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Comparison of the Old and New - Novel Mechanisms of Action for Anti-coronavirus Nucleoside Analogues

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Abstract: Over the past two and a half years the world has seen a desperate scramble to find a treatment for SARS-CoV-2 and COVID. In that regard, nucleosides have long served as the cornerstone to antiviral treatments due to their resemblance to the naturally occurring nucleosides that are involved in numerous biological processes. Unlike other viruses however, it was found early on during the search for drugs to treat SARS-1 and later MERS, that the coronaviruses possess a unique repair enzyme, an exonuclease (ExoN)^[3] which rendered nucleoside analogues useless, thus negating their use.^[4] During the current outbreak however, as both well-known and new nucleoside analogues were investigated or reinvestigated as a possible cure for SARS-CoV-2, several novel and/or lesser-known mechanisms of action were uncovered. This review briefly describes these mechanisms.

Keywords: nucleosides · nucleotides · RdRp · chain termination · lethal mutagenesis · translocation



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viruses and due to their flexibility, can retain their potency when confronted with binding site point mutations. Dr. Seley-Radtke is currently the President of the International Society for Antiviral Research (ISAR), as well as a past President for the International Society for Nucleosides, Nucleotides & Nucleic Acids (IS3NA).

1. Introduction

Nucleoside analogues have long served as the basis for developing effective treatments and cures for many diseases, including those caused by cancers, parasites, and bacteria, but most nota-

bly, against many different viruses.^{[1] [2]} Because even the smallest change to the basic nucleoside scaffold can have profound effects, this has inspired many to design a wide variety of structural modifications, including multiple modifications to the same scaffold. Currently there are more than 30 FDA-approved nucleoside/nucleotide analogues approved for use. Moreover, as the field has progressed and more complex modifications have been pursued, new and more complex mechanisms of action have been discovered, with some nucleoside analogues working by more than one mechanism.

2. Chain termination

For years it has been well known that most nucleoside analogues targeting viral polymerases, following conversion by kinases to their active triphosphate form, work by competitive inhibition of the viral polymerases, and in most cases, chain termination, either obligate, non-obligate/delayed or pseudo-obligate.^[1,2,5] Each involves a key 3'-position of the nucleoside's sugar, as well as potential steric considerations of neighboring positions (Figure 1).^[5]

As shown in Figure 2, obligate chain termination occurs with nucleosides lacking the 3'-hydroxyl group. This group is absolutely necessary for the next incoming nucleotide to attach to, thereby extending the growing nucleic acid chain. Some examples

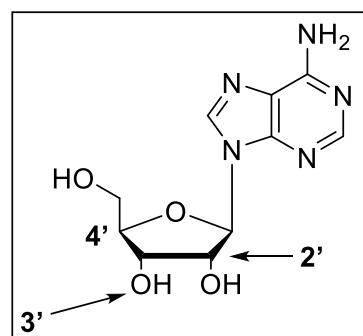


Figure 1. Key mechanistic positions.

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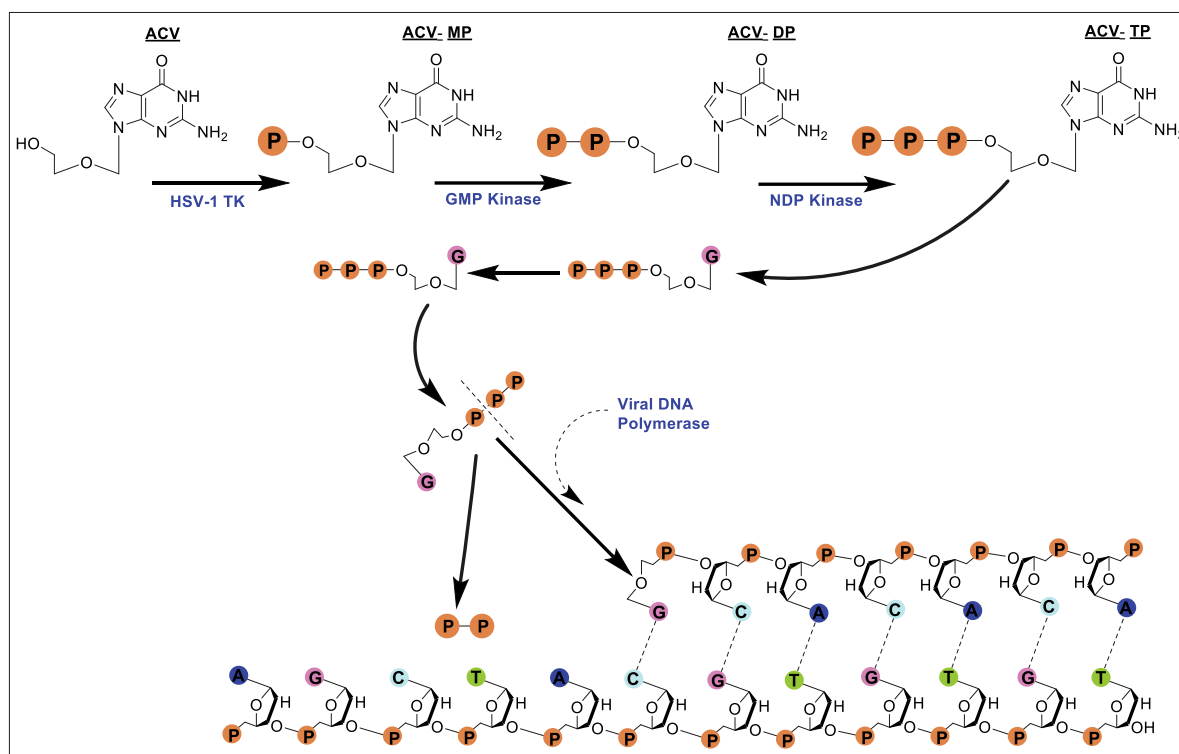


