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Targeting viral genome synthesis as broad-spectrum approach against RNA virus infections

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Abstract

Zoonotic spillover, i.e. pathogen transmission from animal to human, has repeatedly introduced RNA viruses into the human population. In some cases, where these viruses were then efficiently transmitted between humans, they caused large disease outbreaks such as the 1918 flu pandemic or, more recently, outbreaks of Ebola and Coronavirus disease. These examples demonstrate that RNA viruses pose an immense burden on individual and public health with outbreaks threatening the economy and social cohesion within and across borders. And while emerging RNA viruses are introduced more frequently as human activities increasingly disrupt wild-life eco-systems, therapeutic or preventative medicines satisfying the "one drug-multiple bugs"-aim are unavailable. As one central aspect of preparedness efforts, this review digs into the development of broadly acting antivirals *via* targeting viral genome synthesis with host- or virus-directed drugs centering around nucleotides, the genomes' universal building blocks. Following the first strategy, selected examples of host *de novo* nucleotide synthesis inhibitors are presented that ultimately interfere with viral nucleic acid synthesis, with ribavirin being the most prominent and widely used example. For directly targeting the viral polymerase, nucleoside and nucleotide analogues (NNAs) have long been at the core of antiviral drug development and this review illustrates different molecular strategies by which NNAs inhibit viral infection. Highlighting well-known as well as recent, clinically promising compounds, structural features and mechanistic details that may confer broad-spectrum activity are discussed. The final part addresses limitations of NNAs for clinical development such as low efficacy or mitochondrial toxicity and illustrates strategies to overcome these.

Keywords

Nucleoside analogues, RNA polymerase, inhibitors, replication

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Introduction

When a novel virus acquires the ability to efficiently transmit between humans, it poses an imminent pandemic risk.[1,2] Among (re-)emerging RNA viruses, the influenza A virus takes on a prominent role: New strains can and have evolved *via* reassortment of gene segments within co-infected hosts (antigenic shift)[3] and continual mutations in the viral genome (antigenic drift) can lead to vaccine mismatches[4] and resistance to existing drugs.[5,6] Influenza A virus strains are classified by their surface glycoproteins hemagglutinin (H), which is required for virus attachment, and neuraminidase (N), required for virus budding. Of these proteins, 18 (H) and 11 (N) different subtypes have been identified and they determine the virus' tropism and pathogenicity. Since the beginning of the 20th century, five major flu pandemics have occurred starting with the 1918 flu pandemic ("Spanish flu"), caused by an H1N1 virus, that led to devastating disease with global death tolls of an estimated 50 million people,

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accompanied by massive socio-economic impact.[7,8] This was followed by the emergence of a novel, H2N2-strain which caused 1–4 million deaths in 1957 ("Asian flu") and only 11 years later, the emerging H3N2 virus brought about the 1968 flu pandemic ("Hong Kong flu") that had a similar impact. In 1977, there was an outbreak ("Russian flu") of what is believed to be a re-emerged H1N1 virus and in 2009, a reassortant H1N1 influenza A virus caused the first flu pandemic of the 21st century ("Swine flu"), only 90 years after the devastating 1918 pandemic.[9] This virus (H1N1pdm09), as well as the H3N2 strain are still circulating in the human population causing seasonal outbreaks. Aquatic birds harbor a vast reservoir of avian influenza A viruses (AIVs), which can evolve to highly pathogenic avian influenza A viruses (HPAIVs) and cause severe outbreaks with huge economic cost in farmed poultry.[2,10] Occasionally, humans are infected with avian strains by zoonotic spillover, e.g. in the context of poultry farms,[1,9,11]and numerous events have been reported where people were infected with HPAIV strains H5N1 and H7N9 through close contact with infected animals or *via* contaminated environments which led to severe disease with high fatality rates.[9] Although thus far, sustained human-to-human transmission has not been observed, HPAIVs might acquire this ability *via* antigenic drift and/or shift.[9] The introduction of a novel influenza virus with the ability to efficiently spread among humans into the immuno-naïve human population would naturally lead to massive spread and severe disease,[2] potentially cause high fatality rates and have drastic socio-economic consequences. Accordingly, HPAIVs are attributed a high pandemic risk[2] and in addition to surveillance, other preparedness efforts are warranted.

Many other RNA viruses continue to repeatedly cross the species barrier from animal to human, and once spillover has occurred, some achieve efficient human-to-human transmission and can thus cause outbreaks of different size. For instance, among the numerous hemorrhagic fever viruses, *Filoviridae* have repeatedly been introduced from wildlife reservoirs causing severe disease and high mortality in humans.[12] The 2014 Western Africa epidemic was the largest Ebola virus outbreak yet, recording almost 30,000 infections with more than 11,000 resulting in death, according to WHO statistics.[13] Further, a large variety of negative strand RNA ((–)ssRNA) viruses belonging to the order of *Bunyavirales*, such as the Andes (hantavirus),[14] Lassa (arenavirus),[15] and Crimean-Congo hemorrhagic fever virus (nairovirus),[16] cause frequent disease outbreaks of different size *via* cross-species transfer in many parts of the world. Another example of a viral zoonotic disease gaining attention is caused by Nipah virus, a member of the *Paramyxoviridae*. It causes severe disease in humans with high case fatality rates of up to >70% and with its broad host range, Nipah virus is able to infect and spread among different animals as well as humans, hence its classification as a public health concern.[17,18] Among the positive strand RNA ((+)ssRNA) viruses, different coronaviruses have recently emerged from animal reservoirs and caused severe outbreaks of respiratory disease, such as SARS, MERS, and, importantly, the COVID-19 pandemic, the largest pandemic since the 1918 flu.[19] The causative agent, SARS-Coronavirus-2, was only recently introduced into the human population and is continuing to spread while this review is being written. It spreads *via* the respiratory route and has already had most devastating effects on the lives of people around the world with recorded case numbers in the double-digit million range (August 2020); in consequence, it crashes economies and threatens international piece and cooperation. While vaccine candidates are being developed and public health measures are implemented, rapid pandemic response is needed to limit the spread and impact of the virus. Treatment for those suffering from the disease is hampered by the lack of effective antiviral medication; thus, WHO's solidarity[20] as well as a multitude of other recently launched clinical trials are aimed at identifying efficient treatments among available drugs that had previously demonstrated *in vitro*- or *in vivo*-potency against related viruses.[21]

These examples dramatically highlight the urgent need for better and more effective preparedness measures and with it, research to develop broad-spectrum antiviral drugs is gaining momentum. This review examines strategies that ultimately target viral nucleic acid synthesis to stop viral infection. Generally, viral RNA polymerases are considered error-prone and most RNA viruses do not possess any error-correcting function, hence they acquire a comparably high number of mutations during genome replication.[22] As a consequence, these viruses evolve rapidly and therefore, they're considered a high risk for zoonotic disease transmission. Adding to this risk, they can quickly become resistant to treatment or escape vaccine-induced immunity by the same mechanism.[23,24] However, this trait can also be exploited to defeat a virus and hence, the viral polymerase sometimes is referred to as its "Achilles heel".[24] Following this approach, viral infection can be treated with compounds that either indirectly or directly block viral nucleic acid synthesis, or those that drive the virus' mutation rate over a threshold and thereby stop the production of infectious virus (often referred to as lethal mutagenesis).[22,23] Virus-targeted examples for such compounds are nucleobase, nucleoside or nucleotide analogues (NNAs) that – after being activated to

their corresponding non-natural nucleoside 5'-triphosphate (NTP) form *via* host cell pathways – are "wrongly" incorporated into the viral genome by the viral polymerase. Host-targeted strategies include compounds that block cellular pathways for the production of natural NTPs, thereby depriving the polymerases of their natural substrates – an established approach which is used in anticancer chemotherapy as well. The following sections will examine both these strategies in more detail.

Targeting viral RNA synthesis *via* inhibition of the host *de novo* nucleotide pathways

Upon infection, viruses release their cargo into the host cell and use some of the cell's own machinery and metabolites to replicate their genome, produce viral progeny and infect new cells. Thus, in a host-targeted approach, the inhibition of certain enzymes or factors of the infected cell can stop the virus from replicating. And since different viruses rely on the same host cell processes, broad-spectrum activity may be achieved. Further, host-targeted compounds benefit from a high barrier to resistance since they aren't under the control of the error-prone viral replication machinery that often leads to drug-evasion of fast-evolving viruses.[25,26] Especially RNA-viruses, owing to their short generation times,[22,27] require high amounts of NTPs from the host's cellular pool to sustain the rapid replication of their genome in acute infection. Hence, by creating an imbalance in the cells' own NTP pool, the number of mutations that the viral RNA genome acquires during replication can be increased or, simply by depriving the enzyme of its substrates, viral genome synthesis can be stopped altogether. As with all host-directed strategies, however, a balanced inhibition of the cellular pathway is required to maintain cell viability while efficiently blocking the infection.[26] With regard to nucleotides, mammalian cells not only rely on *de novo* biosynthesis but also harbor salvage pathways that recycle purine or pyrimidine nucleotides from e.g. nucleic acid degradation products. While the salvage pathways are able to sustain cell viability, they may not supply sufficient amounts of NTPs to allow fast proliferation of e.g. malignant cells or support viral replication; thus, in addition to antiproliferative therapies, the inhibition of the *de novo* pathways for nucleotide biosynthesis constitutes a broad-spectrum antiviral strategy.[28,29]

Blocking de novo *pyrimidine nucleotide biosynthesis*

In the *de novo* pyrimidine nucleotide biosynthetic pathway, dihydroorotate dehydrogenase (DHODH) takes on a central role and its inhibition reduces cellular UTP and CTP,[30] and consequently also dCTP and TTP concentrations (Figure 1).[32] A very potent inhibitor of DHODH, Brequinar (Figure 1), has been investigated as potential anti-cancer as well as immunosuppressant drug; however, data from clinical trials suggest that this drug is unable to efficiently inhibit DHODH in solid tumors while causing severe side effects systemically.[33] These unfavorable therapeutic properties so far excluded Brequinar from gaining approval. One example of a less potent yet more balanced DHODH inhibitor in clinical use is Teriflunomide (Figure 1), an immunosuppressive drug that is applied in the treatment of Multiple Sclerosis. Similarly, its prodrug Leflunomide is applied in Rheumatoid Arthritis[28] and both compounds, along with other DHODH inhibitors, have gained attention as potential broad-spectrum antivirals.[34] Because they're targeting a cellular enzyme that is essential in covering the high (d)NTP demand of replicating viruses, these compounds are able to inhibit not only diverse RNA, but also retro- and DNA viruses.[26,35] However, so far none of them have successfully completed clinical development as anti-infectives. One limiting factor to their *in vivo* efficacy may lie in the high plasma levels of uridine, which can enter cells *via* specific transporters and thus quickly compensate for DHODH inhibition, thereby counteracting the drugs' effect.[28] Still, efforts to develop broadly acting antivirals *via* targeting DHODH continue and a recent study reported the discovery of two promising DHODH inhibitors S312 and S416 (Figure 1) with low *in vivo* toxicity starting from a virtual screening.[34] Both compounds were able to inhibit the replication of different influenza A viruses, Zika- and Ebola virus, as well as, importantly, SARS-CoV-2.[34]

Blocking de novo *purine nucleotide biosynthesis*

Because of its crucial role in the *de novo* biosynthesis of guanosine nucleotides, inosine monophosphate dehydrogenase (IMPDH) represents another well-known host-directed target that is clinically exploited in not only anticancer and immunosuppressive, but importantly also in antiviral therapy (Figure 2).[37] Similar to the above discussed DHODH inhibitors, compounds that block IMPDH deplete cellular purine nucleotides and thus show antiproliferative and immunosuppressive activities, such as the highly potent mycophenolic acid (MPA).[38–40] Yet, even though MPA and its analogues also potently inhibit the replication of diverse viruses *via* this mechanism,[41–43] thus far, their clinical use in antiviral therapy appears limited due to toxicity and unfavorable metabolism leading to rapid inactivation of MPA.[44] Another compound that reduces

