as follows: FluA, influenza A virus; FluB, influenza B virus; CCHFV, Crimean-Congo hemorrhagic fever virus; RVFV, Rift Valley fever virus; LASV, Lassa virus; EBOV, Ebola virus; RSV, respiratory syncytial virus; RV, rabies virus; MERS-CoV, Middle East respiratory syndrome coronavirus; SARS-CoV, severe acute respiratory syndrome coronavirus; NoV, norovirus; PV, poliovirus; HAV, hepatitis A virus; ZIKV, Zika virus; DENV, dengue virus; HCV, hepatitis C virus; EV71, enterovirus 71; YFV, yellow fever virus; CHIKV, chikungunya virus; NiV, Nipah virus.

Application of drug treatment changes the selective pressures taking place between competing genotypes: where the wild type virus would have a fitness advantage without treatment, with treatment, mutants with a lower fitness at baseline that are less affected by a given drug are then able to propagate [24]. On the other hand, resistance is also important in understanding the mechanism of action of a given drug. Specific mutations give insight on specific drug–protein interactions, indicating which residues may be required for a molecule's activity. A useful step in the rational design of antivirals is therefore the determination of mechanisms of action as well as identifying possible resistance mutations.

2. Viral RNA-dependent RNA polymerases

The RNA-dependent RNA polymerase is responsible for viral genomic replication and transcription, making it a key player in the viral lifecycle [25]. Given the universal requirements for RdRps across all RNA viruses,

there are not only important similarities but also differences in structure and function of these enzymes [19]. Despite how these proteins are often defined by the polymerase activity found in the conserved RdRp domain, there may also be additional functional domains which are responsible for other catalytic activities [26–29]. The tertiary structure typically found in the core RdRp domain is commonly likened to a right hand, with distinct structurally conserved subdomains for the fingers, thumb, and palm, all constituting a common catalytic site of RNA synthesis [25,30–32]. Within the RdRp domain is a set of seven (A through G) sequence motifs [32]. These motifs play a direct role in the catalytic activity of the RdRp domain, generating a strong selection for sequence and structural conservation (Fig. 2) [32]. The RdRp motifs themselves can be found separated in the primary structure, with their residues found in both the finger and palm subdomains, with the palm housing the majority of the motifs (A through E) involved in NTP substrate and catalytic metal coordination for catalysis [32]. The thumb subdomain, conversely, lacks consistently observed RdRp motifs, but is commonly implicated in holding and positioning the nucleic acid substrate during catalysis [32].

The fingers of most RdRps contain motifs F and G with motif F residues forming the NTP entry channel. Motif G residues guide single-stranded RNA template toward the active site while the N-terminal region of the motif G facilitates the exit of the double-stranded RNA from the active site [33]. In some, the "fingertips," extended β strands, come in direct contact with the thumb, enclosing the active site and RdRp catalytic residues [25,32]. Significant changes can be associated with distinct conformations, indicating that the dynamic spatial relationship between individual subdomains and domains is required for function [32].

Regarding specific motifs, foremost is the triad of motif A, C, and D, which are considered critical for polymerase activity [25,30,32]. The mechanism of NTP incorporation into the growing RNA strand is metal ion-dependent, with two divalent metal ions coordinated by aspartate residues found in both motif A and C [25,30,32]. The same residues also are involved in positioning incoming NTPs [25]. Motif D also participates in the polymerase mechanism—the conserved lysine or histidine directly acts as a general acid, interacting with the PPi leaving group via deprotonation [25,34,35].

Motif B plays a significant role in discriminating between dNTPs and NTPs, ensuring the correct substrate is incorporated into the growing RNA strand [31]. Motif E also plays a similar role in facilitating interactions

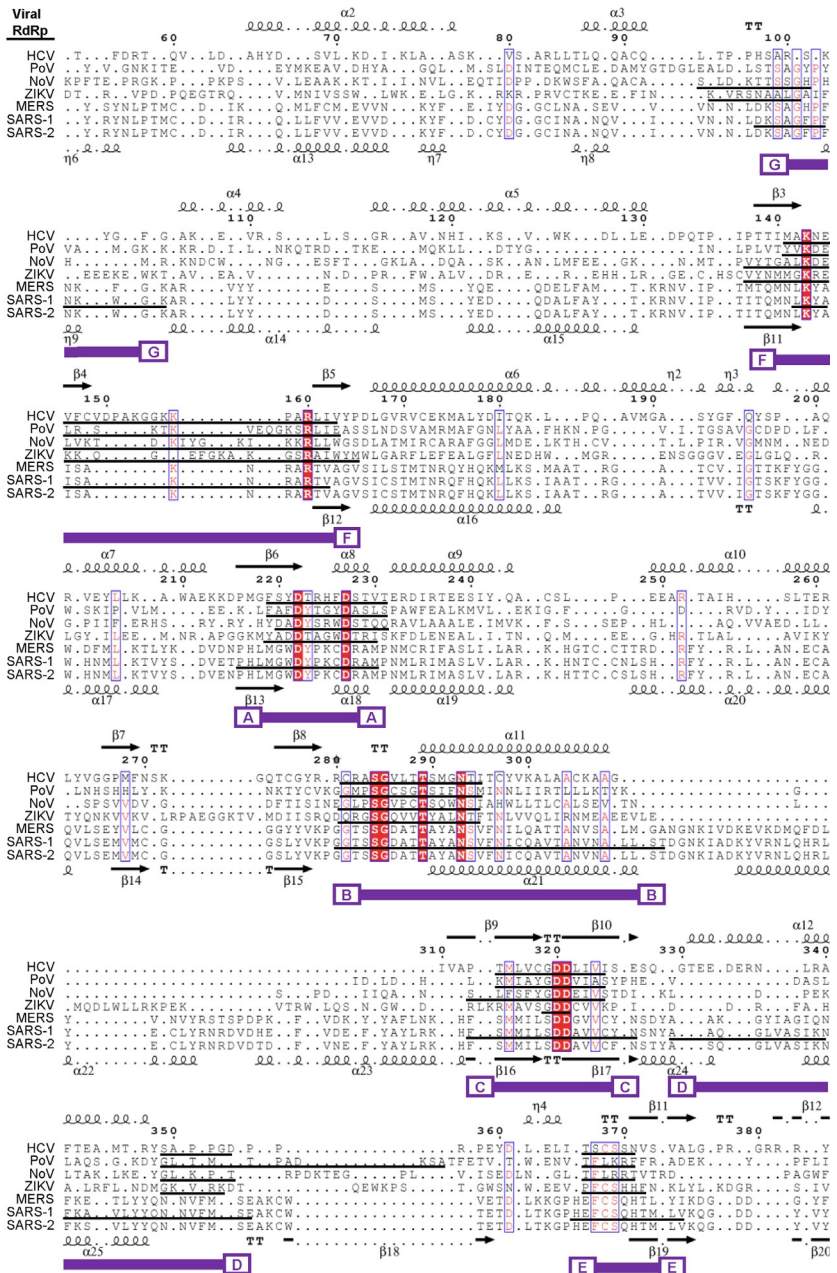# Structural conservation of RdRp motifs of selected (+)RNA viruses

Fig. 2 Structure-based alignment of select positive-sense genome RdRps. Sequences are aligned based on structural identity, with secondary structural elements of HCV (top) and SARS-2 (bottom) indicated. The following pdb files were used to generate the structure-based alignment: 4wta (HCV), 3o19 (PoV), 3h5y (noV), 5u0b (ZIKV), 6nur (SARS-CoV), and 6yyt (SARS-CoV-2). MERS nsp12 structural model was generated in SWISS-MODEL homology modelling pipeline [32a]. Due to the large nature of the proteins, segments containing RdRp motifs were aligned, rather than the entire protein. Conserved sections containing RdRp motifs A through G are indicated underneath in purple. Black underlined sequences correspond to motif sequences as defined in the literature. The structural alignments were conducted using Chimera UCSF software and visualized in Espript 3.0 [277,278].

between substrates by positioning the $3'$ group of the primer for further extension [25]. Motifs F and G are the only motifs found within the finger subdomains [32]. Motif F plays a role in manipulating incoming NTPs likely via interactions with the template, whereas the role of motif G is less conserved [25,32]. In some RdRps, motif G's role in binding the template and coordinating polymerase translocation has been suggested [36].