Figure 2. Obligate chain termination mechanism as shown with acyclovir. Figure adapted from Deval et al.^[5]

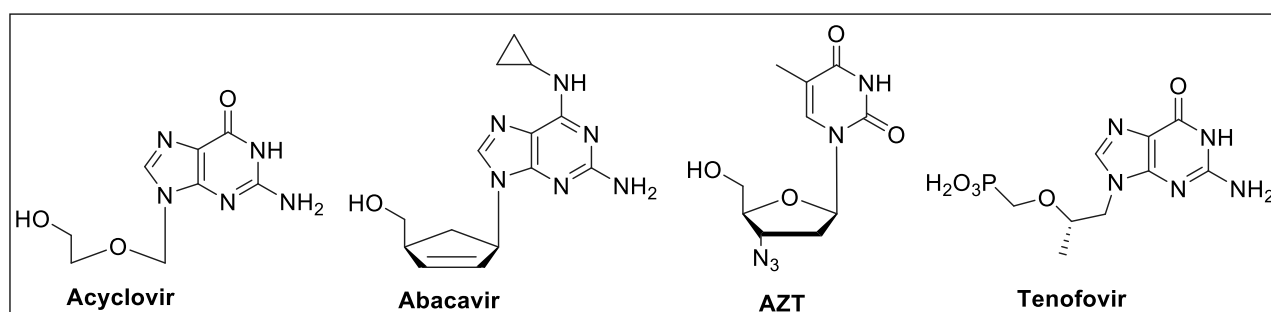


Figure 3. Obligate chain terminators.

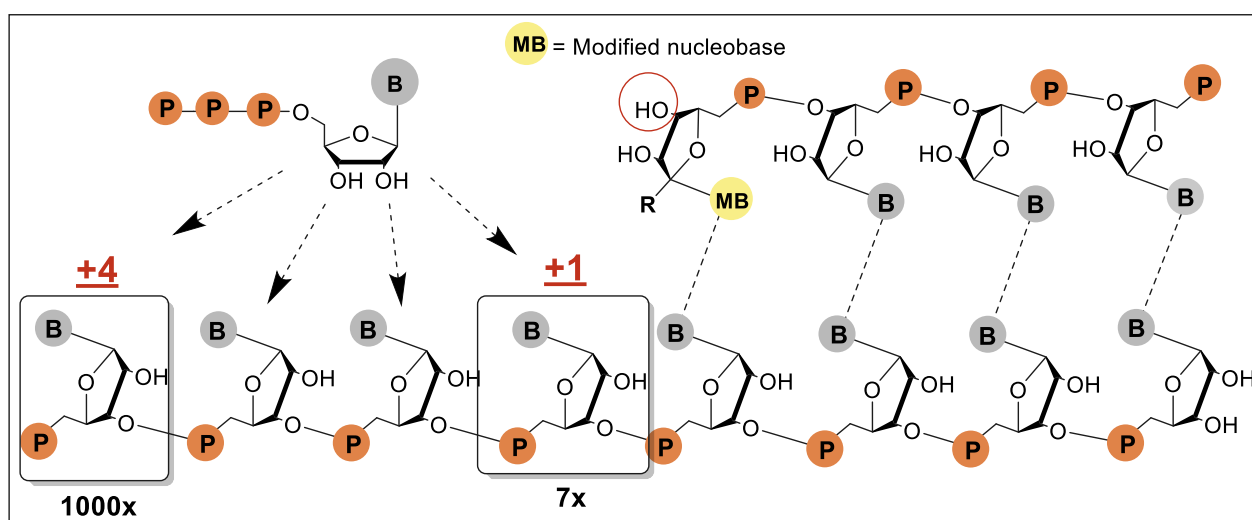


Figure 4. Delayed/non-obligate chain termination mechanism. Necessary 3'-OH of modified nucleoside circled with red circle. Figure adapted from Deval et al.^[5]

of these include acyclovir (ACV, Figure 3), which targets the herpes viral DNA polymerase, while AZT, abacavir, and tenofovir (Figure 3) all target the viral reverse transcriptases (RT) and are used against HIV and HBV.^[5]

In contrast, nucleosides that work by delayed or non-obligate chain termination do possess a 3'-hydroxyl group that can lead to additional incorporation of incoming nucleotides. Due to sterics at neighboring positions however, each subsequent addition occurs more slowly, until after two to four incorporations, no more additions occur (Figure 4).^[5] This is thought to be due to a kink forming in the growing nucleic acid chain, which eventually leads to a non-functional nucleic acid. One example is entecavir (sold as Baraclude), a viral RT competitive hepatitis B inhibitor (Figure 5).^[6,7] Newer nucleosides working by this mechanism include sofosbuvir^[8,9], remdesivir (GS-5734)^[10–12] which is sold under the brand name Velkury, and the corresponding parent nucleoside GS-441524^[13], which is used for feline coronaviruses (Figure 5). All three of these inhibit viral RNA-dependent RNA polymerases (RdRps).