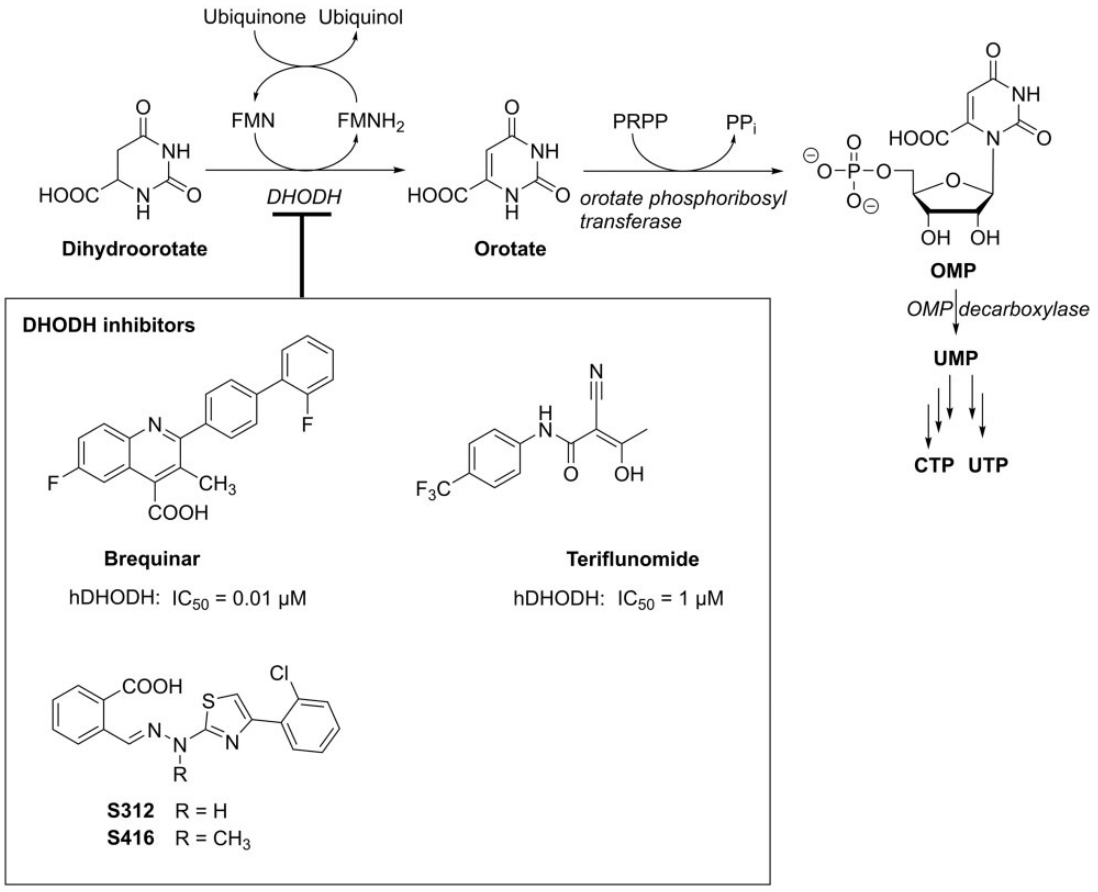

Figure 1. The central role of DHODH in the *de novo* pyrimidine nucleotide synthesis, two of its known inhibitors, brequinar (not currently approved mainly due to its narrow therapeutic window) and teriflunomide (also used in its prodrug form leflunomide for treating autoimmune disorders), and two recently reported inhibitors, S312 and S416. IC_{50} values are half-maximal inhibitory compound concentrations reported in Knecht and Loffler.[31] PRPP – 5-phosphoribosyl pyrophosphate; FMN – Flavin mononucleotide; OMP – Orotidine 5'-monophosphate.

intracellular GTP levels builds on a different molecular scaffold and is widely applied as antiviral in the clinic: Ribavirin (RBV) is a nucleoside analogue that, once converted by host adenosine kinase to the 5'-monophosphate (RBV-MP), mimics IMP and competitively inhibits IMPDH; its binding to the enzyme, however, is much weaker when compared to the uncompetitive inhibitor MPA (Figure 2).[36] RBV is approved for the treatment of respiratory syncytial virus (RSV) infections and, in combination with pegylated interferon-α, RBV has been standard treatment for chronic hepatitis C virus (HCV) infections before the introduction of direct-acting antivirals. While RBV has shown activity against various other and, importantly, highly different viruses in cell culture or animal models,[45] its therapeutic use remains limited. In addition to uncertain or low efficacy in the clinic,[46] RBV can cause severe side effects, such as hemolytic anemia, and teratogenic effects have been observed in animal models.[47,48] Still, as currently neither specific nor broad-spectrum drugs are available for many RNA virus infections, RBV often is the only available option in cases of severe disease or epidemic scenarios. For instance, RBV is used for treating viral hemorrhagic fevers caused by Lassa[49–51] or Crimean-Congo hemorrhagic fever virus[52]; all the while its efficacy remains uncertain.[46] Further, RBV's mechanism of action is still extensively discussed[53–55] and appears to differ for different viruses and under different experimental settings.[56–61] Targets include the here discussed host pathway of *de novo* purine biosynthesis,[56,62] leading to a reduced purine nucleotide pool, as well as downstream effects such as polyamine depletion *via* the induction of spermidine/spermine acetyltransferase-1 SAT1.[61] Additionally, RBV-5'-triphosphate (RBV-TP) can directly target the viral polymerase and its incorporation into the genome causes lethal mutagenesis[63,64] (discussed in more detail in a later section), or it can inhibit RNA capping.[65,66] While it is difficult to discern these different mechanisms of action, the overall observed *in vivo*

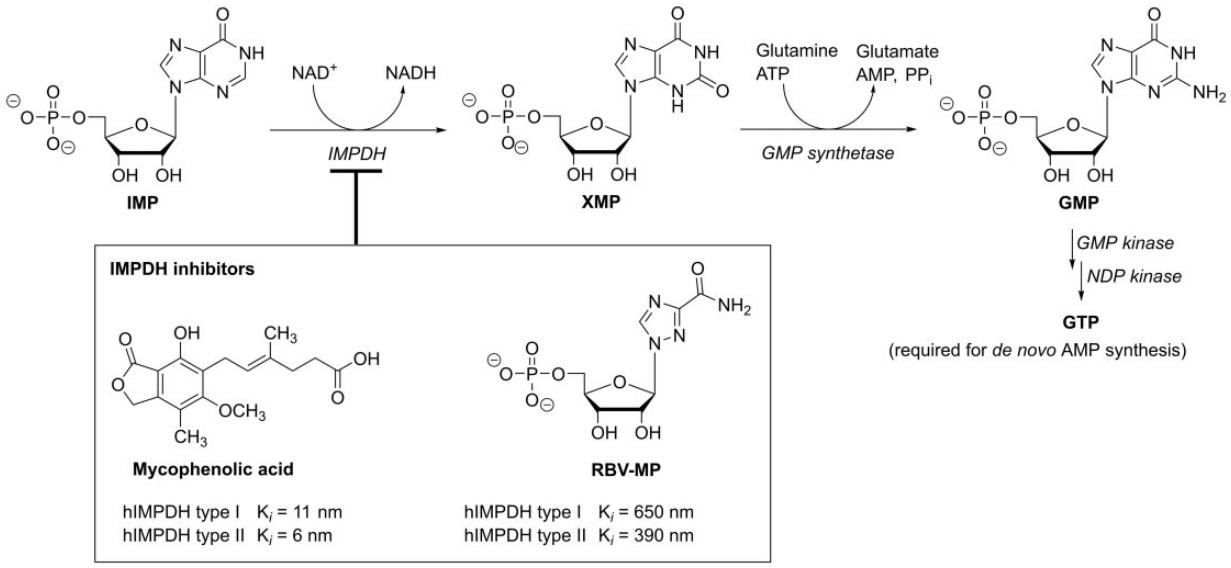

Figure 2. The central role of IMPDH in the *de novo* purine nucleotide synthesis and two of its clinically used inhibitors, MPA (its prodrug MPA-mofetil is used as immunosuppressive drug) and RBV (shown in the active 5'-monophosphate form). Inhibitory constants (K_i) are reported in Hager et al.[36] XMP – Xanthosine 5'-monophosphate, NDP – nucleoside 5'-diphosphate.

antiviral effect may often be a combination. In fact, different mechanisms may even be synergistic, as GTP depletion, for instance, may reduce competition and thus favor RBV-incorporation by viral polymerase.[53]

While RBV is the most prominent broad-spectrum antiviral nucleoside analogue, many derivatives have been developed and tested over the past decades. EICAR-MP, the 5'-ethynyl-derivative of RBV-MP (Figure 3), is also an IMP-analogue and inhibitor of IMPDH,[67] with even greater potency than RBV in its cytostatic as well as broad-spectrum antiviral effects.[68] In contrast to RBV and reminiscent of 6-chloropurine ribonucleotide,[69] EICAR irreversibly inhibits IMPDH by covalently alkylating an active-site cysteine residue in a Michael-type addition.[70] And with RBV's clinical impact as anti-HCV treatment, more analogues based on this molecular scaffold continue to emerge, such as ETAR or IM-18,[71,72] aiming for the development of pan-flavivirus inhibitors with enhanced potency or reduced cytotoxicity.[73]

In RBV and some of its close analogues, the carboxamide-moiety in the nucleobase-part (Figure 3) with its rotational flexibility enables the analogues to mimic not only inosine and guanosine but also adenosine nucleotides; hence, these compounds are recognized by different enzymes of host purine metabolism (in Figures 2 and 3, note that adenosine kinase catalyzes RBV's monophosphorylation while RBV-MP inhibits IMPDH as IMP analogue; this feature also results in what was termed 'ambiguous base pairing' which will be discussed in the following section). Moreover, as can be seen from the examples of tiazofurin and benzamide riboside (Figure 4), the carboxamide-moiety can also lead to compounds being recognized as analogues of the natural nicotinamide nucleotide (NMN, Figure 4): Tiazofurin and benzamide riboside monophosphate are converted to the NAD^+ analogues TAD and BAD, respectively, by host nicotinamide/nicotinic acid adenylyltransferase (Figure 4). As such they bind to IMPDH in place of the natural cofactor NAD^+ and thus inhibit the dehydrogenation reaction.[74,75]

However, so far, RBV remains the major drug being widely applied in the clinic against various RNA virus infections. For host-directed drugs it is crucial to balance the desired antiviral effect and the inevitable toxicity for the host; thus, the strongest inhibitor might not be the best antiviral candidate. Still, since many RNA virus infections are non-chronic and require only short-term treatment, some degree of toxicity might be tolerable – and especially in face of newly emerging RNA viruses with pandemic potential, host nucleotide synthesis remains a valuable target for developing drugs that can exert a general antiviral effect.

Targeting viral RNA synthesis with direct-acting antiviral nucleobase, nucleoside and nucleotide analogues

RNA viruses universally encode an enzyme that is responsible for synthesizing RNA, the RNA-dependent RNA polymerase (RdRp).[76] This enzyme

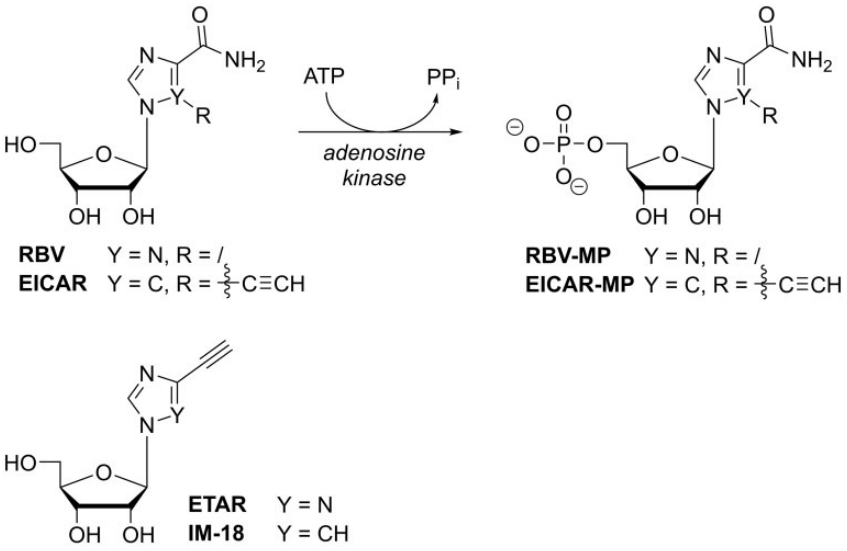

Figure 3. Metabolic activation of RBV and EICAR to their 5'-monophosphates by host adenosine kinase and chemical structures of ETAR and IM-18.