3. Picornaviruses

Picornaviruses represent a diverse family of small, nonenveloped positive-sense RNA viruses with genomes less than 10 kb in size, many of which are known to cause human disease [37,38]. Significant picornaviral pathogens include poliovirus (PV), hepatitis A virus (HAV), coxsackievirus, human rhinovirus, foot-and-mouth disease virus (FMDV), several numbered enteroviruses, and norovirus [39–44]. Pathogenic members of this family typically infect intestinal tissues, causing gastrointestinal symptoms [39–44]. Some demonstrate the ability to spread elsewhere, as with HAV and poliovirus, spreading to the liver and neuronal tissues, respectively [39,42]. Work surrounding PV, the prototypical picornavirus, stretches back close to a century [45]. The severity of the disease poses a powerful scientific impetus—while most cases resolve as acute gastrointestinal infections, a minority of cases occur such that the virus infects neuronal tissue, leading to temporary or permanent paralysis [46,47].

Efforts toward managing PV have centered around vaccination. The first PV vaccine, developed by Jonas Salk and adopted in 1955, was based on inactivated viral particles [48]. The vaccine was soon field tested nationally in the US on a massive scale and was found to be effective [49,50]. Soon after, an orally administered variant based on a live attenuated strain of the virus was developed by Albert Sabin [48,50]. Due to several advantages, vaccination efforts have mainly relied on the oral Sabin vaccine [51]. However, one key difference is the capability of Sabin's vaccine to revert attenuation and cause disease like the wild type of the virus [52–54]. Interestingly, as we approach global eradication of PV, a significant concern is the potential for outbreaks triggered by the orally administered vaccine and elimination of already circulating revertant vaccine strains [53,55].

Due to the many years spent working toward its eradication, several aspects have been intensely studied. The polymerase of PV, 3D^{pol} serves as a model of positive-stranded monomeric RdRps, with the first

incomplete and later complete structures nearing two decades in age [56,57]. Typical of many viral RdRps, 3D^{pol} shows a core fold resembling a right-hand, complete with palm, finger, and thumb subdomains [57,58]. Found within the RdRp core as well are the expected polymerase motifs [58,59]. Of particular note is the template entry channel found within the fingers, a highly dynamic region that was initially unresolved in the original structure [60]. Like many RNA viruses, the mutational rate of PV is remarkably high [22,61]. With a body of work pointing out that polymerase fidelity itself not solely responsible, one likely source is the lack of proofreading capability present in 3D^{pol} [62,63]. Consequently, the high rate allows for the formation of "quasispecies" due to the variety of viral progeny, leading to a diverse genetic "cloud" of viruses present, even in a single infected individual [64].

Perhaps due to the success of the PV vaccine over the past 70 years, there are currently no approved antiviral treatments for PV [65,66]. However, there does still exist a need due to circumstances where the use of a vaccine has been limited [67]. Backing this is a significant body of work examining the effects of antivirals on PV, much of which has informed our understanding of basic RNA virus biology: RdRp nucleoside inhibitors (NIs), RdRp template channel-interacting compounds, and capsid-binding compounds have shown inhibitory activity against PV [68–71]. Of specific note is the well studied nucleoside compound ribavirin (RBV), a broad spectrum guanosine-like analog [72]. Possibly explaining the broad spectrum of activity are the several mechanistic activities shown by RBV, including modulating cellular GTP concentrations, modulating host T-cell responses, inhibiting RNA capping, and directly interacting with viral RdRps by either affecting enzyme activity or inducing lethal mutagenesis of viral genomes through G to A and C to U transition mutations [72,73]. Mechanistic studies performed with PV and HCV demonstrate the ability of RBV to be incorporated at sites templated by cytidine or uridine residues, and when found in the template, allow for incorporation of cytidine or uridine [74,75]. One likely explanation is the rotational flexibility of the carbon-carbon bond connecting the amide group of the molecule to the 5 atom heterocycle base mimic [76]. Depending on the rotation of this bond, RBV can mimic the base pairing characteristics of either adenosine or guanosine (Fig. 3) [74]. Of significance is the direct activity of RBV upon PV 3D^{pol}, where the increase in mutational rate drives the RdRp beyond the tolerable threshold into error catastrophe, rendering the produced genome nonviable [77].
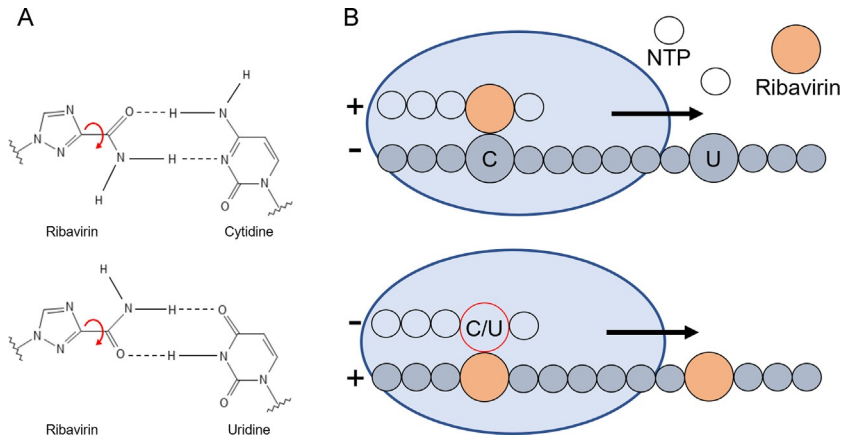
Fig. 3 Mechanism of action of ribavirin on poliovirus 3D^{pol}. (A) Base pairing capability of ribavirin with cytidine and uridine. Based on the rotation of the bond between the 5-carbon heterocycle and the amide group, ribavirin is capable of base pairing with either uridine or cytidine. (B) Cartoon depiction of ribavirin incorporation and mutagenesis in resulting rounds of replication. Ribavirin can be incorporated at both cytidine and uridine-templated positions. When the ribavirin-containing strand is then used as a template, cytidine or uridine may be added against ribavirin-templated positions [74]. *Panel (A): Adapted from S. Crotty, et al., The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen, Nat. Med. 6 (12) (2000) 1375–1379.*

Perhaps the strongest evidence for lethal mutagenesis as a possible ant–iviral mechanism is the emergence of a RBV resistance–conferring mutation that counteracts these effects. In PV 3D^{pol}, the mutation G64S emerges and increases polymerase fidelity, lowering the overall mutational load below that of the tolerable threshold [78]. While this mutation in isolation may seem beneficial to the virus, it is associated with lower fitness [79]. For some time after the initial discovery, the mutation was believed to result in lower fitness as a direct result of the lower mutational rate in the absence of RBV, presumably by decreasing quasispecies diversity [64,79]. Recent work suggested that the decreased fitness of PV 3D^{pol} G64S is due to a slower, more careful polymerase rather than decreased genetic variety [80,81]. Later work by Kempf et al. described how L420, a thumb motif residue, was critical in allowing for WT levels of recombination, with L420A heavily lowering the frequency of recombination (Fig. 4) [82]. Recent work has solidified the connection between recombination and RBV resistance: While the G64S mutant does demonstrate a higher fidelity over WT, its RBV resistance is dependent on polymerase recombination, with L420A abrogating its resistant phenotype [83].
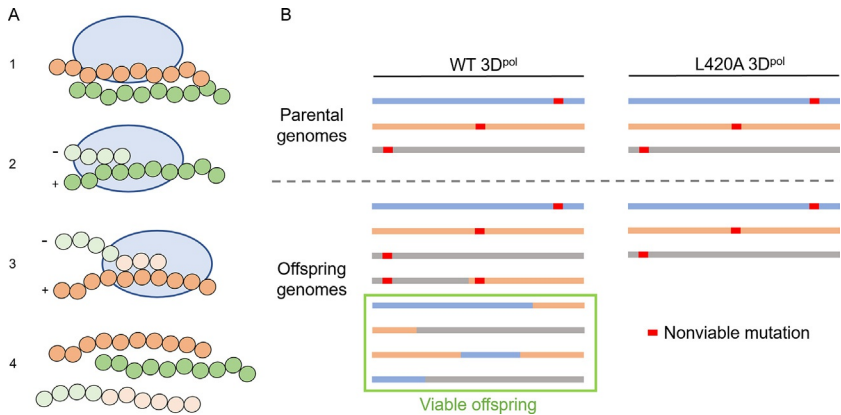
Fig. 4 Outline of poliovirus recombination. (A) Stepwise outline of the generation of a recombinant genome from two parental genomes. (1) A cell is infected with two genomes harboring sequence differences. (2) The RdRp of poliovirus, 3D^{pol}, begins to replicate one of the genomes. (3) 3Dpol transfers over to a new template from a differing genome without aborting replication, producing a genomic copy that contains sequences from both templates. (4) The final product yields a new genome distinct from the parental material. (B) Mechanism of recombination-mediated avoidance of lethal mutagenesis as compared between wild type and a recombination-deficient mutant (L420A) as suggested by Kempf et al. [83]. Despite only having initially nonviable genomes, wild type virus can establish entirely viable genomes via recombination between nonviable material. With a mutant polymerase that exhibits greatly reduced recombination rates, the generation of viable offspring is limited. *Panel (A): Adapted from B.J. Kempf, et al., Picornavirus RNA recombination counteracts error catastrophe, J. Virol. 93 (14) (2019).*