Sofosbuvir is part of a cure for hepatitis C and remdesivir was initially investigated against a number of viruses including Ebola^[11,12] and MERS^[11]. Interestingly, in the case of remdesivir, it allows for three additional nucleosides to be incorporated into the growing RNA chain by the RdRp of SARS-CoV-2, but five by the RdRp for Ebola.^[14] Sofosbuvir is a cure for HCV and sold under the brand name Sovaldi but is also used in combination with velpatasvir and sold under the name Epclusa. Also, both sofosbuvir and remdesivir are McGuigan ProTides, and while sofosbuvir can be delivered orally, remdesivir must be administered by IV in a hospital setting.

Finally, nucleosides that work by the pseudo-obligate mechanism also possess a 3'-hydroxyl, but due to substituents on adjacent carbons, which cause steric hinderance, lead to immediate cessation of the growing chain.^[5] Examples of pseudo-obligate terminators include the 4'-modified nucleosides such as azvudine (also known as FNC)^[15], islatravir^[16], balapiravir^[17], and AL-335^[18] (Figure 6).

3. Lethal mutagenesis

Another mechanism of action for several nucleosides that are currently being investigated for use against SARS-CoV-2, but that has not seen much success, is lethal mutagenesis. Lethal mutagenesis focuses on the ability of a nucleoside analogue to increase the error rate of rapidly replicating RNA viruses such that it overwhelms the cellular repair mechanisms, eventually driving the virus to extinction.^[19] This is accomplished using nucleosides that have the ability to base pair ambiguously, sometimes known as universal bases, thus causing base pairing mismatches, which

in turn lead to mutations that increase each round until the virus can no longer function.^[19] One of the first nucleoside analogues to be identified to work via this route was ribavirin (Figure 7).^[20]

Ribavirin exhibits pleotropic effects on cells, however the mechanism leading to ribavirin's rather limited antiviral effects *in vivo* have yet to be fully elucidated, and in fact, many different modes of action have been attributed to ribavirin's antiviral effects.^[20] Ribavirin's ability to form base pairs mimicking either adenosine or guanosine leads to the mismatches. Unfortunately, some of the nucleosides, for example favipiravir, that work by this mechanism have also shown significant toxicity.^[21]

In that regard, a second nucleoside, which is administered as the heterocyclic base and is transformed into the nucleoside following *in vivo* ribosylation, is favipiravir (Figure 8). Favipiravir has been shown to work by both lethal mutagenesis as well as chain termination.^[22] In some studies however, favipiravir exhibited toxicity in animals, thus raising concerns for use in humans.^[23] Despite this, favipiravir is approved for use against certain types of influenza^[22], however only in Japan, but is currently being evaluated in numerous clinical trials around the world against SARS-CoV-2. One such study showed it was quite effective and safe, and exhibited high NTP incorporation rates, as well as high error rates, thus representing an "Achilles' Heel" that could be exploited.^[24]

More recently another broad-spectrum antiviral nucleoside that has been around for some time and also works by lethal mutagenesis is N-hydroxycytidine (NHC) now known as molnupiravir (Figure 9). Molnupiravir recently received emergency approval for use against SARS-CoV-2 and COVID; however, the FDA approved it by a slim margin, likely due to it exhibiting only a 30% reduction in hospitalization. Molnupiravir possesses a hydroxylamine group at the 4-position of the base, thus allowing it to be recognized as either cytidine or uridine, again, leading to mismatches.^[25] There is significant controversy over the use of molnupiravir, given the risks for cancer, but more importantly, toxicity to both developing fetuses and male germline cells, as well as its lack of efficacy and the availability of other drugs that not only work much better, but are not associated with toxicity.^[26]

4. Nucleoside analogues exhibiting multiple mechanisms

Another nucleoside that was approved for emergency use against SARS-CoV-2 and COVID is remdesivir. Remdesivir was first studied for use against Ebola, and was in fact, used in several compassionate use cases, but was later abandoned after several monoclonal antibody treatments showed significantly better results. Not just limited to Ebola, Remdesivir exhibits broad spectrum activity against SARS-1, MERS, and more recently respiratory syncytial virus, as well as several other viruses, thus was immediately investigated for potential activity when the current

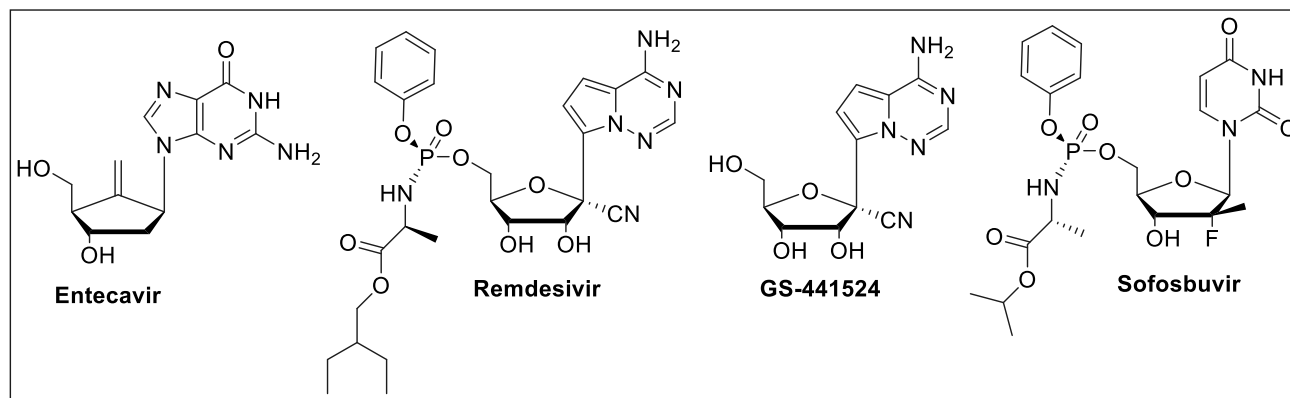


Figure 5. Delayed/non-obligate chain terminators.