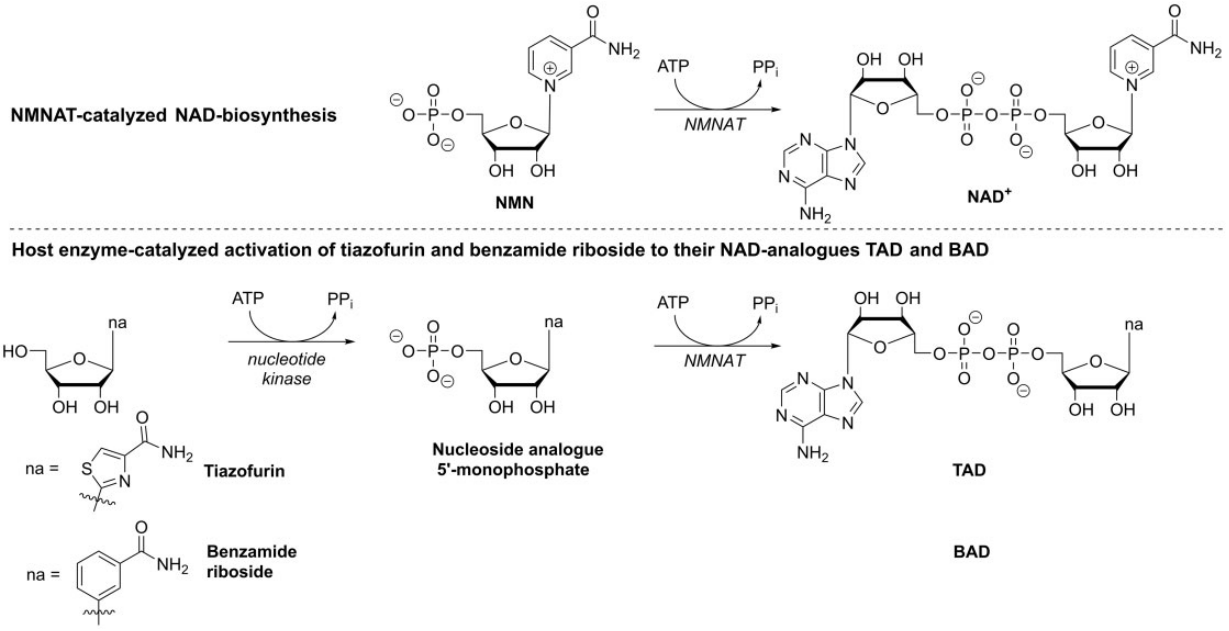

Figure 4. Host enzyme-catalyzed metabolic activation of tiazofurin and benzamide ribonucleoside to their NAD^+-analogues TAD and BAD by first 5'-monophosphorylation and subsequent dinucleotide formation, catalyzed by nicotinamide/nicotinic acid adenylyltransferase (NMNAT). NMN – nicotinamide ribonucleotide, na – nucleobase analogue.

catalyzes the phosphodiester-formation between the 3'-hydroxyl group of the priming (oligo)ribonucleotide and the α-phosphate of the incoming ribonucleoside 5'-triphosphate using RNA as template during genome replication as well as transcription (Figure 5). Viral RdRps work fast and are generally considered low fidelity enzymes: during genome synthesis, they incorporate the wrong nucleotide with a frequency of around 1×10^{-4} mutations per site for every round of replication (i.e. one mistake every 10,000 nucleotides).[24,27,77] Without additional proof-reading capacity, this results in progeny virus that each differ by 1–2 nucleotides from their parent.[24] In contrast, some larger DNA viruses as well as the cellular replication machinery harbor proof-reading and DNA repair enzymes and thus, mutation rates are much lower.[24] In RNA viruses, the overall low fidelity during genome replication leads to a relatively diverse virus

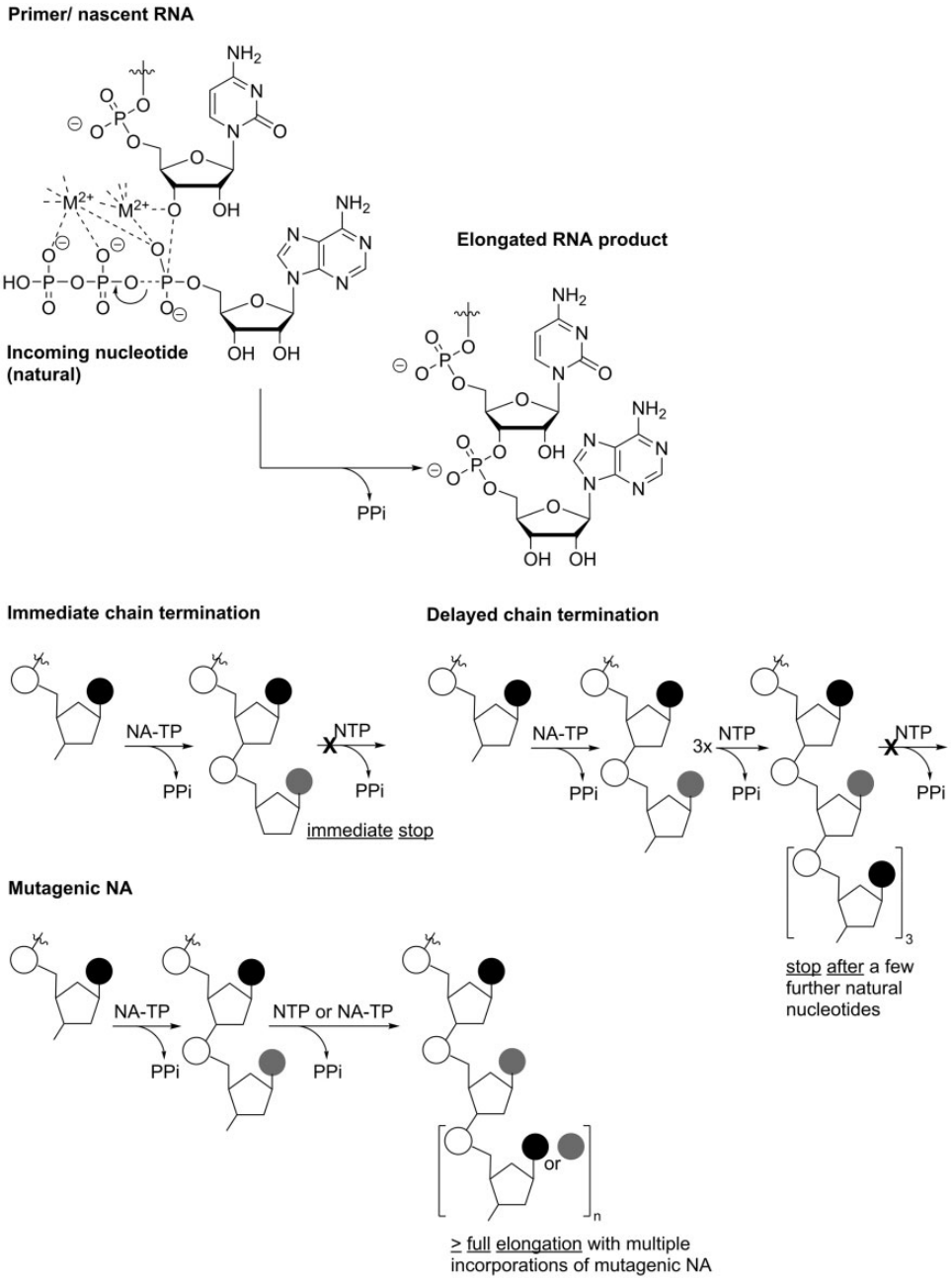

Figure 5. Polymerase-catalyzed RNA elongation. Top: The 3'-end of the nascent RNA attacks the α-phosphate of the incoming NTP, which is activated by M^{2+}-coordination, and forms the phosphate diester after release of pyrophosphate (PP_i). Bottom: Schematic of different mechanisms of action of NNAs. NA-TP – nucleoside analogue triphosphate.

population ('quasi-species'), and hence, RNA viruses possess the ability to quickly adapt to environmental changes. Consequences include the viruses' ability to overcome bottlenecks in transmission,[78] to evade the immune response, become resistant to drugs,[24] and to uphold or even increase pathogenicity when compared to higher-fidelity mutants.[79] On the other hand, this trait offers a possible weakness that can directly be addressed by drugs, analogues of the natural NTP substrates, to block the virus from replicating and stop the disease it causes.[22]

The structural features of different viral RdRps have been described and while this is a very active field of research, reviews providing extensive information on this topic are available.[80,81] Briefly, like all nucleic acid polymerases, the 3D-structure of RdRp enzymes (or enzymatic domains, as in some viruses, larger proteins harbor several enzymatic activities in distinct

sites) resembles a right hand with palm, finger and thumb subdomains. Although overall RdRp sequences can differ extensively, the folding of seven large protein segments (including motifs A-G) that make up about 75% of the total enzyme are highly similar for different viral RNA polymerases and have thus been termed 'homomorph' segments.[82] What's more, domains that are directly involved in nucleotide selection and catalysis even show high sequence conservation across different RNA virus families. Hence, the core of the enzyme, the active site that is formed by motifs A-E and that resides in the palm subdomain, seems to maintain high similarity among RNA viruses.[83,84] This may be attributed to an early emergence of RdRp from a common ancestor.[85] Consequently, since RdRp activity is essential for viral replication and the polymerase's structure is highly conserved across viral families, its active site represents an attractive target to develop virus-directed drugs that inhibit not only one specific but multiple RNA viruses and can thus serve as broad-spectrum antiviral treatments.[86] Moreover, in terms of resistance development, several studies indicate that mutations in the RdRp enzymatic core domain concomitantly lead to attenuated phenotypes *in vivo*.[87–89] Still, so far only few NNAs have been developed that show moderate to good antiviral activities across multiple virus families. This underlines the intricacy of this mission that may be caused by the influence that RdRp amino acids remote from the active site have on nucleotide incorporation fidelity.[77] Nonetheless, RdRp is the only enzyme that RNA viruses universally encode. Examples of broadly-active NNAs that act at the RdRp active site have been identified and will be introduced in this chapter alongside those NNAs that have shown to act on a rather narrow group of viruses. Their structural features as well as mechanisms of action will be discussed.

Targeting the viral polymerase, non-natural nucleosides and nucleotides have long been at the core of antiviral therapies. These drugs were among the first to treat HIV-infection and AIDS, where they remain the backbone of combination antiretroviral therapy and are even used as pre-exposure prophylaxis (PrEP), attesting to their efficient and safe profile.[90] To exert their effect on viral nucleic acid synthesis, nucleobase, nucleoside or nucleotide analogues (NNAs) have to be processed by intracellular host enzymes to their corresponding 5'-triphosphates. These function as analogues of the natural (d)NTP substrates and can thus be incorporated into the nascent RNA or DNA by the viral polymerase. Depending on their chemical structure as well as the context in which the analogues are incorporated, nucleic acid synthesis may be terminated directly after (immediate chain termination) or once a few additional (natural) nucleotides were added (delayed chain termination; Figure 5). Furthermore, nucleic acid synthesis may continue and even reach completion despite incorporation of an analogue but the analogue may cause an increase in mutational frequency such that no more infectious virus can be produced – a process that was termed lethal mutagenesis (for an insightful review on the different mechanisms by which antiviral NNAs inhibit nucleic acid synthesis, see Deval[91]) The following sections will discuss and compare some examples of antiviral NNAs that exert their antiviral effect *via* one of these mechanisms within the context of potential broad-spectrum activity. As, historically, major efforts in the development of anti-RNA virus NNAs have centered on finding new and effective treatments for HCV infection, many of the discussed compounds were originally sourced from anti-HCV-programs.