Similar results have been observed in related picornaviruses and with similar compounds: experiments performed with FMDV demonstrate RBV resistance mutations, some also located in the template channel, and some associated with increased fidelity [84–89]. The same high fidelity, RBV resistance phenotype has been observed in enterovirus 71 [90]. Work again with FMDV examining 5-fluorouracil also showed potential for mutagenic compounds other than RBV, with similar high–fidelity mutants being identified [91,92]. The observation that N-6-substituted purine analogs also demonstrate broad spectrum mutagenic effect but are attenuated by PV 3D^{pol} G64S further suggests a common mechanism of RBV [93,94].

Recent work, leveraging advances in assay capabilities, has also highlighted a class of compounds with a previously unknown mechanism of action against PV (and later against coronaviruses, below) [95]. Using magnetic tweezers

capable of measuring the force of tension across an RNA template between a magnetic bead and a glass coverslip, Dulin et al. demonstrated that pyrazine-carboxamide pseudobase containing nucleotide analogs such as favipiravir and the related T-1106 can act to induce backtracking, causing the polymerase to slide backward and pause, eventually leading to inhibition [95]. Using susceptible and resistant mutants to ribavirin, which contains a similar pseudobase to the above, they demonstrate nonoverlapping mechanisms between ribavirin and their compounds capable of inducing backtracking.

4. Hepatitis C virus

Hepatitis viruses represent a phylogenetically diverse grouping, unified by their ability to infect the liver [96–99]. Hepatitis B, C and D viruses exist as significant pathogens due to their ability to manifest chronic infections which, if untreated, may lead to cirrhosis, liver failure, and liver cancer [96–99]. Despite its discovery close to 30 years ago, vaccine development for hepatitis C virus (HCV) has so far been unsuccessful, with no approved or effective vaccine for any genotype to date [100]. While the most recent attempts have established a response and result in lowered viral load, they do not protect from chronic disease [101]. Thankfully, the standard of care for HCV has progressed to a broadly successful clinical treatment, demonstrating how incremental steps forward, pharmacologically, may lead to an effective treatment [102]. With over 150 million people worldwide infected chronically, understanding mechanisms of resistance tied to successful compounds allows for us to preserve such treatments as we progress [103].

HCV contains a positive-sense, single-stranded RNA genome of ~9700 bases, producing all its proteins via a single open reading frame [104]. Currently there are seven circulating HCV genotypes (1–7), with approximately 30% of the genome differing between genotypes [105,106]. Genotype 1 constitutes close to half of all HCV cases globally, and historically has been the most resistant to treatments [107,108]. As genotypes themselves differ significantly, the barrier may be larger or smaller depending on those differences, allowing certain genotypes to establish resistance with fewer mutations [109]. Additional factors such as the generation of quasispecies in infected individuals as well as intrinsic bias in mutational rates also contribute to the generation of resistance associated mutants, with some being detectable at baseline prior to treatment [110–113].

A major antiviral target of HCV is the RdRp, nonstructural protein 5B (NS5B). The structure of this monomeric polymerase was solved near the

end of the millennium independently by several groups [114–116]. These structures and later work demonstrated NS5B contained several unique motifs such as a thumb subdomain β-loop insertion and C-terminal membrane anchor, in addition to the expected RdRp motifs and right-handed tertiary structure [117]. The enclosed nature of NS5B was also remarked upon, with the active site of the RdRp enveloped in the finger and thumb subdomains, including the mentioned thumb β-loop [117]. Rearrangements during initiation of polymerase activity by these components has been shown to be indispensable for efficient activity, outlining a requirement for dynamic conformational shifts [118,119].

Early treatments for HCV infection centered around the use of RBV alongside pegylated interferon alpha, which remained the standard of care into the early 2010s [120,121]. RBV has many purported mechanisms, with no one clear effect driving its efficacy against HCV [122]. Evidence does exist to indicate that RBV acts directly upon the polymerase as a mutagenic agent, and/or as an inhibitor of RNA synthesis, similar to its effects on poliovirus, however the lack of concrete resistance-associated mutations in NS5B point to other mechanisms [74,75,121,123,124].

A major step forward in the treatment of HCV was the development of sofosbuvir (SOF), a highly effective uridine analog approved for HCV treatment in 2013 [125,126]. Mechanistically, SOF-triphosphate acts as a substrate of NS5B, leading to its incorporation into the growing RNA genome after modification from its prodrug to its active form, eventually resulting in chain termination (Fig. 5) [127,128]. Theories primarily supported by structural data suggest that SOF, once incorporated, spatially blocks the attack of additional nucleotides, effectively blocking further elongation [117]. Data from similar molecules suggests this steric clash occurs between incoming nucleotides and the bulky 2′-methyl group [129]. Notably, SOF was observed to be an effective treatment regardless of HCV genotype [130,131]. While resistance mutations have been noted for SOF, they arise in surprisingly low abundance and are typically not observable at baseline prior to treatment [111,132–134]. The primary mutation associated with SOF resistance, S282T, is found within the active site of NS5B (Fig. 6) [117,131]. The mutation would be expected to cause a conflict between the side chain of T282 and SOF, limiting SOF incorporation [117]. Potentially due to the nature of the mutation and its proximity to the active site, S282T is associated with significant loss of fitness in the virus, with resistant genotypes lacking the ability to persist in individuals after SOF treatment [132,135]. Other mutations have been associated with SOF resistance. L159F, also located in the vicinity of the active site, has been implicated
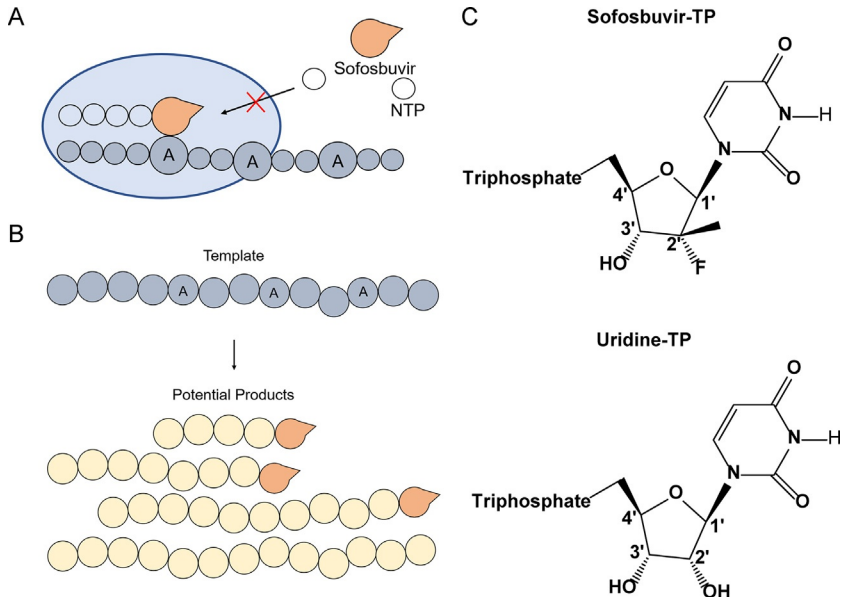
Fig. 5 Mechanism of action of sofosbuvir (SOF) against HCV NS5B. (A) Incorporation of SOF into the growing RNA strand prevents additional NTP incorporation. Sofosbuvir can be incorporated at positions templated by adenosine, mimicking the natural substrate, uridine [127,128]. After incorporation, due to steric clashes between SOF and incoming NTPs, no further elongation is possible [117]. (B) Schematic of potential products of chain termination as caused by SOF incorporation. At each position with a template adenosine, SOF incorporation may result in chain termination, rendering that round of replication or transcription nonviable. (C) Structure comparison of SOF (uridine analog) and uridine. Of note is the fluoro- and methyl-group moieties found on the 2′ ribose carbon of sofosbuvir.
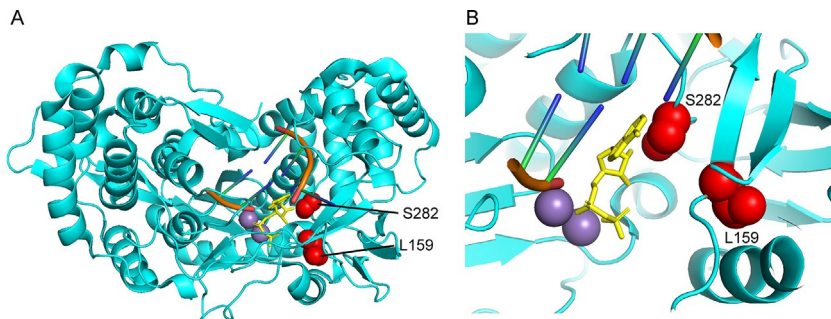
Fig. 6 Structural insights into sofosbuvir resistance mutations found in hepatitis C virus NS5B (4WTJ). (A) Overview of NS5B structure with template and elongating RNA strands (orange), incoming ADP (yellow), manganese ions (purple), and two amino acid residues for which sofosbuvir resistance mutations exist for (red, S282T, L259F). (B) Zoomed view of the spatial position of S282, L159, and the incoming ADP nucleotide. As suggested by Appleby et al., due to the proximity of residue 282 to incoming nucleotides, the T282 mutant may sterically clash with the 2′ methyl group found on the functional sofosbuvir molecule, prohibiting incorporation [117]. Structures oriented in PyMol, PDB: 4WTJ.