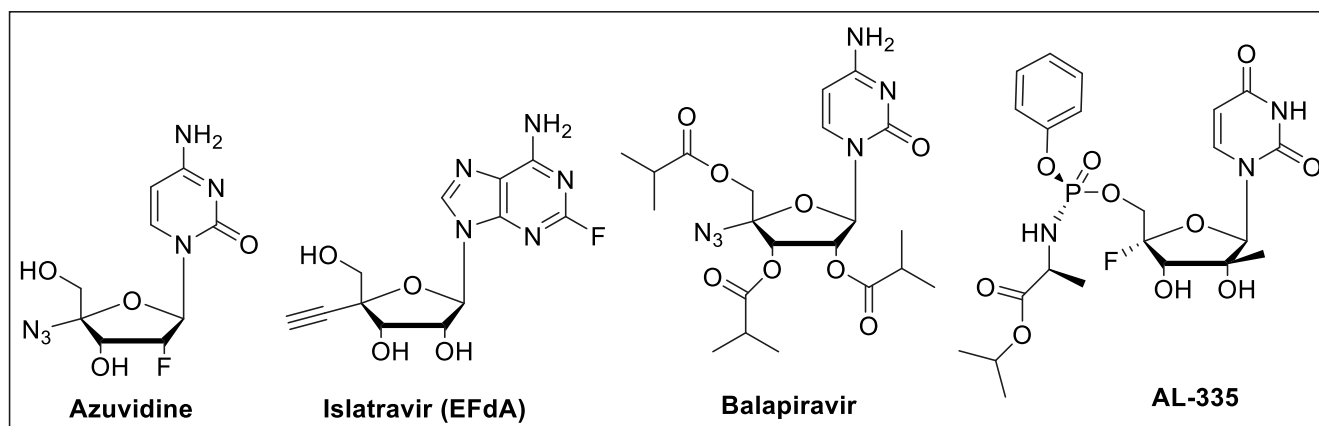


Figure 6. Pseudo-obligate chain terminators.

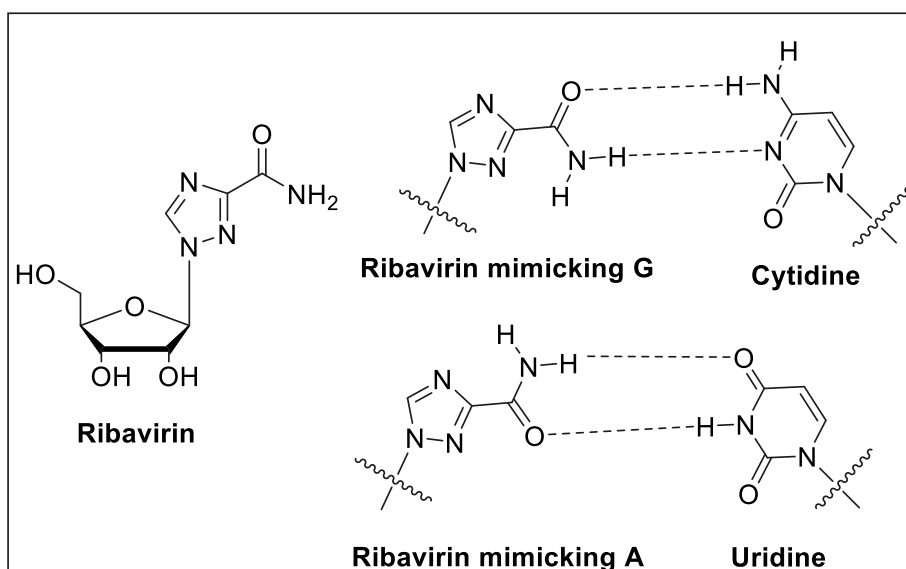


Figure 7. Ribavirin mismatch base pairing.