Chain terminators

2'-Ribose modified analogues. In terms of hepatitis C virus infection, the backbone of treatment has long relied on a combination of pegylated interferon-α (PEG-IFNα) and ribavirin (RBV), a nucleoside analogue already discussed in the previous section. RBV can address multiple targets including the viral RNA polymerase, but suffers from relatively low efficacy (sustained virologic response rate of approx. 30%) paired with severe toxicity,[48] which is especially grave in the long-term treatment that chronic HCV infection requires. Consequently, considerable efforts into the development of more potent and safer, direct-acting nucleoside analogues resulted in the approval of sofosbuvir,[92] achieving sustained virologic response rates of up to 90% after a 12-week treatment course in combination with PEG-IFNα and RBV.[48] Higher response rates of up to 100% were even seen with combinations of sofosbuvir and another direct acting antiviral, thus making it possible to fully dispense RBV from HCV-treatment.[48] Sofosbuvir's chemical structure resembles the natural nucleotide uridine 5'-monophosphate differing only at the 2'-position of the ribose-moiety and additionally containing the phosphoramidate prodrug unit (Figure 6). The prodrug form protects the nucleotide from phosphatases and masks the negative charge of the phosphate so that it can diffuse through cell membranes (pronucleotides, prodrugs of nucleotides, are discussed in more detail in the final section of this review). Ultimately, the prodrug groups are cleaved in a host enzyme-induced cascade reaction to release the 'naked' sofosbuvir monophosphate (Figure 6). Although, theoretically, incorporation of the sofosbuvir nucleotide would allow further elongation of the nascent RNA, once incorporated, the 2'-deoxy-2'-α-fluoro-2'-β-*C*-methylribose motif seems to distort

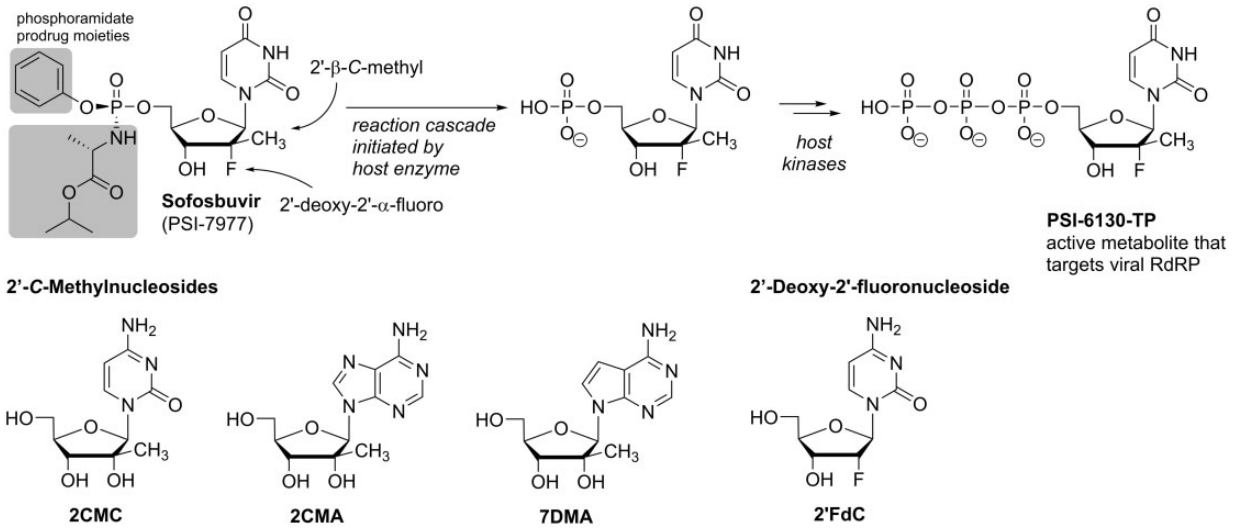

Figure 6. Chemical structures of 2'-*C*-methyl- and/or 2'-deoxy-2'-fluororibose-modified nucleoside analogues including anti-HCV pronucleotide sofosbuvir (phosphoramidate prodrug moieties highlighted in grey) and schematic of host cell metabolism to sofosbuvir's active triphosphate form PSI-6130-TP.

the structure of the RNA and thus prevents elongation (non-obligate chain termination).[86,93] While this particular motif is unique among clinically advanced antiviral NNAs and preferred only by HCV polymerase,[93,94] the 2'-*β*-*C*-methylribose modification has been recognized to equip NNAs with antiviral activity against not only HCV but a multitude of pathogens from various (+)ssRNA virus families.[95,96] These include flaviviruses, such as yellow fewer, dengue, west nile,[97] zika,[98] and tick-borne encephalitis virus,[99] that are mainly transmitted by mosquitos and repeatedly cause outbreaks of disease that can have highly debilitating outcomes.[100–103] Further, 2'-*C*-methylcytidine, 2'-*C*-methyladenosine and 7-deaza-2'-*C*-methyladenosine (2CMC, 2CMA, 7DMA; Figure 6) have demonstrated potency as broad-spectrum inhibitors of noro-, rota- and sapovirus infection (belonging to *Calici-* and *Reoviridae*, the latter are double stranded RNA viruses), which are the leading etiological agents causing viral diarrhea in children.[104] The spectrum of activity further includes members of the *Picornaviridae*,[105,106] another (+)ssRNA virus family.

As is reasonable to assume, the RdRps within the group of (+)ssRNA viruses are phylogenetically closer and thus more conserved than when comparing those from (+) and (−)ssRNA viruses.[107] In fact, it appears that 2'-*C*-methylribose-modified NNAs don't achieve comparable potency against (−)ssRNA viruses. Hence, this motif may contribute to the development of an antiviral nucleoside or nucleotide analogue with a broad antiviral spectrum among (+)ssRNA viruses, some of them, importantly, being pathogens with major significance to public health around the world such as members of the *Flavi-*, *Picorna-*, and *Coronaviridae*. The same strategy may, however, not expand to (-)ssRNA viruses.[105]

In this regard, a recent RdRp-based phylogenomic analysis reconstructed the evolutionary relationships among RNA viruses and concluded a phylogenetic tree of five major branches. (+)ssRNA viruses are spread about three of these branches, in part overlapping with double strand RNA viruses. In fact, it appears that dsRNA viruses have evolved from (+) ssRNA viruses on two different occasions at least while, in contrast, (−)ssRNA viruses may have evolved from one distinct group of dsRNA viruses. Importantly, (−)ssRNA viruses are limited to only one branch.[76] Being based on RdRp-sequences, this analysis again underlines that this viral enzyme may be a promising target to also inhibit various (−) ssRNA viruses in a "one drug-multiple-bugs"-approach. Still, in contrast to RdRp-targeted antiviral candidates against members of the (+)ssRNA viruses, some of which are in advanced clinical development or have even been approved for treatment (greatly driven by efforts to treat and cure HCV infection), similar progress has lagged behind for (−)ssRNA viruses. Within this group, intensive efforts have been put into drug development against influenza virus infections; yet, approved drugs so far predominantly target entry and release of the virus, are thus highly specific and prone to resistance development.[108] (Recent advancements include the approval of baloxavir marboxil (Xofluza), an inhibitor of the influenza virus polymerase PA-subunit that catalyzes the cap-snatching reaction to initiate translation;[109,110] a

structurally and functionally similar viral enzyme activity is used by other (−)ssRNA viruses such as *Bunyaviridae*,[111] hence, baloxavir or similar compounds targeting viral mRNA synthesis may have some broader spectrum of activity.) Importantly, with favipiravir (T-705) a compound that ultimately targets the influenza virus RdRp core enzymatic activity has been approved in Japan as treatment during outbreaks of new or re-emerging influenza virus where other drugs are not effective (Favipiravir is discussed in more detail in the following subsection). Briefly, it has broad antiviral activity but also suffers from low *in vivo* efficacy and potential teratogenicity. While chain termination has been observed in biochemical assays, favipiravir treatment predominantly seems to lead to the accumulation of mutations in the viral genome (Figure 5).

Still, in terms of non-mutagenic, direct-acting NNAs to broadly target (-)ssRNA virus replication, focused tests as well as screening campaigns have identified some potent inhibitors. Among these were 2'-deoxy-2'-fluororibonucleosides (Figure 6) that, in comparison to the nucleoside analogues discussed above, lack the 2'-*C*-methyl motif. However, they contain a 2'-fluorine substituent that is also present in Sofosbuvir and that sustains the 3'-endo-conformation typical for ribonucleotides, thus ensuring that these analogues can be recognized by RNA polymerases. While these compounds proved highly active against a panel of (−) ssRNA viruses (including influenza[112] and various hemorrhagic fever viruses[113,114]) with especially 2'FdC demonstrating broad and potent inhibition, clinical advancement is hampered by concerns regarding toxicity, probably because these compounds interfere with host nucleic acid synthesis (mitochondrial toxicity is a common limitation for antiviral drug development with NNAs and is discussed in the final section of this review).[115] Biochemical assays with the CCHFV L-protein (harboring the RdRp active site) have further shown that not only the 5'-triphosphate of 2'FdC but also that of 2'-amino-2'-deoxycytidine is a potent, CTP-competitive inhibitor of viral RNA synthesis[116]; yet, whether this applies to other (−)ssRNA viruses and whether it translates into antiviral activity in infectious assays remains to be determined.

C-Nucleosides. Remdesivir (RDV, GS-5734) is another highly promising example for nucleotide analogues with potential broad-spectrum antiviral activity, importantly spanning (+)ssRNA and (−)ssRNA viruses, that has recently attracted much attention. RDV, similarly to Sofosbuvir a McGuigan-type phosphoramidate pronucleotide, contains the non-natural nucleoside GS-441524 (Figure 7).[117,118] This compound originated from a screening campaign of an NNA library against, among others, filoviruses and gained attention after the large West Africa Ebola virus outbreak due to its highly promising inhibitory potency in cell-based and animal studies. In fact, RDV then became part of a clinical trial during the following outbreak of Ebola virus in the Democratic Republic of Congo that started in 2018. However, the RDV treatment arm was discontinued after interim analysis due to superior performance of monoclonal antibodies regarding mortality outcomes.[119] In addition to anti-filovirus activity, RDV, or its underlying nucleoside analogue GS-441524, are potent inhibitors of various members of the *Flavi*-,[117] *Corona*-,[120] *Paramyxo*- and *Pneumoviridae*,[121] hence showing a broad spectrum of antiviral activity including both (+) and (−)ssRNA viruses. With the ongoing and devastating COVID-19-pandemic, RDV has once more moved into the spotlight: It has demonstrated activity against various coronaviruses including SARS-CoV-2 in simple Vero- and primary cell-based models as well as in animal experiments[120,122,123] and has been granted approval by the FDA in October 2020 for the treatment of COVID-19 in patients 12 years of age and older. In terms of chemical structure, the nucleoside core of RDV resembles adenosine and includes modifications both at the nucleobase and the ribose-moiety (Figure 7). The ribose 1'-cyano modification is important for target-specificity of the compound, as the simple, 1'-H *C*-nucleoside suffers from significant cytotoxicity.[118] RDV's broad antiviral spectrum is based on its nucleoside 5'-triphosphate metabolite (i.e. GS-441524-TP) being recognized by diverse viral RdRps. Biochemical assays with polymerase complexes from MERS-CoV, SARS-CoV and SARS-CoV-2 have shown that its incorporation into viral RNA is even favored over the incorporation of the natural adenosine nucleotide.[124] In agreement with experimental data, modeling revealed that after RdRp-catalyzed incorporation of the RDV nucleotide into nascent viral RNA, where the 1'-cyano modification is easily accommodated, three subsequent (canonical) nucleotides can be added. Then, however, a steric clash between the protein and the 1'-cyano group leads to deterioration of the RNA structure, which precludes further nucleotide addition and consequently causes delayed chain termination after four translocation events (Figure 5).[125] However, to analyze the mechanism of action of nucleotide analogues *via* their effect on elongation kinetics, SARS-CoV-2 RdRp-catalyzed RNA-synthesis was studied recently on a >1000 nt long RNA template and in the presence of saturating NTP concentration using magnetic tweezers.[126] The study compared the kinetic signatures of the enzyme in the presence of GS-441524-TP with those in the presence of the immediate chain terminator 3'-deoxy-ATP and