with S282 due to their close structural proximity and the emergence of L159F mutations among low responding SOF patients [136,137].

In addition to compounds that mimic natural substrates of NS5B, several nonnucleoside inhibitors (NNIs) that act upon the RdRp allosterically have been developed and explored. These compounds bind to surface pockets found on NS5B and are categorized based on their site of binding and the mutations associated with that site [138]. Currently there are five binding sites that have been identified and targeted: two thumb sites (T1 and T2), two palm sites (P1 and P2), and one site associated with the β–loop (B1) [139,140]. Structural work examining the conformational landscape of NS5B suggests that NNIs may share a uniform mechanism, blocking the transitions between the initiation and elongation associated conformations, inhibiting polymerase activity [141,142]. When compared to NIs, the barrier for resistance to develop is much lower [143,144]. Indeed, many NNI compounds are genotype restricted, with some resistance mutations commonly found at baseline [143,144].

The standard of care for treating chronic HCV infection has evolved greatly over time, starting with RBV and pegylated interferon alpha treatments [122]. Limitations of such a treatment centered on rates of treatment success around 50% and significant side effects suffered by patients [122]. Current standard of care is determined depending on several factors, including HCV genotype, if the patient is treatment naive, the degree of liver cirrhosis, the presence of any chronic liver disease, and the presence of chronic viral coinfections [145]. Thanks to the degree of therapeutic development since the days of RBV/interferon treatment, response rates with directly acting antivirals can approach nearly 100% [146–148].

5. Respiratory syncytial virus

Respiratory syncytial virus (RSV) belongs to the *Mononegavirales* order of nonsegmented negative strand RNA viruses and is a member of Pneumoviridae family of pneumoviruses [149]. An approximately 15 kb single-stranded RSV genome coding for 11 proteins is encapsidated with the viral nucleoprotein and is associated with the viral RNA polymerase complex inside an enveloped viral particle [149].

RSV infection is omnipresent in children as the leading cause of acute lower respiratory infections in infants and a life-threatening pathogen to the elderly and immunocompromised [150–152]. Prophylactic developments were severely slowed due to complications in clinical trials of an early

vaccine, with no vaccine currently available today [153,154]. Palivizumab, a humanized monoclonal antibody against the F glycoprotein, was approved for prophylaxis of RSV infections in 1998, however, it has recently been reported that its effect on RSV transmission rates is limited [155]. The only antiviral agent that is approved for treatment of RSV infections is ribavirin (RBV), a broad-spectrum antiviral nucleoside analog—unfortunately, the efficacy of RBV when used to treat RSV is poor [156–158].

Considering the recent successes with anti-HIV, -HCV, and -SARS-CoV-2 antivirals targeting viral nucleic acid polymerases, RSV RNA polymerase represents an attractive target for the development of novel nucleoside analogs. The functional core of the RSV RNA polymerase consists of two subunits, a ~250 kDa large polymerase (L) and a tetramer of the ~27 kDa phosphoprotein cofactor (P), both of which are present in the viral particle [159–161]. Recent cryo-EM structures revealed that the RSV RNA polymerase exists as a complex of one L subunit with P tetramer adopting an asymmetric tentacular arrangement [160,161]. L protein is a multifunctional enzyme catalyzing RNA transcription, capping and polyadenylation of transcripts, as well as genome replication each mediated by the L specific subdomains [161].

RNA transcription and genome replication is conducted by the RdRp domain of the L protein, which contains the highly conserved motifs [32]. While both nucleoside and nonnucleoside inhibitors have been in the development over the recent years [162], two cytidine nucleoside analogs are of particular interest: molnupiravir (EIDD-2108) and lumicitabine (ALS-8176) (Fig. 7): molnupiravir has a modification on the base moiety, while lumicitabine contains a sugar moiety modified at 2′ and 4′ positions [151,163]. Both compounds are intracellularly metabolized to their triphosphate forms to compete with their natural counterpart CTP for nucleotide incorporation by viral RNA polymerases [163,164].

Molnupiravir is a broad-spectrum antiviral that is currently undergoing clinical trials against SARS-CoV-2 infection. It has been shown to act as a mutagen, promoting G-to-A and C-to-U transition mutations in coronaviruses and influenza virus [163]. Interestingly, in the context of RSV only A-to-G transition mutations have been observed [163]. While the mechanism of action (MOA) of molnupiravir against SARS-CoV-2 has been recently elucidated (Fig. 8B) [165,166], its MOA against RSV remains unknown.