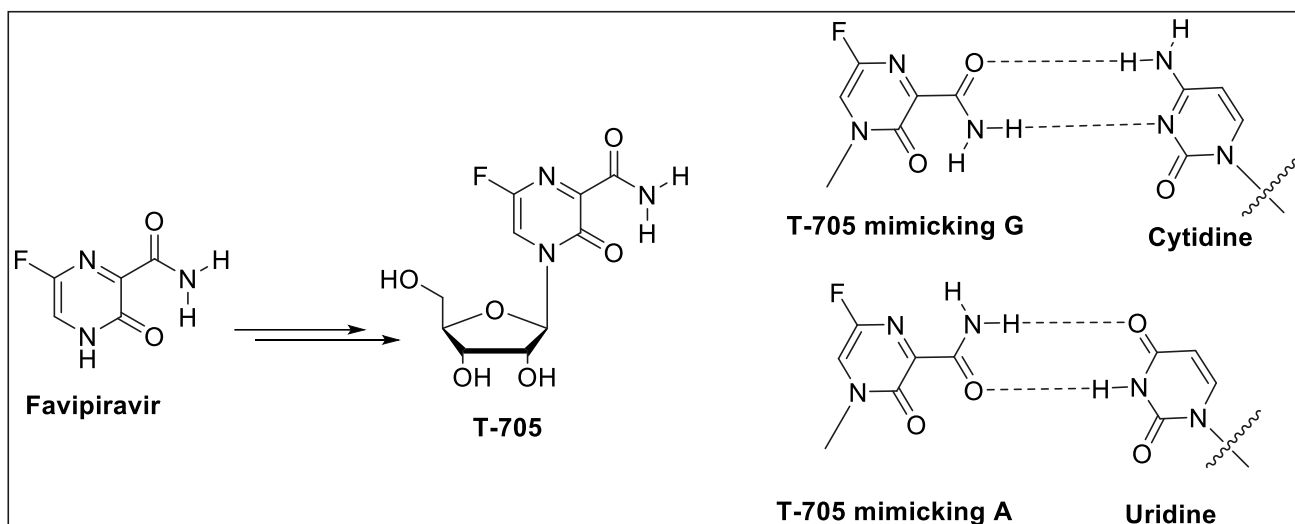


Figure 8. Favipiravir/T-705-TP mismatch base pairing

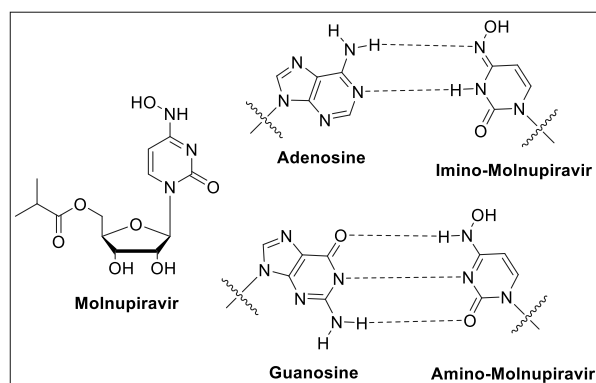


Figure 9. Molnupiravir's tautomeric mismatch base pairing.

pandemic broke out.^[10] Limited by its lack of oral bioavailability, thus requiring administration in a hospital setting, new treatments have been pursued.

As mentioned earlier, it was known from the previous studies against Ebola and MERS that remdesivir works by delayed chain termination, with additional nucleotides being incorporated prior to chain termination. During the course of the early investigations against SARS-CoV-2 however, it was found that remdesivir also works by a second mechanism, described as pausing or stalling. Several groups have studied remdesivir and the stalling mechanism (Figure 10)^[27,14], including Seifert et al., who utilized a single molecule approach.^[28] Employing a magnetic tweezer

approach, they were able to investigate the stalling mechanism as well as how the SARS-CoV-2 RdRp is interacting with remdesivir.^[28] Their conclusions are that inhibition of SARS-CoV-2 involves both the primer strand and the template.^[27]

In that regard, similar to remdesivir's chain termination mechanism, following incorporation the chain is extended by three additional nucleosides, however at this point incorporation pauses or stalls, but then restarts and the chain continues to grow (Figure 10).^[28] This stalling occurs due to a translocation barrier caused by a steric clash between the 1'-cyano group and serine-861 in the nsp12 polymerase.^[14] When Ser-861 was mutated, the stalling did not occur, suggesting this particular mutation is critical for that mechanism. It has also been observed that both stalling and chain termination can be overcome by increasing the nucleotide pools. This led Tchesnokov et al.^[27] to speculate whether or not remdesivir caused template dependent inhibition (Figure 10). When remdesivir is present in the template RNA strand, pairing to UTP is hindered due to improper positioning of remdesivir because of a steric clash with alanine-558. But when an increase in the next nucleotide triphosphate occurs, the inhibition of the RdRp complex is overcome. The resistance-associated Val557Leu mutant enzyme helps decrease the concentration of nucleotide triphosphate required for incorporation, therefore producing low level resistance. Notably, internal incorporated remdesivir molecules appear to be protected from excision by the unique coronavirus exonuclease (ExoN), which has rendered numerous nucleoside analogues useless against the coronaviruses.^[27]

Similarly, islatravir (EFdA) shown in Figure 6, also works by multiple mechanisms, including pseudo-obligate chain termination as mentioned above, but also by several others including