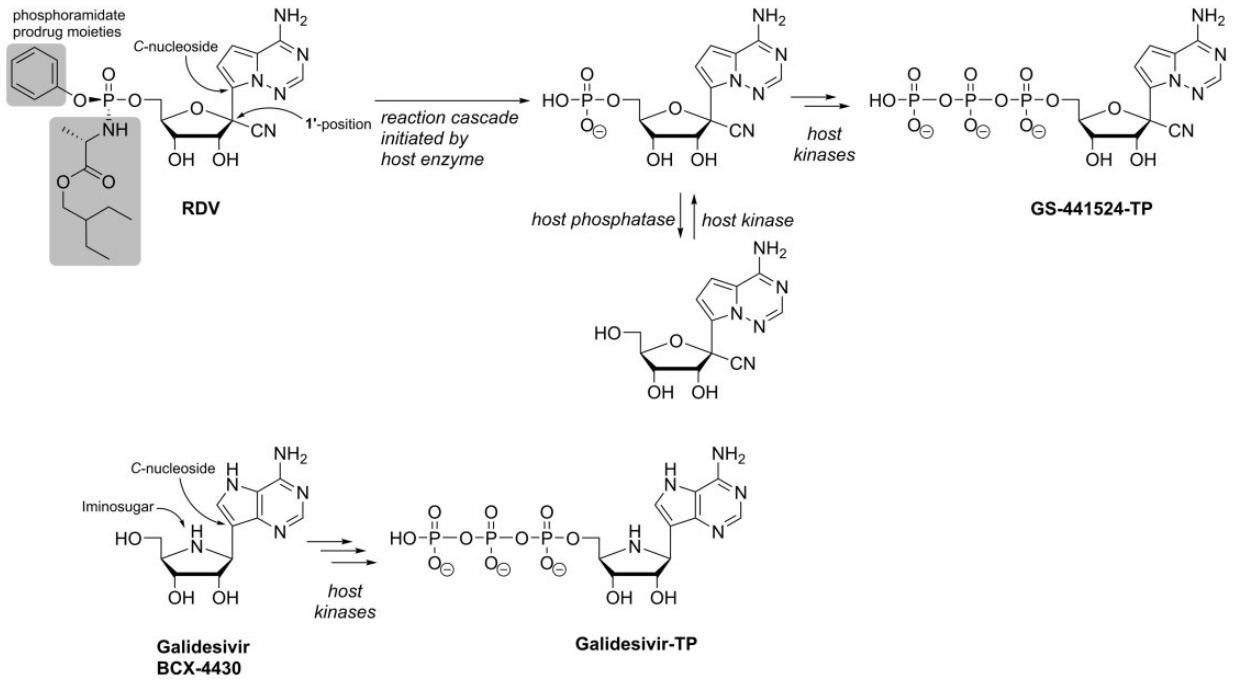

Figure 7. Chemical structures of broad-spectrum antiviral NNAs Remdesivir (phosphoramidate prodrug moieties highlighted in grey) and Galidesivir. Host cell metabolic activation yields the active TP-forms that the RdRp incorporates into nascent viral RNA, which leads to delayed chain termination (or stalling).

surprisingly, the results disagreed with chain termination as RDV's mechanism of action. Rather, they revealed that final product length was largely unaffected in the presence of GS-441524-TP, yet, the median replication time was increased while nucleotide incorporation rates were unchanged. Hence, using stochastic models, the authors concluded that instead of prematurely terminating RNA synthesis, the incorporation of RDV-nucleotide transiently stalls SARS-CoV-2 replicase (the authors noted that polymerase stalling may be interpreted as termination events in gel-based assays).[126] Importantly, having a comparably large genome (30k nt), coronaviruses are distinct from most other RNA viruses in that they harbor exonuclease activity in their nsp14 protein (ExoN). ExoN performs proof-reading by removing wrongfully incorporated nucleotides (or analogues) and thus restricts many NNAs from being active against the wild-type coronaviruses.[127,128] In the case of RDV, while the drug's efficacy is somewhat sensitive to proof-reading by ExoN (i.e. the antiviral potency is higher in ExoN$^-$-CoV mutants compared to the wild-type virus),[129] this is to a much lesser extent than for other NNAs.[125,130] As modelling has shown, ExoN readily accommodates the RDV nucleotide in the 3'-terminal position of the RNA strand; yet, the 1'-cyano moiety precludes its proper positioning for hydrolysis.[125] In combination with the delayed chain termination or stalling mechanism, this may be the reason for RDV's sustained antiviral potency against wild-type CoVs.

With the imino-*C*-nucleoside galidesivir (BCX-4430; Figure 7), yet another adenosine analogue has shown some promise for future treatment of various RNA virus infections. Galidesivir is known as a potent transition state analogue inhibitor of the purine nucleoside phosphorylase (PNP) from the protozoan parasite *Trichomonas vaginalis*[131] and it further inhibits *Leishmania* parasites *via* blocking their nucleoside hydrolase, an important enzyme in the purine salvage pathway that these purine auxotrophic pathogens rely on.[132] In chemical structure, it resembles the antiparasitic immucillins ImmH and ImmG and has thus also been termed immucillin-A (ImmA). Importantly, antiviral screening campaigns identified galidesivir as an inhibitor of diverse members of (+) as well as (−) ssRNA virus families, such as *Flavi-*, *Picorna-*, and *Coronaviridae*, *Filo-*, *Bunya-*, *Arena-*, *Ortho-* and *Paramyxoviridae*.[133] In terms of antiviral mechanism of action, galidesivir does not inhibit mammalian PNP,[131] thus contrasting the antiparasitic properties. Instead, it targets viral RNA polymerases, which incorporate galidesivir nucleotide *via* its triphosphate form into nascent viral RNA.[133] Similar to what is observed with RDV in gel-based assays, nucleic acid synthesis then stops after incorporation of another two natural nucleotides (delayed chain termination, Figure 5).[133] While in primary hepatocytes galidesivir is readily

converted into galidesivir-5'-triphosphate (Galidesivir-TP; Figure 7) by host kinases, in immortalized cell lines, especially Vero cells, Galidesivir-TP attains only low levels.[133] It was suggested that this may limit the observed antiviral potency of the compound in typical screening campaigns and thus lead to underestimation of galidesivir's potential regarding potency and spectrum of antiviral activity – a limitation that is often observed with NNAs and that can be addressed employing prodrug strategies (which will be discussed in the final section of this review). Importantly, the imino-*C*-nucleoside galidesivir seems to be devoid of significant cell toxicity up to concentrations that largely exceed antiviral IC_{50}'s.[133]

The examples of remdesivir and galidesivir impressively show that broad antiviral activity covering not only different viruses of the same family but even spanning diverse members of (+) and (−)ssRNA virus families (including even *Coronaviridae* which harbor proof-reading activity) can be achieved with chain terminating NNAs (although recent analyses challenge this mechanism of action for RDV).[126] Importantly, at the same time these compounds exhibit favorable toxicity profiles, hence optimally positioning them for clinical development. Still, metabolic activation to the ultimately active nucleoside analogue triphosphate may be limited in certain cells and tissues. Thus, to ultimately being successful as broad-spectrum antiviral candidates, technologies to bypass inefficient metabolic activation may become essential.

Lethal mutagens

Apart from chain termination, NNAs can exert their antiviral effect by extensively causing mutations during replication, for instance when they behave ambiguous in their base-pairing properties. Since RNA viruses already exhibit low fidelity during genome synthesis, additional mutations rapidly render progeny virus unfit and may thus stop the infection.[22,24,27] This strategy has first been described as lethal mutagenesis in the context of HIV.[134] In terms of chemical structure, such mutagenic nucleotides require only minor modifications to the nucleobase and none to the ribose-moiety; thus, it is conceivable that such compounds promise to be very broadly active. Nonetheless, analogues certainly need to achieve some specificity for viral over cellular targets to not cause extensive toxicity, which may require additional modifications (one major problem may be mitochondrial toxicity which is discussed further below). Furthermore, mutagenic NNAs appear favorable over many chain terminating NNAs in terms of resistance development.

Carboxamide-substituted nucleobases. With its diverse mechanisms of action, RBV is also a prominent and extensively studied example of a nucleoside analogue, that – once converted to RBV-triphosphate and then incorporated into viral RNA – induces lethal mutagenesis.[63] Its mutagenic potential is explained by the flexibility of the carboxamide substituent at the nucleobase: Rotation around the C(3)-C(=O)-bond allows RBV to mimic guanosine (and inosine, see previous section) or adenosine, thus templating ambiguously for C or U (Figure 8).[53] Still, as discussed above, the predominant mechanism by which RBV exerts its antiviral effect remains highly debated and probably differs for different viruses and model systems. Nonetheless, to induce lethal mutagenesis, multiple incorporations of the analogue during viral nucleic acid synthesis are required (Figure 5) and it is conceivable that GTP depletion *via* IMPDH inhibition (as discussed above) enhances RBV's chances of incorporation. Hence, these two distinct mechanisms may actually work synergistically. RBV's efficacy in the clinic, however, remains suboptimal and RBV treatment can cause severe side-effects, e.g. hemolytic anemia *via* accumulation of its phosphorylated form in erythrocytes,[135] and teratogenic effects have been observed in different animal models.[136–138]

T-705 (favipiravir) is a nucleobase analogue that, just like RBV, contains a carboxamide substituent (Figure 8). Accordingly, once incorporated into viral RNA, the carboxamide's rotational flexibility allows T-705 to template for both C and U and thus induce mutations, which has been demonstrated both *in vitro* and *in vivo*.[139–143] Therefore, T-705 must first be converted by host enzymes to the triphosphoribosylated form (T-705-RTP; Figure 9), which is then recognized as a substrate by various viral polymerases.[144–146] In addition to acting as a mutagen, biochemical assays also suggested that the incorporation of T-705-ribonucleotide into nascent viral RNA may lead to chain termination or polymerase back-tracking.[144,147,148] Consequently, similar to RBV, T-705 has been attributed different mechanisms of action based on *in vitro* work, yet there's strong evidence from animal experiments that this compound in fact induces lethal mutagenesis.[139–144,147,148] Additionally, in contrast to RBV, no or only very low inhibition of cellular IMPDH has been observed with T-705.[141,149] In line with this, cells treated with high concentrations of T-705 appear to experience virtually no toxicity. This, however, is contrasted by the finding that T-705-RTP is an efficient substrate for human mitochondrial RNA polymerase (POLRMT), which typically causes toxic effects of antiviral NNAs.[150] So far, a comprehensive explanation for these contradicting data has not been presented; however, in animal experiments, embryotoxic

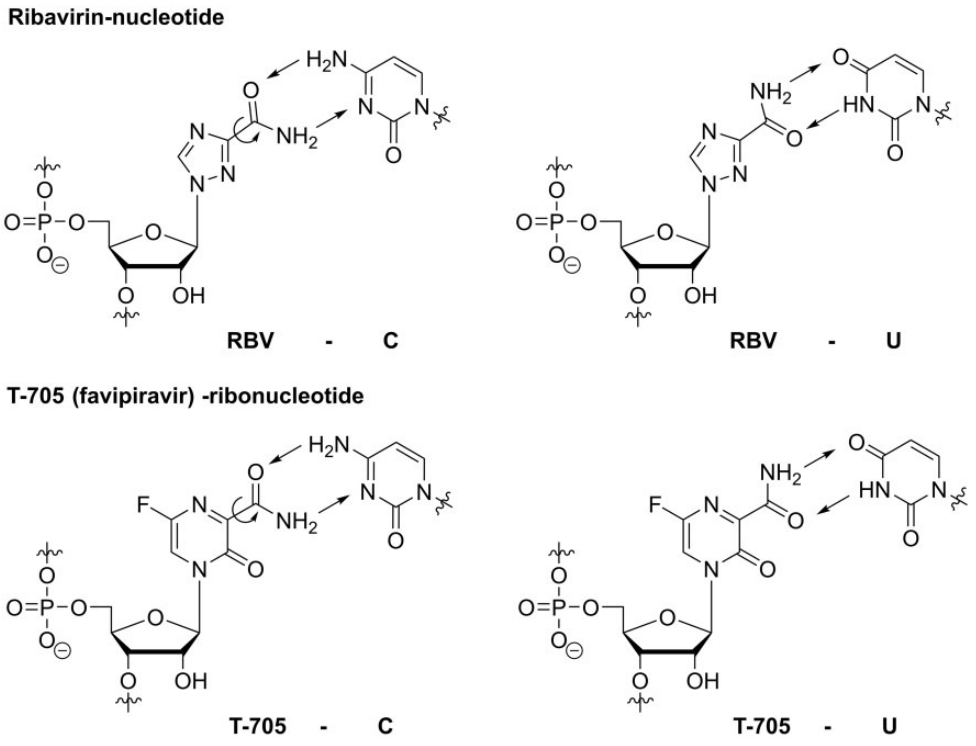

Figure 8. By rotation of the carboxamide moiety, RBV-nucleotide and T-705-ribonucleotide can base-pair with and thus template for both C and U. Hence, they can exert their antiviral effect through inducing mutations after being incorporated multiple times during viral RNA synthesis. Circled arrows indicate rotation of C-C-bond; straight arrows represent H-bonds.