Lumicitabine is the only RSV-specific nucleoside inhibitor that has reached the stage of clinical trials. ALS-8176 is an improved bioavailability
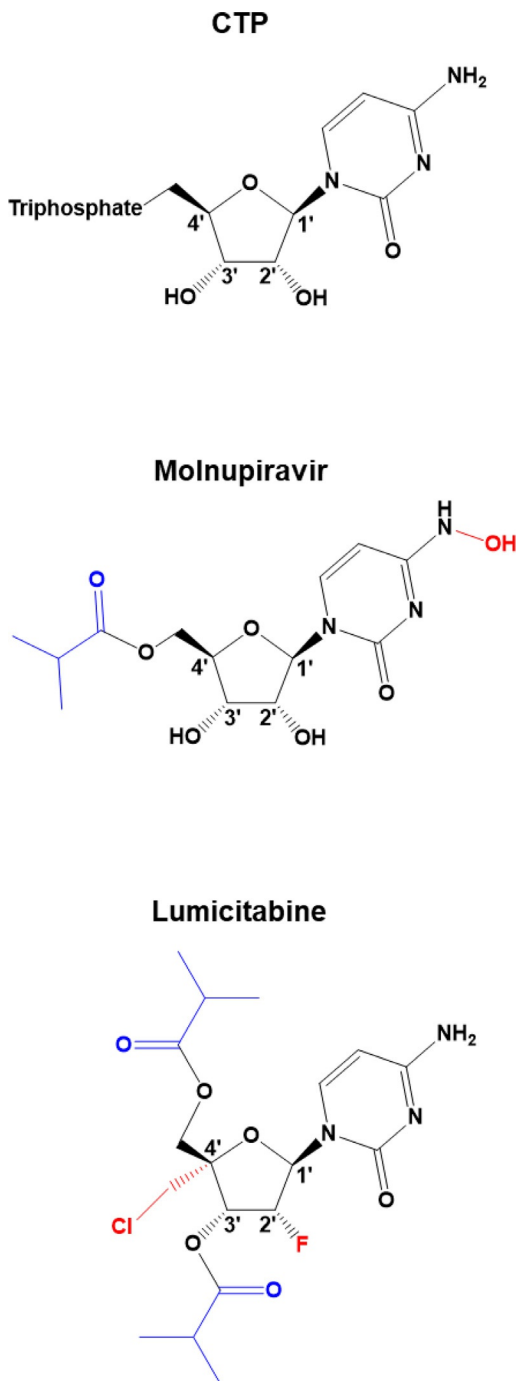
Fig. 7 Chemical structures of CTP analogs. Features highlighted in red illustrate the portion of the molecules that mediate the inhibitory effect on the viral RNA synthesis. Features highlighted in blue illustrate modifications that increase bioavailability of the analog.
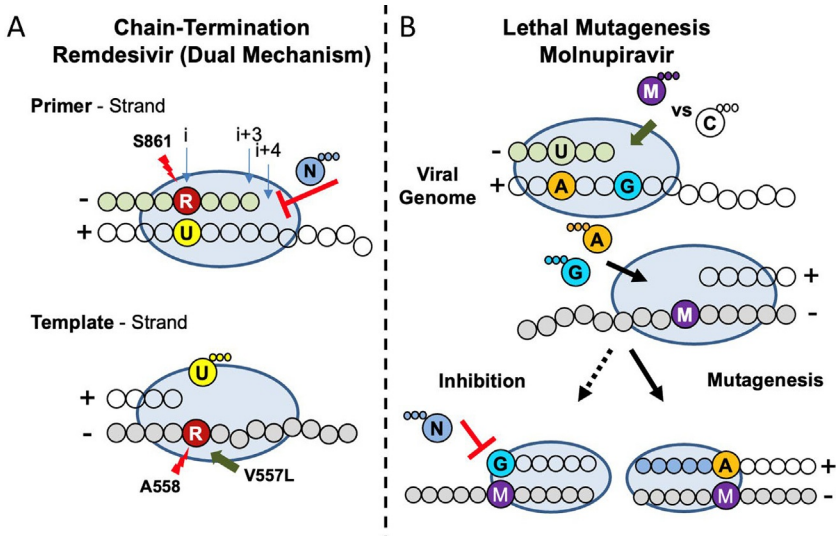
Fig. 8 Model of the mechanism of action of RDV and molnupiravir against SARS-CoV-2 RdRp. (A) RDV functions as a chain terminator preventing RNA synthesis during both primer and template strand synthesis. (Top) Incorporation of RDV-MP (red) by the viral polymerase (blue oval) opposite UMP in the template (yellow/clear bubbles) results in a steric clash with the residue S861 and incomplete inhibition. (Bottom) The primer is eventually extended and is later used as a template. A clash between RDV-MP and the backbone of A558 is responsible for the reduction in UTP (U) binding. V557L, previously identified *in vitro*, can compensate for this clash promoting low level resistance. (B) Molnupiravir incorporation results in mutagenesis promoting incorrect base-pairing. (Top) Molnupiravir (purple) primarily base-pairs with GMP (blue), once extended the subsequent template strand promotes ATP (orange) binding opposite molnupiravir explaining lethal mutagenesis observed in cell culture as a result of A-to-G and U-to-C transitions. Biochemical evidence showed evidence of GTP (blue) binding opposite molnupiravir in the template, but this resulted in minimum inhibition and extension of this product would not result in a mismatch. *Adapted from C.J. Gordon, et al., Molnupiravir promotes SARS-CoV-2 mutagenesis via the RNA template, J. Biol. Chem. (2021) 100770. E.P. Tchesnokov, et al., Template-dependent inhibition of coronavirus RNA-dependent RNA polymerase by remdesivir reveals a second mechanism of action, J. Biol. Chem. 295 (47) (2020) 16156–16165.*

version of the parent drug ALS–8112, which was found to have inhibitory effects on RSV replication *in vitro* [167]. ALS–8112 is a cytidine analog with sugar moiety modifications, with the triphosphate version (ALS–8112–TP) being recognized by the RSV RNA polymerase as a substrate for RNA syn–thesis, albeit about 10–fold less than the natural counterpart CTP [167]. Incorporation of the compound into the extended RNA primer caused immediate chain termination due to the inability to incorporate subsequent

nucleotides [168]. In addition, the compound was not RSV strain specific [168]. Lumicitabine and ALS-8112 resistance-conferring mutations have been characterized and often found together in replicon assays: M628L, A789V, L795I, and I796V located in the conserved motif B of the RdRp domain [168].

When analyzed in isolation, A789V was the only mutation capable of conferring resistance but was still unable to match the phenotype of the quadruple mutant [169]. Structurally, A789V may be significant due to its position near the catalytic motif C as well as the residue D700 in the motif A, however the overall protein structure suggests that all the mutations together may play a role in modifying the active site and facilitating nucleoside discrimination [160,161].

Early clinical data with Lumicitabine indicated that it was highly efficacious in decreasing viral load and speeding viral clearance with the additional observation of no resistance mutants arising because of treatment [169,170]. Unfortunately, further clinical trials were stopped due to treatment–related neutrophil abnormalities [171].

6. Coronaviruses

The *Coronaviridae* are a diverse family of positive-sense, single stranded RNA viruses with a genome approximately 30 kb in size—the largest genome of any known RNA virus [172,173]. *Coronaviridae* are comprised of four genera: *alphacoronavirus*, *betacoronavirus*, *gammacoronavirus*, and *deltacoronavirus*. Alpha- and betacoronaviruses are only capable of infecting mammals, while the host range for gamma- and deltacoronavirus also includes avian species [174,175]. There are seven known coronaviruses capable of causing disease in humans predominantly as a result of infection in the upper respiratory and gastrointestinal tract [174,176]. Consequential outcomes due to coronavirus infection vary from the common cold to severe respiratory damage that can ultimately be fatal [176,177]. The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [178], the causative agent of COVID-19, put coronaviruses in the global spotlight. However, previous outbreaks of SARS-CoV and Middle East respiratory syndrome CoV (MERS-CoV) foreshadowed the pathogenic potential possessed by coronaviruses [179,180].

One outcome of the COVID-19 has been the display of technological advancements resulting in a rapid and expansive pipeline of vaccine candidates [181]. As of June 2021, three vaccines have received Emergency Use

Authorization (EUA) from the Food and Drug Administration (FDA). Pfizer–BioNTech [182] and Moderna [183] are mRNA vaccines which demonstrated an efficacy 95% and 94% respectively in protecting against COVID-19 infection [184,185]. Johnson & Johnson, an adenovirus vector that is unable to replicate, received EUA after exhibiting an efficacy rate of 66.1% during ongoing phase 3 trials [186,187]. AstraZeneca another adenovirus vaccine, has received approval in Europe and Canada based on the pooled results of four independent clinical trials, efficacy was determined to be approximately 70% [188,189]. Conversely to vaccine development, antibody cocktails also received EUA for the treatment of outpatients [190], and the nucleotide analog remdesivir has been approved for COVID-19 treatment [191].