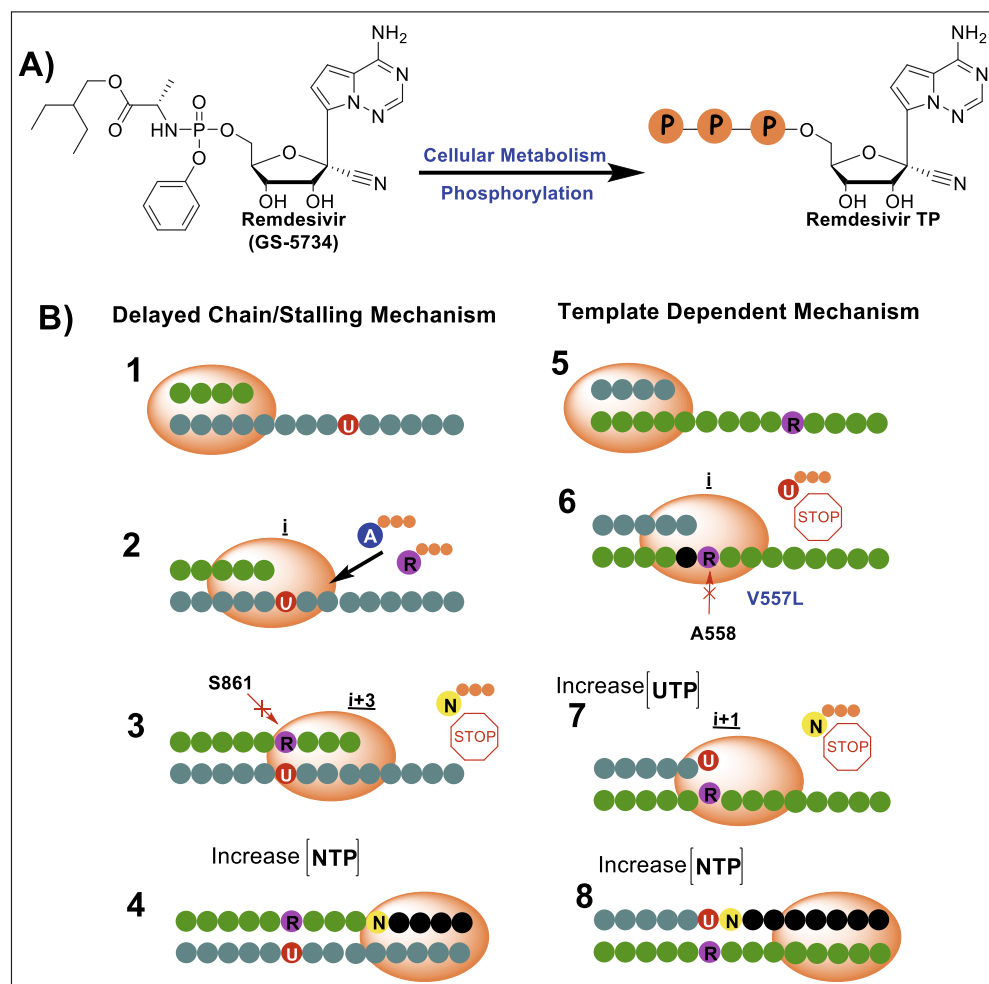


Figure 10. Remdesivir dual mechanism of action. **A)** Conversion of Remdesivir to its active triphosphate form. **B)** Delayed chain/stalling mechanism of Remdesivir (steps 1-4). Template dependent mechanism (steps 5-8). Dark green circles represent the priming strand; gray circles represent template strand; orange oval is the RdRp complex; Remdesivir triphosphate (pink circle with small orange circles); ATP (blue circle with small orange circles); Nucleotide TP (yellow circle with small orange circles); UTP (red circle with small orange circles); stop sign represents inhibition of subsequent nucleotide addition; read through of RNA strand (black circles). Figure adapted from Tchesnokov et. al.^[27]

translocation, delayed chain termination, as well as misincorporation leading to mismatched primers that cannot be excised.^[16] Notably, islatravir was the first nucleoside to be named as a nucleoside reverse transcriptase translocation inhibitor, or NRTTI.^[16]

Interestingly, each of the distinct features of islatravir are responsible for key aspects of its exceptional potency and effectiveness, as well as the unique mechanisms of action (Figure 11). For example, the fluorine on the nucleobase protects the amine group from deamination by adenosine deaminase and the presence of the 3'OH leads to increased recognition by the kinases.^[16] However, it is the 4'-ethynyl group that is responsible for the unique mechanisms associated with this nucleoside. First, it is involved in highly favorable interactions in a hydrophobic pocket in the RT binding site. Then, after incorporation into the primer, the ethynyl group is responsible for the translocation inhibition of the extended primer, by hindering binding and incorporation of subsequent nucleotides.^[16,29] In addition, depending on the particular sequence, islatravir has been shown to be a delayed chain terminator, with the ethynyl group causing a steric clash with the "primer grip" region of the RT.^[16,29]

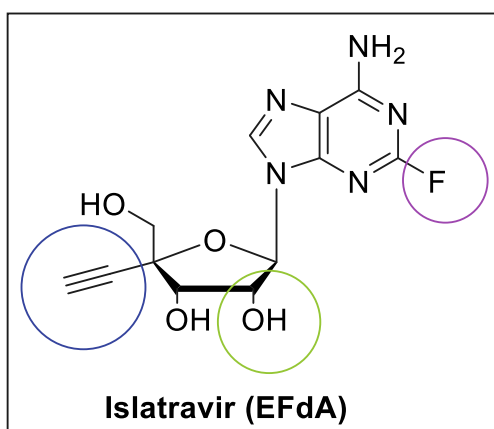


Figure 11. Islatravir's strategic structural modifications.

Another nucleoside analogue developed by Atea Pharmaceuticals that has been studied for potential use against SARS-CoV-2 is AT-527 or AT-511. AT-527 is the hemi-sulfate salt, AT-511 is the neutral form, and AT-9010 is the triphosphate (Figure 12).^[30] AT-527/511 possesses a McGuigan ProTide similarly to sofosbuvir and remdesivir, and its sugar moiety is identical to that of sofosbuvir, possessing a 2'-fluoro, 2'-methyl substitution. This modification prevents correct alignment of the next incoming nucleotide, which in the published figure was another molecule of AT-9010, but in cells can be any nucleoside triphosphate, thus leading to immediate (pseudo-obligate) chain termination. AT-527 initially showed potent activity against Hepatitis C^[31] and was immediately looked at for use against SARS-CoV-2. AT-527 was advanced through to Phase III clinical trials for SARS-CoV-2^[32], however Atea halted the Phase III trial (<https://clinicaltrials.gov/ct2/show/NCT04889040>) due to a lack of efficacy and is currently reassessing a path forward.