effects have been described for T-705[108] and warrant caution regarding its clinical use. Still, T-705 has shown great promise as a broad-spectrum, orally bioavailable antiviral: After it was reported as an inhibitor of influenza virus[151] it was found active *in vitro* and *in vivo* against a vast number of emerging viruses including diverse hemorrhagic fever viruses.[152–155] Favipiravir has subsequently been trialed during outbreaks of Ebola virus[156] as well as SARS-CoV-2 (for an extensive overview of T-705's antiviral activities see recent reviews[157–159]) From these studies, it appears that T-705 is most active against influenza viruses, which are members of the (−)ssRNA *Orthomyxoviridae*, and antiviral effects against (+)ssRNA viruses appear to require higher doses.[160,161] Yet, due to the different model systems, these comparisons may in some cases be difficult to interpret. In this regard, we and others have shown that the efficiency of T-705's metabolic activation, which is required to generate the active -RTP form (Figure 9), may differ strongly in different cells and consequently, inhibitory concentrations not only depend on the virus that studied but also on the cell type that is targeted.[162–165] Additionally, T-705 suffers from chemical instability once it has been incorporated into a ribonucleoside: we found that in buffered aqueous solution of physiologic pH, T-705-ribonucleoside readily decomposes and the rate of decomposition increases with increasing pH.[166] In fact, this may not only limit observed potency but also represent yet another antiviral mechanism with decitabine or AzaC being prominent examples (discussed below). In contrast to T-705, its non-fluorinated analogue T-1105 (Figure 9) proved stable under aqueous conditions. Moreover, T-1105 has demonstrated greater potency to inhibit influenza virus in MDCK cell-based assays and the corresponding ribonucleoside 5'-triphosphate (T-1105-RTP) competes with the purines ATP or GTP during viral RNA synthesis more efficiently than T-705-RTP, which has been shown in the context of human norovirus,[145] influenza virus[167] as well as SARS-CoV[146] polymerase. As mentioned above, however, inefficient metabolic activation to its triphosphate form also limits T-1105's antiviral potency and hence, this compound suffers from drastic differences in efficacy when studied in different cell types,[165] which may somewhat conceal potential broad-spectrum antiviral potency. As mentioned above, both RBV as well as the active forms of T-705 and T-1105 contain an unmodified *ribo*-glycon. Hence, in terms of countering toxic effects, one study reports the combination of the 2'-*C*-methylribose motif with the nucleobase analogue T-1105 to prevent POLRMT-targeting of the ribose-unmodified T-1105-RTP (Figure 9).[145] Although, compared to the

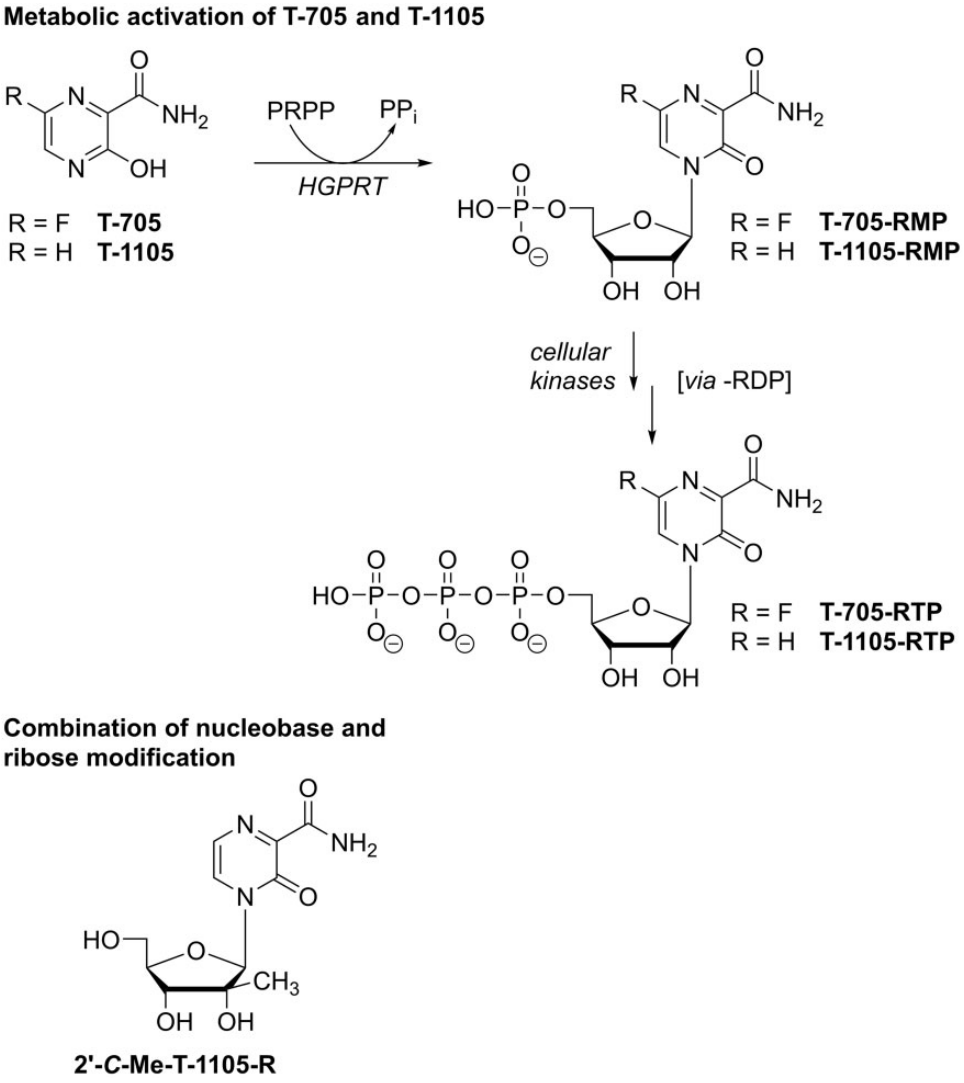

Figure 9. Top: Metabolic activation of T-705 and its non-fluorinated analogue T-1105 to the active ribonucleoside 5'-triphosphate (-RTP) metabolite. PRPP – 5-phosphoribosylpyrophosphate; PP_i – pyrophosphate; HGPRT – hypoxanthine guanine phosphoribosyl transferase. Bottom: Chemical structure of 2'-*C*-methyl-modified ribonucleoside of nucleobase analogue T-1105.

ribose-unmodified version, the 5'triphosphate form of this analogue proved less potent in inhibiting norovirus polymerase, its recognition by POLRMT was also much less efficient,[145] demonstrating that small chemical modifications can greatly reduce off-target effects.

Other features of nucleoside analogues that induce a mutagenic effect. Another ribonucleoside analogue that has demonstrated antiviral potency against influenza and diverse other RNA viruses *via* inducing mutations is *β*-D-*N*4-hydroxycytidine (NHC; Figure 10).[168–173] Importantly, this compound is also active against a broad panel of coronaviruses including SARS-CoV-2[174,175] and its 5'-*iso*propylester EIDD-2801, an orally bioavailable prodrug, has advanced to clinical development during the COVID-19 pandemic.[176–178] Through its tautomerism and rotational isomerism (Figure 10),[179] NHC can replace both pyrimidine nucleosides uracil and cytosine, i.e. it templates for A and G and thus induces lethal mutagenesis.[168] Similar to remdesivir and galidesivir - yet contrasting other mutagenic NNAs[128] –NHC's potency is only minorly affected by coronaviral ExoN activity despite its natural *ribo*-glycon; the mechanism behind NHC's ability to evade CoV proof-reading has not been elucidated so far. Importantly, in terms of drug evasion, NHC behaves similar to other mutagenic NNAs: passaging experiments did not generate robust resistance in influenza-,[173] corona-[174] or other RNA viruses.[168,172]

In addition to rotation of a small substituent at the nucleobase, also *syn*-/*anti*-isomerization at the gylcosidic bond can result in ambiguous base-pairing.

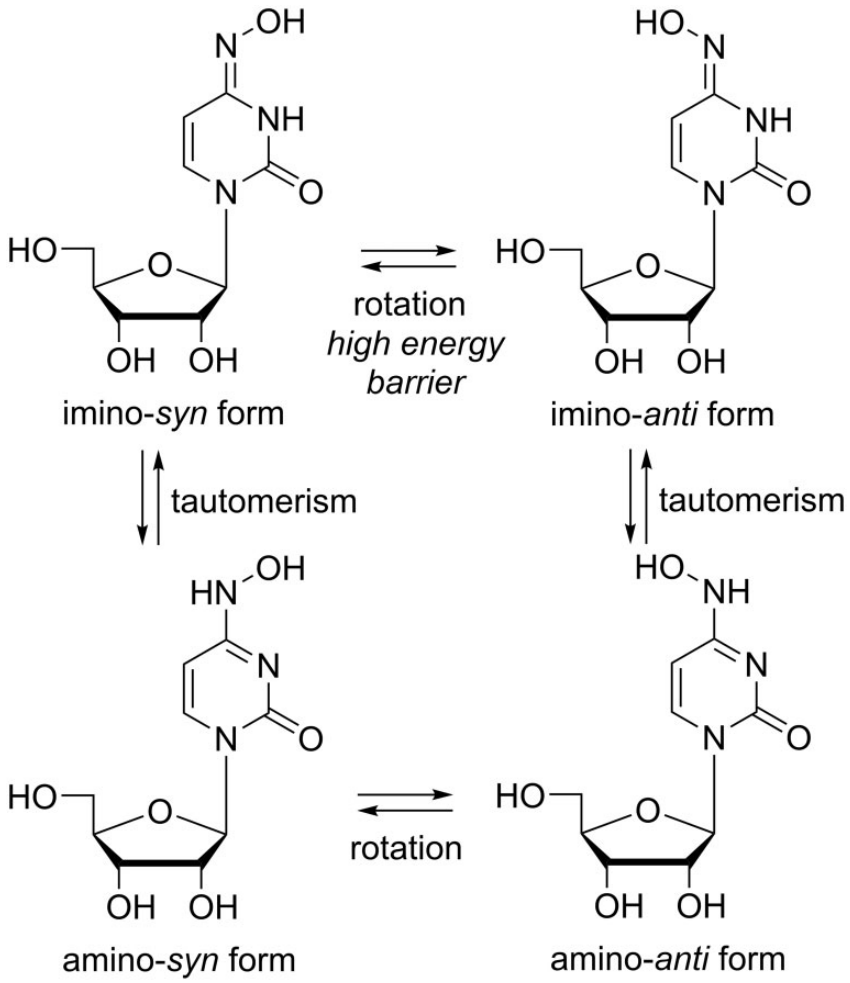

Figure 10. Chemical structures of the imino- and amino-forms of the mutagenic nucleoside analogue β-D-*N*4-hydroxycytidine (NHC).