Upon host cell entry, ORF1a and ORF1ab of the viral genome is translated and a −1 ribosomal frameshift enables the production of two distinct polyproteins, 1a and 1ab [192]. The proteolytic processing of 1a and 1ab result in two polyproteins comprised of nonstructural protein (nsp) 1–11 and 1–16 respectively [193,194]. Posttranslational assembly results in the formation of a replicase transcriptase complex (RTC), driving RNA synthesis and supporting viral propagation [193,195]. The large genome puts an extended pressure on the replication machinery during RNA synthesis, thus coronaviruses require higher replication fidelity as compared to other RNA viruses [196,197]. Two crucial subunits within the RTC responsible for fidelity are nsp12 and -14, the RdRp and the $3'$ to $5'$ exonuclease (ExoN), respectively [194,196,197]. The RdRp drives RNA synthesis while the ExoN functions as a proof-reader repairing errors in nucleotide incorporation [196,197]. The vital role of the RdRp and its highly conserved nature among coronaviruses makes it a logical target for therapeutics to prevent viral propagation [195,198]. The proofreading capability of the ExoN presents a plausible evolutionary development to support the replication of their large genome [199]. Consequently, the excision of incorporated nucleoside analogs by the ExoN adds an additional layer of complexity for therapeutic development [199,200].

Additional components crucial to RNA synthesis by the RdRp are nsp7 and -8, often implicated in processivity to support the replication of their large genome [21,201–203]. The exact role of nsp7 and -8 remains relatively undefined, however biochemical and structural data suggests nsp8 could serve to function as a primase or a $3'$-terminal adenylyltransferase [204–207]. The structure of a RdRp complex with nsp7 and -8 has been solved for of SARS-CoV and SARS-CoV-2 identifying a stoichiometric ratio of 1:2:1 for nsp7, -8, and -12 respectively [33,207–210].

The antiviral activity of nucleoside analogs ribavirin [211,212], BCX4430 [213], gemcitabine hydrochloride [214], and acyclovir fleximer [215] have been investigated *in vitro* against SARS-CoV and MERS-CoV, while mizoribine was tested solely against SARS-CoV [216]. A shared characteristic among these therapeutics is a low antiviral effect, indicating the potential to treat coronavirus infection is low. In addition to being readily excised, ribavirin's rate of incorporation was significantly lower than its natural counterpart GTP ($K_m = 136\,\mu M$ vs $1.5\,\mu M$) [200]. The poor incorporation efficiency of ribavirin observed biochemically coincides with a modest antiviral effect in cell culture studies previously mentioned. Taken together, the requirements for a nucleotide analog to elicit an antiviral effect is twofold, relying on efficient incorporation while going unrecognized by the ExoN to avoid excision.

Favipiravir has demonstrated an antiviral effect against SARS-CoV-2 *in vitro* with an inhibitory concentration in the high micromolar range [217]. The mechanism of favipiravir remains ambiguous, but previous biochemical, cell culture and structural work suggests lethal mutagenesis as the main MOA [217,218]. Biochemical studies investigating incorporation efficiency of favipiravir by the SARS-CoV-2 RdRp are contradictory [217–220], furthermore it appears to be ill-equipped to outcompete its natural counterpart for incorporation [220]. One possible explanation may reside in recent data showing that the SARS-CoV-2 RdRp is capable of backtracking [221]. As favipiravir and related compounds have been shown to trigger backtracking and inhibition upon incorporation upon incorporation by the PV RdRp 3D^{pol} [95], a similar mechanism may be at play during incorporation by the SARS-CoV-2 RdRp.

Remdesivir (RDV, formerly GS-5734) is a nucleotide analog that, when metabolized to its triphosphate form (RDV-TP), mimics the natural nucleotide adenosine triphosphate (ATP) with 1′-C-nucleoside bond and a 1′-cyano-substitution [222]. RDV is broad-spectrum in nature showing an antiviral effect *in vitro* and *in vivo* against Nipah virus (NiV), respiratory syncytial virus (RSV), and Ebola virus (EBOV) [222–225]. Biochemical assays demonstrated RDV-TP can compete with its natural counterpart, ATP, for incorporation by the RdRp of Ebola resulting in delayed chain-termination [226]. Moreover, the therapeutic potential of RDV was tested in a randomized, controlled trial in 2019 during the Ebola outbreak in the Democratic Republic of the Congo (DRC), but it was ultimately determined two different antibody therapies proved to be more effective [227].

RDV has undergone extensive investigation in its ability to inhibit the RdRp of CoV on route to being the first FDA approved antiviral treatment for COVID-19 [191]. Approval was in large part due to a randomized

clinical trial by the National Institute of Allergy and Infectious Disease (NIAID), concluding RDV treatment reduced median time to recover by 5 days [228]. However, another randomized clinical trial performed by the World Health Organization (WHO) did not arrive to the same conclusions [229]. This inconsistency could be linked to discrepancies in experimental design, both of which failed to measure the viral load [230]. Initial investigation demonstrated an EC_{50} in the submicromolar range [231,232], and reduced the viral load of SARS-CoV and MERS-CoV in a murine model [232,233]. The efficacy of RDV was demonstrated further when treatment of MERS-CoV infection in a rhesus macaque model decreased viral replication [234]. The potency of RDV as an antiviral against SARS-CoV-2 was demonstrated in human airways epithelial (HAE) lung cells as well as in mice infected with a SARS-CoV expressing the polymerase of SARS-CoV-2 [235]. RNA synthesis assays determined the SARS-CoV-2 RdRp complex incorporated RDV-TP approximately three times more efficient than its natural counterpart ATP [220]. Following incorporation of RDV-TP in the nascent RNA strand RNA synthesis arrest occurs following the incorporation of three nucleotides (i + 3), demonstrating the first MOA delayed chain termination (Fig. 8A, top) [220]. A biochemical mutagenesis assessment revealed that delayed chain termination is a result of a steric clash with the 1′-cyano-group of the incorporated RDV-TP and the hydroxyl group of S861 [236]. Moreover, increased nucleotide concentrations can diminish the intermediate i + 3 product suggesting newly synthesized negative sense copies of the viral genome contain an incorporated RDV (RDV-MP) [236]. This presents the second mechanism, template-dependent inhibition (Fig. 8A, bottom) [236]. When RDV-MP is embedded in the template strand, incorporation of complementary UTP is severely compromised requiring an increased concentration to promote RNA synthesis beyond the RDV-MP blockade [236]. This obstruction can be attributed to a clash between the backbone of residue A558 with the SARS-CoV-2 RdRp and the 1′-cyano-group of RDV-MP [236].

Serial passages of β-CoV model murine hepatitis virus (MHV) with increasing concentrations of the RDV resulted in a resistant phenotype with two amino acid mutations (F476L and V553L) within the RdRp [231]. These residues are conserved within the CoV RdRp, and when aligned, correspond to F480 and V557 of SARS-CoV, and F481 and V558 of MERS-CoV (Fig. 9) [231]. Induced mutagenesis in this fashion further solidifies that the mode of inhibition by RDV is imposed on the RdRp of CoV [231]. V557L is positioned in motif F while F480L is not associated
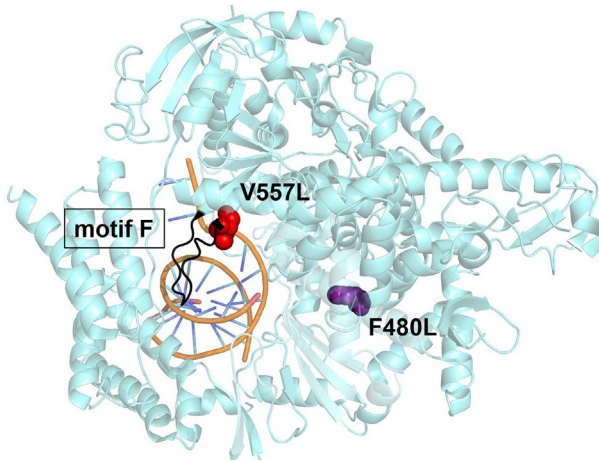
Fig. 9 Structural representation of the SARS-CoV-2 RdRp (nsp12) containing the resistance conferring mutations, 7BV2. Two RDV resistant mutations selected for *in vitro* with the coronavirus model murine hepatitis virus (MHV) align to V557L (red) and F480L (purple) in SARS-CoV-2. Both residues are located in the finger subdomain, V557L is associated with structural motif F (black). This motif is implicated with nucleotide incorporation and contacting the 5′ end of the template. Computational and biochemical studies have associated A558 with template-dependent mechanism of RDV, neighboring V557L counteracts this effect resulting in low-level resistance. The distal location of F480L from the active site and V557L suggest it does not have a direct role in RDV resistance as it relates to RNA synthesis. Structures oriented in PyMol, PDB: 7BV2 [208]. *Adapted from M.L. Agostini, et al., Coronavirus susceptibility to the antiviral remdesivir (GS-5734) is mediated by the viral polymerase and the proofreading exoribonuclease, mBio 9 (2) (2018) e00221-00218.*

with any structural motif [198,231]. Both residues are within the fingers subdomain of the CoV RdRp responsible in forming the passage that supports incoming nucleotides [231,235]. The adjacent location of V557 to A558, the residue likely responsible for template–dependent inhibition [236], presents an interesting dynamic for a potential mechanism of resistance. When studied biochemically, the single mutation, V557L, within the SARS-CoV-2 RdRp conferred the low-level resistance previously observed in cell culture [236]. The mechanism of resistance was specific to RDV-MP in the template as decreased UTP concentration was needed to promote RNA synthesis beyond RDV-MP [236]. V557L had no effect on RDV–TP incorporation efficiency or delayed chain termination [236]. RDV resistance studies with EBOV identified the mutation F548S conferring low level resistance [237]. F548 resides in motif F, although its location is not identical to V557, this

demonstrates a potential unifying broad spectrum mechanism of RDV as it targets structurally equivalent regions within the RdRp [237].