Interestingly however, during the initial studies on AT-527's activity, it was discovered that AT-527 works by a dual mechanism, albeit much different than remdesivir. In that regard, unique to coronaviruses, the nsp12 of SARS-CoV-2 and other coronaviruses contain two functional domains, including the RdRp and a nidovirus RdRp-associated nucleotidyltransferase (NiRAN), which primes the RdRp for RNA synthesis. Atea's studies have shown that three molecules of AT-9010 occupy two different sites

in the binding sites – one incorporated in the RdRp, another in the channel, and finally, a third in the NiRAN binding site (Figure 13).^[32,33]

In that regard, Shannon et al.^[34] showed that the first AT-9010 is incorporated in the active site at the 3' end of the RNA strand, with a second AT-9010 stalled in the channel, leading to the aforementioned chain termination. Finally, a third molecule of AT-9010 is bound in the NiRAN site. Their studies also showed that the guanine base is in a flipped orientation in contrast to native NTPs, occupying a previously unidentified cavity. Notably, AT-9010 outcompetes all native NTPs for the NiRAN binding site, thus inhibiting its nucleotidyl-transferase activity. Another interesting aspect, AT-9010 also appears to be resistant to excision by the ExoN unlike many other nucleosides.^[32]

Finally, a very recent entry on the horizon involves a very unique mechanism of action for a novel liver-targeted nucleoside developed by Antios Therapeutics that has shown potent activity against HBV, ATI-2173 (Figure 14).^[35] ATI-2173 is the phosphoramidate analogue of clevudine, and was designed to deliver the 5'-monophosphate to the liver, and once in the liver, is converted to the 5'-triphosphate. What is unique is the mechanism of action – ATI-2173 is a first in class non-competitive non-chain terminating nucleoside. As described above, most nucleosides that target the viral polymerases typically act as competitive chain terminating inhibitors, however ATI-2173 does neither, yet is able to completely shut down all functions of the HBV reverse transcriptase. The compound binds to the active site of the polymerase and subsequently blocks DNA synthesis at all stages including priming initiation and polymerization, despite not being incorporated into the growing chain.^[35]

5. Inhibition of methyltransferases

In contrast to the aforementioned nucleoside analogues all of which target the viral polymerases in one way or another, in 2015 the Seley-Radtke group were the first to report low single digit μM levels of activity against coronaviruses, including SARS-1, MERS, and human CoVs.^[36] Several fleximer analogues of acyclovir (Figure 15) exhibited potent activity, while acyclovir showed no activity up to 1000 μM . Moreover, these same analogues then showed potent activity against Ebola and Marburg,^[37] and nanomolar levels of activity against Dengue and Yellow Fever viruses.^[38] Initially it was thought that they too would exert their antiviral activity as polymerase inhibitors, however it was later shown that instead, they inhibited the viral methyltransferases.^[38] Currently these analogues are being investigated against SARS-CoV-2, however those results will be reported elsewhere as they become available.

6. Conclusions and future outlook

In this short review we have outlined the mechanisms of action for how the various different nucleoside analogue scaffolds work, both old and new. Although these are just a few of the nucleosides that are being explored for possible use against SARS-CoV-2, there are obviously many more under investigation, particularly given the highly creative structural modifications that are currently being pursued. Moreover, now that these new and novel mechanisms have been revealed, it will be interesting to see if previously explored nucleosides may also work by some of these unique mechanisms. Given that it has recently been shown that several of the nucleosides highlighted work by more than one mechanism, particularly those with more complex structures, it is likely that we still have much to learn about just how nucleoside analogues work. Moreover, the more information we have about mechanistic subtleties, the more effective our design efforts will be in developing new and more efficacious broad-spectrum analogues, particularly those that could prove useful for emerging and reemerging infectious diseases.

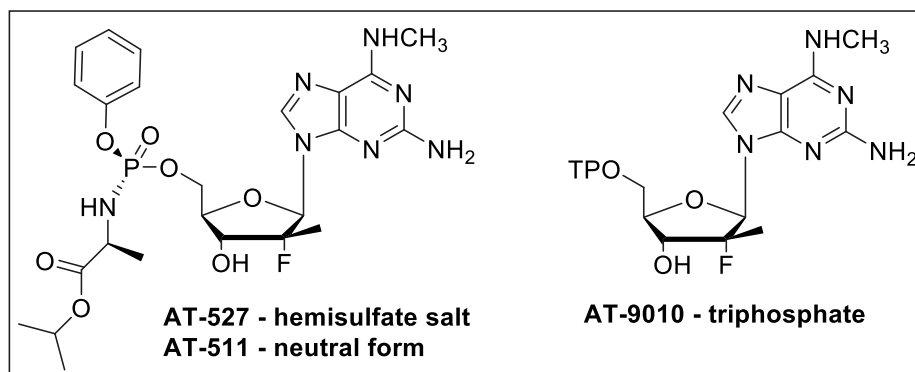


Figure 12. Structures of AT-527/511 and AT-9010.