Janus type nucleosides exhibit different hydrogen bond donor/acceptor patterns at the two different sides of their unnatural nucleobase and thus, by rotating at the glycosidic bond, they're able to mimic different natural nucleotides (Figure 11).[180,181] However, so far this concept has not produced any promising antiviral candidate with broad-spectrum potency against RNA viruses. The concept of rotational flexibility has further been extended to rotation within the nucleobase core: fleximers are nucleoside analogues that bear a split purine base, thus, the imidazole and pyrimidine part are no longer fused but connected *via* a single C-C bond.[182] Originally, this approach was followed to study how increased flexibility in the nucleobase would affect enzyme recognition and binding affinity compared to rigid analogues. It was then found that fleximers can bind to atypical enzymes and that they can overcome point mutations, which usually confer resistance to the rigid nucleoside analogue.[183] Further, broad-spectrum antiviral activity may be achieved through the fleximers' ability to take part in "mutually induced fit".[182] Combining the split purine base with an acyclic glycon (as found in the anti-herpesvirus drug acyclovir) has led to promising activity against members of different RNA virus families, including *Filo-*, *Flavi-,* and *Coronaviridae*.[184–186] Still, the mechanism of action remains subject of studies and although it has been shown that fleximer-triphosphates can be substrates for nucleic acid polymerases,[187] it is still unclear whether the antiviral activity of acyclic fleximers is achieved *via* this mechanism. Mutagenic properties similar to ribavirin etc. have so far not been reported for fleximers, but it is conceivable that this concept – having flexibility at its core – may in the future extent to inducing lethal mutagenesis. Next to rota- or tautomeric properties, ionization can cause ambiguous base-pairing. In 5-fluorouridine (the ribonucleoside of 5-fluorouracil, 5-FU, the most prominent example following this mechanism) the 5-fluoro substituent strongly decreases

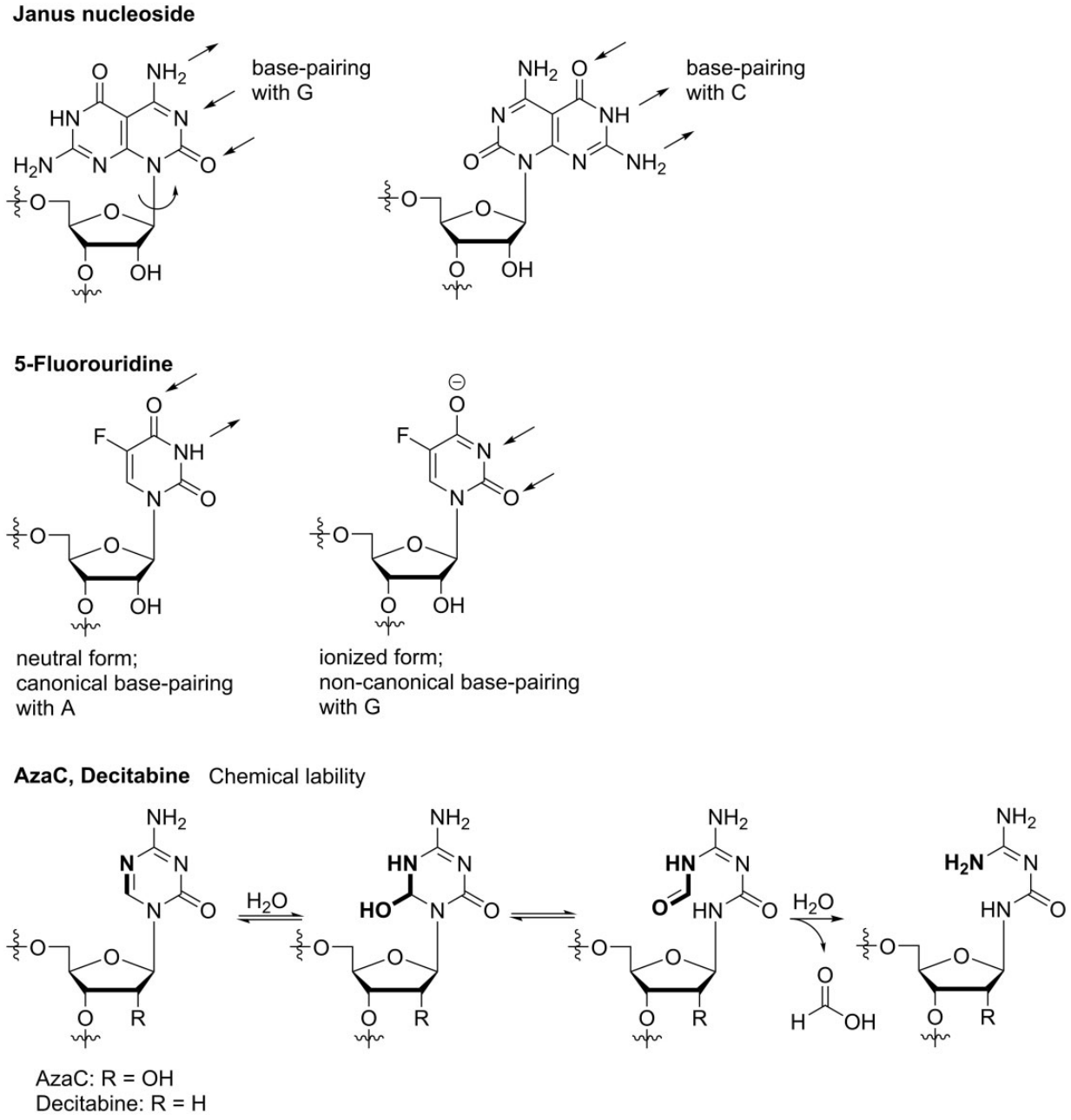

Figure 11. Chemical structures and molecular basis for mutagenic properties of Janus-GC nucleoside, 5-FU and AzaC/Decitabine.

the pKa of the NH-group (or, the OH-group of its tautomeric imino-form, respectively; Figure 11). Hence, in addition to 5-FU forming a natural-like base-pair with A, the ionized form of 5-FU is able to hybridize with G.[188] 5-FU ribonucleotide further inhibits its cellular thymidylate synthase, leading to cell death, and is therefore used in anti-tumor therapy.[189] Finally, mutagenic properties in NNAs can also be the result of chemical instability of the un-natural nucleobase. For instance, in 5-azacytidine (azaC) and its 2'-deoxy-congener decitabine, the triazine is prone to nucleophilic attack and ring-opening by water, followed by deformylation (Figure 11).[190–192] Since phosphorylated metabolites of azaC and decitabine also interfere with cellular processes, these compounds are used in the treatment of myelodysplastic syndrome and leukemia.[193,194] The antiviral effect of azaC through mutagenesis has been demonstrated in the context of HIV,[195] foot-and-mouth disease virus,[196] lymphocytic choriomeningitis virus[197] and coronavirus that lacks

ExoN-activity.[128] However, since these compounds inhibit crucial cellular processes they're so far not being used in the clinic to treat viral infection.

Nonetheless, especially the examples of T-705/T-1105 and NHC give rise to hope that the development of broad-spectrum antiviral drugs based on mutagenic NNAs may be attainable. To achieve this goal, cell-based screening campaigns need to integrate cell models that better represent the various target tissues that are clinically relevant for different RNA virus infections, thus factoring in potential cell type-dependent antiviral potency of the compounds. On the other hand, development efforts will require innovative medicinal chemistry strategies to a) enhance efficacy by overcoming poor intracellular activation and b) limit toxicity by enhancing target selectivity. The following section will briefly introduce examples of such strategies.

Problems of virus-targeted nucleoside analogues in antiviral drug development

Though NNAs play essential roles in the treatment of viral diseases such as hepatitis C virus infections, the development of novel members of this drug class is cumbersome and the danger of failure in advanced clinical stages poses high financial risk. One problem is that *in vitro* antiviral activity may not translate well into *in vivo* efficacy, as, for instance, NNAs have to overcome metabolic limitations in the activation to their nucleoside analogue triphosphate form within the target tissues. Second, *in vivo* toxicity of NNAs that only came to light in advanced clinical phases has in the past led to spectacular failures and high attrition rates. Often, this is due to insufficient selectivity of nucleoside analogue triphosphates that interfere with cellular nucleic acid synthesis such as mitochondrial RNA synthesis.

Low selectivity causing toxicity. Since cells use nucleotides in diverse processes and harbor (DNA and) RNA polymerases that share their catalytic mechanism with viral polymerases, NNAs can cause severe toxicity when they don't selectively target the viral enzymes. Consequently, to become successful antiviral drugs, the active metabolites of NNAs must show sufficient selectivity for viral over host enzymes. Examples from the development of antiviral NNAs against DNA viruses, such as herpesviruses and hepatitis B virus, or the retrovirus HIV have shown that for an NNA to be successful, its NTP-form must have sufficiently low substrate properties for host polymerases when compared to the viral polymerase.[198] Hence, when an NNA-triphosphate inadvertently targets a host polymerase, consequences are severe toxicity and (late) failure of the candidate in clinical development, as exemplified by the anti-HBV nucleoside analogue fialuridine which causes mitochondrial toxicity *via* inhibition of DNA polymerase γ.[199,200] The problem of mitochondrial toxicity has also led to the attrition of diverse NNAs in development for the treatment of RNA virus infections.[115] Ribonucleoside analogue 5'-triphosphates are at risk of being recognized as substrates by host mitochondrial RNA polymerase (POLRMT) and consequently, when analogues disrupt normal mitochondrial RNA synthesis, protein expression is reduced which leads to mitochondrial dysfunction.[115,201] In consequence, it was suggested that target selectivity of nucleoside analogue triphosphates should best be studied at an early *in vitro* stage to avoid failure at more advanced stages in clinical development; available biochemical as well as cell-based assays have been reviewed recently.[202] In a joint effort with early testing, the chemical structure of antiviral NNAs must be optimized so that their triphosphate metabolites are disfavored by cellular enzymes, such as POLRMT, while being good substrates for various viral polymerases. However, biochemical studies have shown that POLRMT is quite promiscuous in terms of NTP substrate specificity.[115] Lately, following spectacular failures in clinical development, POLRMT substrate properties towards non-natural ribonucleoside triphosphates have been studied with regard to compounds from anti-HCV campaigns, thus including nucleoside analogues modified at the 2'- or 4'-ribose-position.[115,150,201] Data for ATP derivatives show that the obligate chain terminator 3'-deoxy ATP is a better substrate for POLRMT than the non-obligate chain terminator 2'-*C*-methyl ATP.[115] Among 2'-modified NTPs, the inhibitory potency of 2'-*C*-methyl purines towards POLRMT-catalyzed RNA synthesis appears to be approx. 5-fold higher compared to pyrimidine derivatives.[201] Interestingly, 2'-deoxy-2'-fluoro-2'-*C*-methyluridine, the nucleoside analogue contained in the highly successful anti-HCV drug sofosbuvir (Figure 6), clearly stood out with its 5'-triphosphate (PSI-6130-TP) showing very low incorporation efficiency and virtually no inhibition of POLRMT-catalyzed RNA synthesis ($IC_{50} > 500\,\mu M$).[115,201] Regarding modifications in 2'- *versus* 4'-ribose position, 2'-*C*-methyl CTP (the active form of valopicitabine) was a better substrate for POLRMT than 4'-*C*-methyl CTP; however, changing the 4'-substituent to an azido group (as in balapiravir's active form) again resulted in enhanced substrate efficiency and pronounced toxicity in the clinic.[115] NNAs that elicit their antiviral effect *via* inducing mutations are generally weaker chain terminators, and for T-705 *in vitro* studies have shown no strong effect on mitochondrial protein expression even though the compound's active

form, T-705 ribonucleoside 5'-triphosphate, was an efficient substrate for POLRMT.[150]

These analyses suggest that POLRMT Figure 12 does not discriminate well against non-natural NTPs containing single ribose-modifications, such as the 4'-azido- or 2'-*C*-methyl motifs. Analogues are incorporated into and hence prematurely terminate the synthesis of mitochondrial RNA transcripts, thus reducing mitochondrial protein levels and causing toxicity. Combining multiple ribose-modifications, as seen for example in sofosbuvir, may decrease the triphosphate's substrate efficiency towards POLRMT and consequently lead to a potential drug with a much better safety profile. Importantly, however, its distinct pattern of ribose-modifications has precluded sofosbuvir from being broadly active. Here, mutagenic NNAs seem the better option, at least judging from the data available so far. Still, these analogues may cause teratogenic effects and since the problem of ribonucleoside analogue triphosphates targeting POLRMT has only recently moved into the spotlight, more studies are clearly needed to establish general conclusions and to be able to anticipate mitochondrial toxicity from the chemical structure of NNAs.

In terms of countering mitochondrial toxicity by chemical strategies that leave the nucleoside analogue unchanged, an innovative and promising approach has been reported recently in the context of the anti-HIV drug d4T. Here, an alkyl-modification attached to the γ-phosphate group of d4T triphosphate greatly decreased its substrate properties towards host DNA polymerases while having a much smaller effect on its substrate properties towards HIV-RT.[203] Thus, the γ-alkyl-modification was able to enhance the selectivity index of the active compounds. If these findings translate also to POLRMT, this strategy holds great potential to generally counter mitochondrial toxicity of any ribonucleotide analogue that is to be developed as broad-spectrum drugs against RNA viruses.