Four additional mutations were identified in the RDV resistant CoV strain none of which were located in the ExoN [231]. Deletion of the ExoN resulted in an increased sensitivity to RDV, indicating nsp14 could participate in the excision of the incorporated RDV [231]. A mechanism of action for the possible excision of RDV by the ExoN has yet to be described in the literature. However, the efficiency of RDV incorporation as well as potential protection due to delay-chain termination could explain why the ExoN is unable completely alleviate RDV induced inhibition [220,238].

Molnupiravir, the prodrug of β-D-N4-hydroxycytidine (NHC), when metabolized to its triphosphate form (NHC-TP) is an effective substrate for the CoV RdRp [165,166]. The potency of molnupiravir is exhibited by the submicromolar EC_{50} values determined against MERS-CoV, SARS-CoV, and SARS-CoV-2 in a variety of cell types [233]. CoV Cell culture data revealed the likely mechanism of molnupiravir is lethal mutagenesis, promoting A-to-G and C-to-U transitions [233,239]. This is a result of NHC-TP functioning as a tautomer existing as both an N-hydroxylamine and an oxime [240]. RNA synthesis assays revealed NHC-TP as a substrate for the CoV RdRp, which is incorporated most efficiently in its hydroxylamine form as a CTP analog opposite a template G [165,166]. Incorporated NHC-MP does not result in chain termination; therefore NHC-MP will be present in subsequently generated copies of the CoV genome [165,166]. Additional biochemical experiments revealed GTP and ATP incorporation is promoted opposite the templated NHC-MP [165,166]. This suggests NHC-MP in the template exists in both tautomeric forms acting as CMP or UMP forming a solidified mutagenic mechanism of inhibition by molnupiravir (Fig. 8B) [165,166].

Sequential passaging of MHV and MERS-CoV in the presence NHC revealed a high genetic barrier for the antiviral resistance [239]. Cross-resistance to NHC was examined with the previously mentioned RDV resistant MHV strain (F476L and V553L) [233]. Interestingly, the known RDV resistant strain of MHV displayed an increased sensitivity to NHC [239]. As opposed to RDV, the ExoN deletion does not impact the antiviral potency of molnupiravir, suggesting NHC-MP is resistant to excision [239]. A biochemical mechanism elucidating the relationship between the CoV ExoN and molnupiravir remains unanswered. The potential inability of the ExoN to excise NHC-MP could be due to RNA synthesis remaining unimpeded following NHC-TP incorporation, going unrecognized, and therefore avoiding excision [165,166].

7. Influenza viruses

Influenza is a severe, contagious respiratory illness caused by infection with influenza viruses. Influenza viruses are segmented negative sense RNA viruses with ~14 kb genomes that together comprise six of the eight members of the family *Orthomyxoviridae* [241]. Four genera of influenza, influenza A, B, C, and D have been identified, two of which, A and B, cause significant morbidity and mortality worldwide [241,242]. The WHO reports that influenza A and B together account for up to 5 million severe illnesses and 650,000 deaths annually [243]. This accounts for a total economic burden of $87 billion per year in the United States alone, despite the availability of effective vaccines and antivirals against both influenza A and B [242,244].

Vaccination is widely considered to be the most effective way to prevent influenza infection [245]. Disease resulting from influenza infection is predominantly the result of infection with either influenza A or B [245]. Therefore, the currently available influenza vaccines consist of a mixture of A and B strains which is determined annually based on global surveillance of the circulating strains [245,246]. Two types of influenza virus vaccine, live attenuated and inactivated vaccine, are currently in use [245]. However, despite the availability of these vaccines influenza still causes significant morbidity and mortality necessitating the development of antivirals against influenza [247].

Antivirals represent an important tool for the treatment and management of influenza infection. Historically, influenza virus infection has been treated using M2 ion channel and neuraminidase inhibitors [248]. Unfortunately, in recent seasons >99% of circulating influenza strains have been resistant to M2 ion channel inhibitors, limiting treatment options [249]. This has necessitated the development of antivirals with novel mechanisms of action. To this end, inhibitors of the viral RdRp have been developed.

The structures of the RNA dependent RNA polymerases (RdRp) of influenza A and B have been solved [250,251]. These structures revealed that the influenza RdRp is as a multifunctional heterotrimer consisting of three proteins PA, PB1, and PB2 [250,251]. PA encodes an endonuclease activity, PB1 encodes the RdRp activity, while PB2 contains 7-methylguanylate cap binding activities [250,251]. As each of these activities are distinct and indispensable, the viral RdRp serves as an attractive drug target. Influenza performs transcription via a well described process known as cap snatching [251–257]. Briefly, PB2 binds host mRNAs via a 5′ m^{7}GTP cap binding domain, bringing them into proximity to PA. The endonuclease domain

within PA cleaves them, producing short, capped primers, which are subsequently extended by the RdRp activity of PB1 to produce capped viral transcripts.

Each aspect of this process has currently been targeted for drug development and compounds targeting each component of the polymerase complex are currently available (Fig. 10). For example, the first–in–class PA endonuclease inhibitor baloxavir marboxil (BXM) was approved by the FDA in December of 2018 for the treatment of uncomplicated influenza in adults [258]. A single dose of BXM results in the alleviation of influenza symptoms
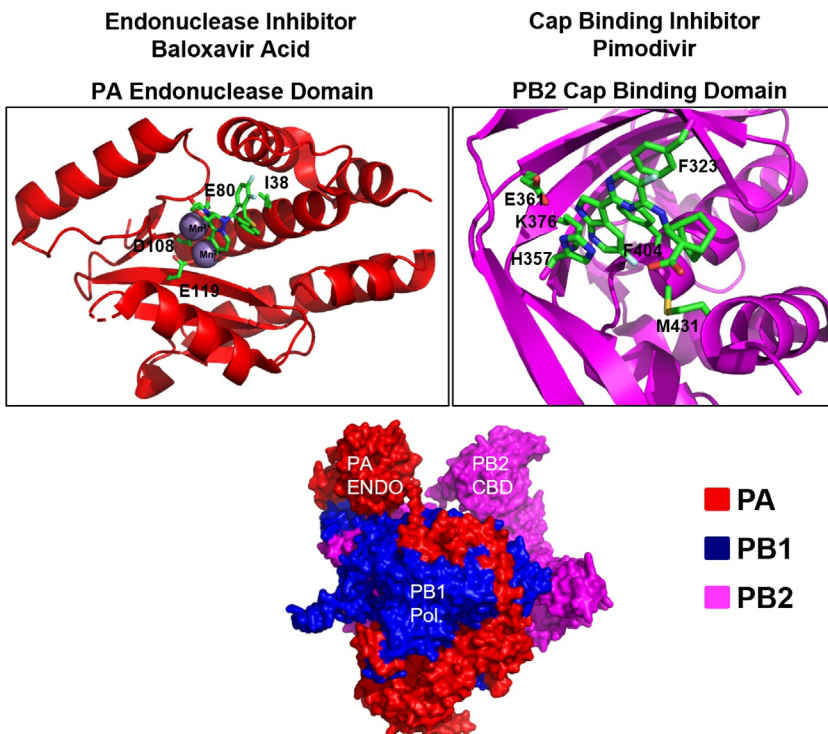