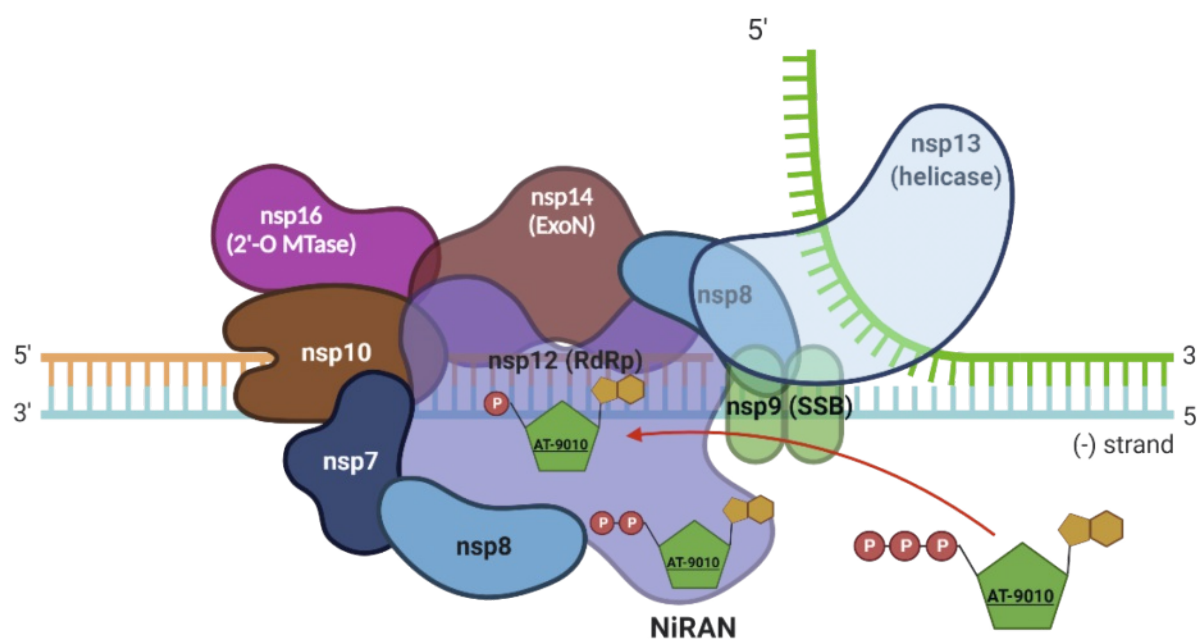
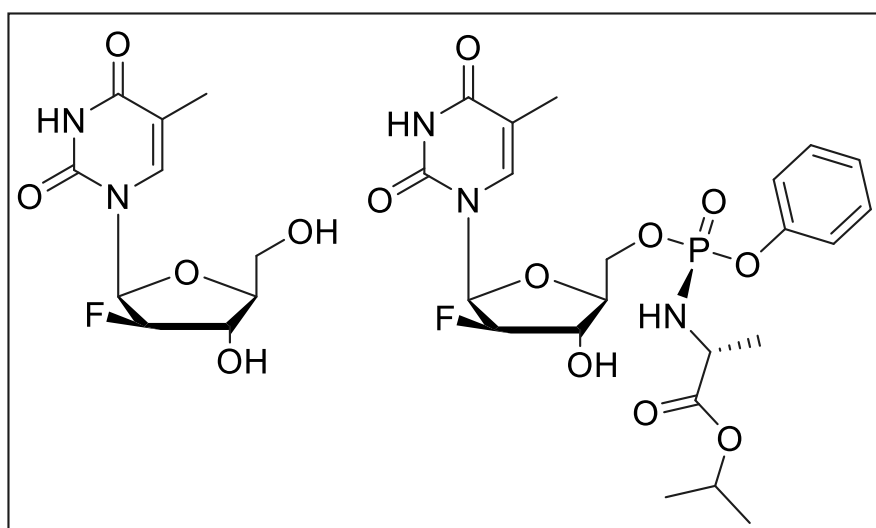
Figure 13. Mechanism of action of AT-527/AT-9010. Figure is depicting SARS-CoV-2 RdRp complex and the incorporation of AT-527/AT-9010. AT-9010 monophosphate in the RdRp, along with an incoming AT-9010 triphosphate in the channel. AT-9010 diphosphate occupies the NiRAN binding site. Figure adapted from Hartenian et al. and made via Biorender.com.^[33]

Figure 14. Structures of Clevudine (left) and ATI-2173 (right).

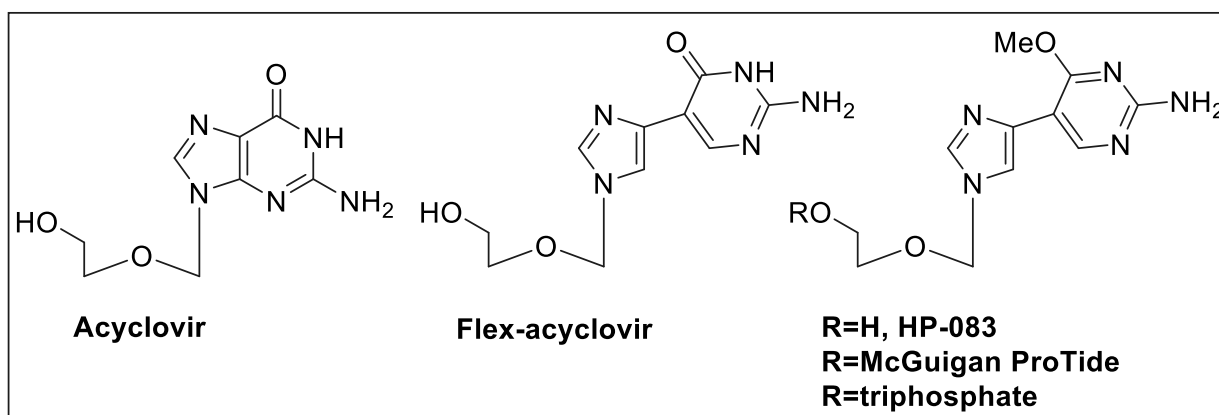


Figure 15. Acyclovir and Flex-Acyclovir analogues.

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