Low efficacy due to inefficient metabolic activation. Nucleobase, nucleoside or nucleotide analogues can only become active on the target viral polymerase once they've been converted into the nucleoside analogue 5'-triphosphate. This intracellular metabolic activation is catalyzed by host enzymes (Figure 13) and can thus suffer from being inefficient or even blocked when analogues are not recognized well as substrates by these enzymes. As mentioned above, these metabolic

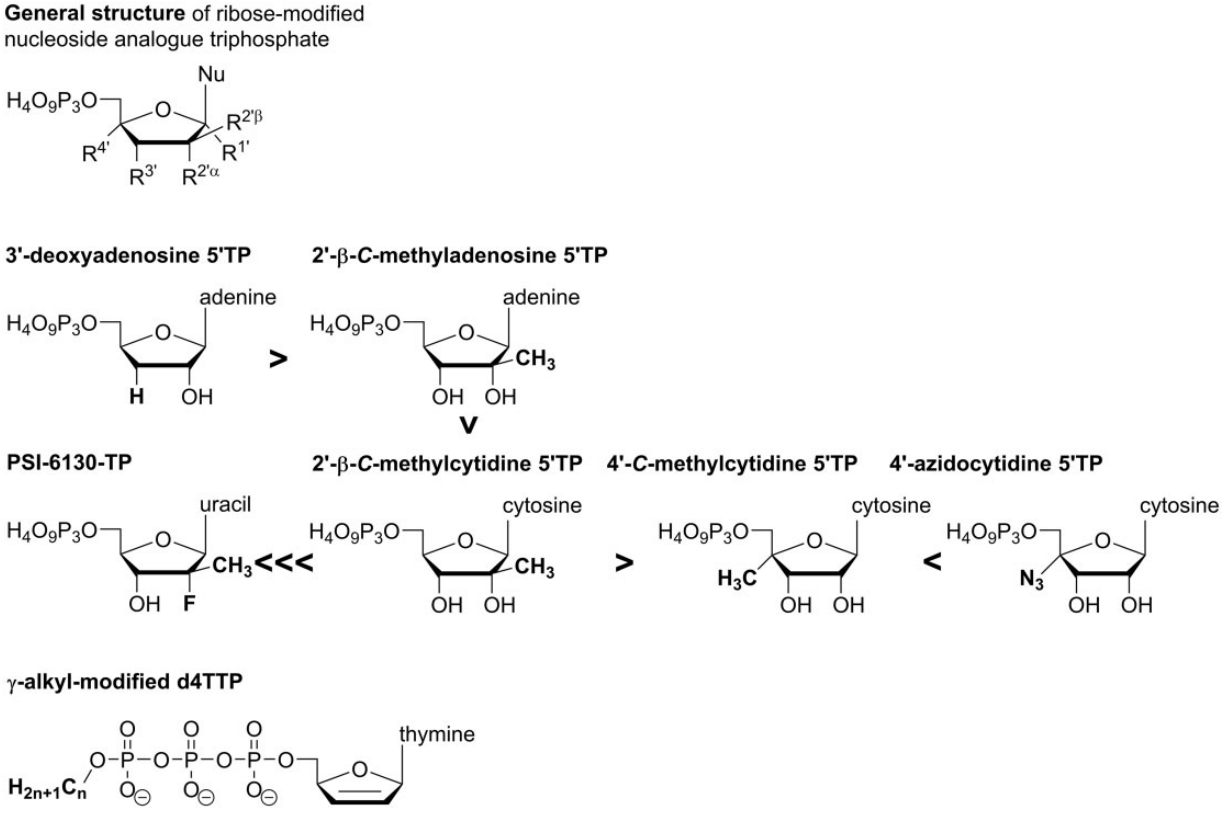

Figure 12. Comparison of chemical structures of 3'-deoxy ATP, 2'-*C*-methyl ATP, PSI-6130-TP, 2'-*C*-methyl CTP, 4'-*C*-methyl CTP and 4'-azido CTP; γ-alkyl-modified d4TTP. Bold arrowheads indicate relative substrate properties towards POLRMT.

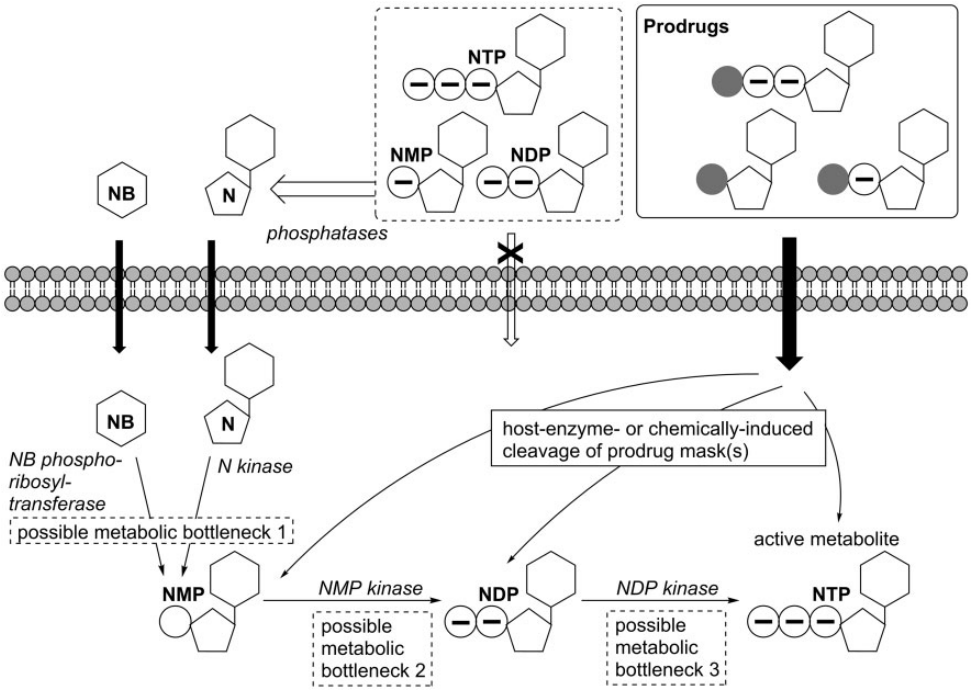

Figure 13. Intracellular activation pathway for nucleobase (NB) or nucleoside (N) analogues and nucleoside 5'-mono- (NMP), -di- (NDP), or -triphosphate (NTP) prodrugs. NB and N can traverse the cell membrane by passive or facilitated diffusion while nucleotides are held back due to their negative charges. Host phosphatases dephosphorylate NMPs, NDPs and NTPs. In the form of prodrugs, these nucleotides are protected from dephosphorylation and the charges are masked; therefore, the prodrugs can pass the cell membrane. Inside the cell, N or NB are metabolized by host enzymes via NMP and NDP to the antivirally active NTP form. Due to insufficient substrate properties of the analogues towards the host enzymes, one or more of these steps may be blocked. Prodrugs are activated intracellularly by ubiquitous host enzymes or chemical reactions and can thus overcome metabolic bottlenecks and deliver the active metabolite.

bottlenecks may not be general but depend on specific cell types and hence, varying antiviral potency depending on the cell type has been reported for various NNAs.[162,163,165] As this cell type-dependency limits the compounds' antiviral spectrum, prodrug systems that deliver the active form not only contribute an important factor to attaining highly potent but also broadly active antivirals.

The active forms, the non-natural NTPs, can't be applied as such as they 1) would quickly be dephosphorylated by host enzymes before reaching their intracellular viral target and 2) would not be able to pass through the cell membrane due to their highly polar, negatively charged properties. Hence, to overcome limiting steps in the metabolic activation of nucleoside analogues, prodrug strategies have been developed, often in the context of HIV or HCV as highlighted in a recent review.[204] In sofosbuvir, for example, the 5'-monophosphate form of the nucleoside analogue is masked as an aryloxy phosphoramidate triester (Figure 6) and those masking groups are cleaved intracellularly *via* a cascade mechanism that is initiated by host carboxypeptidase or -esterase.[205–207] This type of phosphoramidate pronucleotide ('ProTide'), pioneered by Chris McGuigan and colleagues (for a review see Mehellou et al.[208]), was designed to release (non-natural) nucleoside monophosphates inside cells. The same technology is also used in remdesivir and while sofosbuvir specifically targets HCV, remdesivir has gained attention as potential anti-Ebola virus and, importantly, anti-coronavirus treatment. Since cleavage of the prodrug moiety still requires host enzyme-catalyzed steps, the prodrug's efficacy may still be limited by different enzyme content (isoforms and concentrations) in different target tissues.[209] Moreover, in terms of general applicability, it has been shown for other NNAs that the bottleneck can also lie in the later steps of metabolic activation, i.e. the formation of the nucleoside analogue di- or even triphosphate.[210,211] And although many different prodrug systems have been developed for the intracellular delivery of nucleoside monophosphates (a topic which has been reviewed periodically, see literature[212–214]) similar strategies for di- and triphosphates were long believed impossible due to their high charges and the inherent chemical instability of the phosphoanhydride bond(s). However, in recent years, the development of the Di*PP*ro- and Tri*PPP*ro-technologies demonstrated that chemical

delivery systems for modified nucleoside di- and triphosphates are in fact attainable and successful in bypassing late or even all possible metabolic bottlenecks.[215,216] This was impressively demonstrated by "switching" former inactive nucleoside analogues into active antivirals by applying the Tri*PPP*ro-technology.[216] Still, once delivered into target cells, the active nucleoside analogue triphosphate may undergo rapid dephosphorylation by host phosphatases and this may again limit the efficacy of the drug. In this context, the combination of the γ-alkylphosphate modification (mentioned above in the context of lowering toxicity) and a prodrug-moiety, as recently reported for anti-HIV nucleoside analogue d4T triphosphate, appears promising.[203] While the prodrug moiety enables the modified NTP to reach the intracellular space and is cleaved there, the γ-alkylphosphate modification was not only shown to increase the target selectivity but to also preclude dephosphorylation and thus stabilize the active nucleoside analogue triphosphate, thereby enhancing its half-life. Altogether, this strategy may increase the antiviral efficacy of NNAs and widen their antiviral spectra; a promising approach that will hopefully facilitate the clinical development of broad-spectrum NNAs as RNA virus therapeutics in the future.

Conclusion

RNA viruses have been responsible for causing devastating epi- and pandemics and the immediate threat of another global health crisis posed by a newly emerging RNA virus has become dramatic reality once more just recently with the emergence of SARS-CoV-2. Still, effective treatments remain scarce. Clinical development for drugs against diseases that are not yet in the large population is often hampered by limited testing opportunities and by being economically unviable. For newly emerging pathogens, the development of specific drugs or vaccines can only start once the pathogen is identified and model systems are available. In this context, the current SARS-CoV-2 pandemic has dramatically put the spotlight on the urgent need for antiviral drugs that are broadly active. Such drugs that inhibit diverse RNA viruses, either by targeting a common cellular process or a highly conserved viral enzyme, would be most promising as immediately available treatments against newly emerging viruses.

RNA viruses rely on a high supply of NTPs from the host metabolism to efficiently replicate. Drugs that inhibit some of the cellular NTP-producing processes can consequently also block viral replication in general and thus possess broad-spectrum antiviral activity. However, when cellular targets are addressed, toxicity may arise and a balance between efficient antiviral action and tolerable host toxicity must be found. In this context, enzymes of the purine or pyrimidine *de novo* pathways have been identified as potential host targets, as these pathways are required only for fast-dividing cells, such as cells of the immune system or in malignancies, and fast-replicating viruses but not to sustain viability of cells of the normal tissue. Regarding virus-targeted drugs, nucleoside and nucleotide analogues have been in use for several decades and vast antiviral data for many different NNAs is available to date; however, so far, no general pattern for structure-activity relationships emerges. Reasons for this include cell line-dependent metabolic limitations as NNAs need to be converted to their active triphosphate form in the relevant tissue. Consequently, the path towards developing broad-spectrum antiviral NNAs remains misty. Nonetheless, recent progress in the structural biology and biochemistry of viral polymerases, including the achievements in high-throughput as well as mechanistic assays, will help guide future efforts. Together with medicinal chemistry strategies to decrease toxic effects and innovative technologies to bypass metabolic limitations and overcome cell type-dependent potency, these will hopefully yield promising candidates. To counter high attrition rates, especially in advanced clinical development, efforts must further include early pre-clinical test systems for mitochondrial and other toxicity. Consequently, extensive collaboration among scientists from different fields and sectors must be fostered and the efficient translation of basic scientific findings to clinical settings must be supported to ultimately develop broadly-acting antiviral drugs and close this gaping hole in global preparedness.

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