Fig. 10 Structural overview of inhibitors targeting the influenza RdRp. The influenza RdRp is a multifunctional heterotrimer consisting of three subunits: PA, PB1, and PB2. PA encodes the cap snatching endonuclease, PB1 encodes RdRp activity, and PB2 encodes a m^7G-cap binding activity. The binding sites of inhibitors targeting each subunit and selected residues involved in inhibitor binding and resistance are highlighted. Structures adapted from the following and oriented using PyMol: FluA-polymerase heterotrimer 4WSB [251], PA endo with Baloxavir, 6FS6 [261], PB2 with pimodivir 4P1U [273]. Structural snapshots for favipiravir and molnupiravir are not shown as structures of the polymerase bound to these molecules are currently unavailable.

approximately 23–28 h faster than placebo [259]. This is likely because baloxavir is a highly potent, tight-binding, PA endonuclease inhibitor that is capable of inhibiting influenza viruses in the low nanomolar range [258,260]. BXM is a prodrug, the active form of which, baloxavir acid (BXA), binds to divalent metal ions in the PA endonuclease active site preventing catalysis [261]. Baloxavir is active against all known genera of influenza including viruses resistant to neuraminidase inhibitors [258,262]. This, in addition to the fact that BXM can be administered orally as a single dose make BXM highly attractive for clinical use [259]. However, soon after the discovery of BXM resistant strains emerged. The majority of described resistance mutations are substitutions in the I38 position, with I38T/M/F resulting in a 2- to 50-fold increase in EC_{50} with I38T having the largest effect [259,261]. It has been postulated that this reduced susceptibility is due to the fact that I38T may reduce van der Waals contacts with the inhibitor [261]. I38T has also been shown to confer resistance to baloxavir in *in vitro* biochemical assays [260,261]. It has been reported that these mutations have a significant adverse effect on viral fitness so it is unknown how prevalent they may become should the use of BXM become widespread [261]. However, the development of widespread resistance may be possible as the emergence and transmission viruses containing BXM resistance mutations has been documented in clinical trials [259,263].

The RNA synthesis activity of the polymerase has been targeted using nucleotide-base moiety analogs such as favipiravir and NIs as molnupiravir (NHC) [163,264,265]. To date, however, the only analog approved for the treatment of influenza infection is favipiravir, which gained approval in Japan for the treatment of pandemic influenza [265]. Favipiravir is a nucleotide base-moiety analog which, following ribosylation and triphosphorylation by the cellular machinery functions as an inhibitor of the influenza polymerase [266]. Favipiravir has been evaluated in clinical trials and, while the exact mechanism of action requires clarification, it is thought to function through the induction of lethal mutagenesis, although limited biochemical studies have suggested that chain termination can occur after two successive incorporations (Fig. 11) [267–269]. While favipiravir can tautomerize, the keto tautomer is the most stable form at biological conditions and is therefore responsible for the biological activities of the compound [240]. Specifically, favipiravir functions as either an ATP or GTP analog, the successive incorporation of which leads to chain termination (Fig. 11) [268]. Resistance to favipiravir has been generated *in vitro* [270]. The PB1 K229R mutation located within the conserved F motif of the RdRp has been shown to confer resistance to
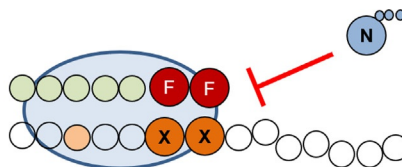
A

B
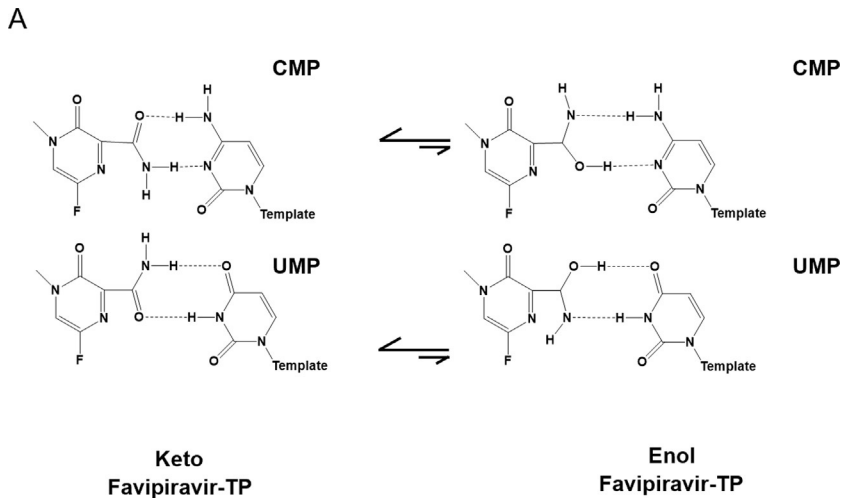
Fig. 11 Mechanism of inhibition of the influenza RdRp by favipiravir. (A) Base pairing of the keto and enol forms of favipiravir with CMP and UMP. (B) Two consecutive incorporations of favipiravir leads to chain termination, preventing successful polymerase activity. X, G or A nucleotides; N, any nucleotide; F, favipiravir. *Panel (A): Adapted from N.R. Jena, Role of different tautomers in the base-pairing abilities of some of the vital antiviral drugs used against COVID-19, Phys. Chem. Chem. Phys. 22 (48) (2020) 28115–28122. Panel (B): Mechanism adapted from Z. Jin, et al., The ambiguous base-pairing and high substrate efficiency of T-705 (favipiravir) ribofuranosyl 5'-triphosphate towards influenza A virus polymerase, PLoS One 8 (7) (2013) e68347.*

favipiravir at the cost of viral fitness [270]. However, this loss of fitness is restored by the PA 653L mutation [270]. Interestingly, resistance to favipiravir has never emerged in clinical trials suggesting that the fitness cost associated with these mutations is likely too great [269].

Several unpublished clinical studies on the efficacy of favipiravir for the treatment of uncomplicated influenza have been performed with variable results [271]. Studies have reported anywhere between a 6 and 14 h decrease in time to influenza symptom resolution vs placebo [271]. This moderate effect, when combined with the fact that teratogenicity has been observed in multiple animal models at doses similar to those used in treatment, limit the utility of favipiravir in the treatment of uncomplicated influenza [271]. However, due to its activity against pandemic influenza and highly pathogenic Avian influenza strains, favipiravir remains clinically significant [272].

Molnupiravir (NHC), a NI targeting the RdRp activity of PB1, has also been evaluated for efficacy against influenza [163,264]. NHC has shown efficacy against influenza A and B in cell culture, mouse, and ferret models of influenza infection with inhibitory concentrations in the nanomolar range [163,264]. Increased frequencies of G to A and C to U transversion mutations were observed suggesting that NHC inhibits influenza via a mechanism involving lethal mutagenesis [163,264]. This is similar to the mechanism which has been recently described for inhibition of SARS-CoV-2 and discussed earlier in this chapter [165]. While efficacy has yet to be demonstrated in clinical trials, given the fact that orally bioavailable prodrugs are available, NHC is a promising candidate for further development [163,264].

The cap binding subunit of the influenza polymerase, PB2, has been targeted by pimodivir. Pimodivir binds to the cap binding domain of PB2 at nanomolar concentrations preventing the binding of capped host mRNAs. This, in turn, prevents cap-snatching and viral transcription [273]. However, pimodivir is only active against influenza A viruses, likely owing to significant amino acid sequence divergence in the cap binding domain of PB2 between different genera of influenza [273]. Resistance to pimodivir has been reported with the Q306H, S324I, S324N, S324R, F404Y, and N510T mutations conferring a greater than 10-fold decrease in susceptibility [274]. Furthermore, resistant strains of influenza containing PB2 substitutions have also emerged in clinical trials examining the efficacy of pimodivir [271,275]. The most common of these is the PB2 M431I variant which has been shown to confer a 57-fold reduction in pimodivir susceptibility in cell culture [271,275]. However, interestingly, in patients treated with a combination of a neuraminidase inhibitor (Tamiflu) and pimodivir the emergence of resistant strains was greatly reduced [271,276].

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