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POST-SCREEN (GREY)

Nucleosides and emerging viruses: A new story

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With several US Food and Drug Administration (FDA)-approved drugs and high barriers to resistance, nucleoside and nucleotide analogs remain the cornerstone of antiviral therapies for not only herpesviruses, but also HIV and hepatitis viruses (B and C); however, with the exception of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), for which vaccines have been developed at unprecedented speed, there are no vaccines or small antivirals yet available for (re)emerging viruses, which are primarily RNA viruses. Thus, herein, we present an overview of ribonucleoside analogs recently developed and acting as inhibitors of the viral RNA-dependent RNA polymerase (RdRp). They are new lead structures that will be exploited for the discovery of new antiviral nucleosides.

Keywords: Nucleoside analogues; Antiviral therapy; (Re)emerging RNA viruses; Broad-spectrum antiviral agents; RNA-dependent RNA polymerase (RdRp)

Nucleoside analogs against DNA viruses

Nucleoside analogs remain the cornerstone of antiviral therapy, with more than 30 drugs approved over the past 50 years.[1,2] 5-Iodo-2′-deoxyuridine (IDU), discovered by W. Prusoff in 1959,[3] is considered to be the first antiviral active against herpes simplex virus (HSV). Subsequently, the discovery in 1971 by G. Elion of acyclovir [9-(2-hydroxyethoxymethyl) guanine], an anti-HSV antiviral, was a major breakthrough because acyclovir was (and still is) the only highly selective antiviral drug with little or no adverse effects on uninfected human cells.[4] The discovery of HIV during the 1980s as the causative agent of AIDS, as well as of 3′-azidothymidine (AZT, zidovudine) have driven the synthesis of numerous nucleoside analogs and their chemistry.[5] These nucleosides include: 2′,3′-dideoxynucleosides (ddNs), such as D4T (stavudine) against HIV; some L-analogs, such as L-dT (telbivudine) against HBV, and 3TC (lamivudine) and FTC (emtricitabine) against HIV; and some carbocyclic analogs of nucleosides (carbanucleosides), such as carbovir and its prodrug abacavir (ABC) against HIV and entecavir against HBV (Fig. 1). A. Holy and E. De Clercq pioneered a second generation of antiviral nucleosides,[6,7] called acyclic nucleoside phosphonates (ANPs), which are a major class of antivirals. Three of these have a broad antiviral spectrum against several DNA and RNA viruses and retrovirus: cidofovir (HPMPC) was particularly effective against herpesviruses [e.g., cytomegalovirus (CMV), HSV-1 and HSV-2, Varicella zoster virus (VZV)]; adefovir (PMEA) and its *bis*(-POM)-prodrug (adefovir dipivoxil) against HBV; and tenofovir (PMPA) and its *bis*(POC)-prodrug (tenofovir disoproxil) and tenofovir alafenamide against HIV and HBV (Fig. 1).

General mechanism of action of nucleosides

Nucleoside analogs (and acyclic nucleoside phosphonates) generally act in their 5′-triphosphorylated form and target viral DNA/RNA polymerase and HIV reverse transcriptase, thereby preventing the formation of viral nucleic acid. However, if the nucleoside triphosphate is the active form, it cannot cross the cell membrane because it is negatively charged; thus, one uses parent nucleosides that, after penetration into the cell, are then transformed into analogs of nucleoside triphosphate by three successive phosphorylations (Nu → NuMP → NuDP → NuTP)

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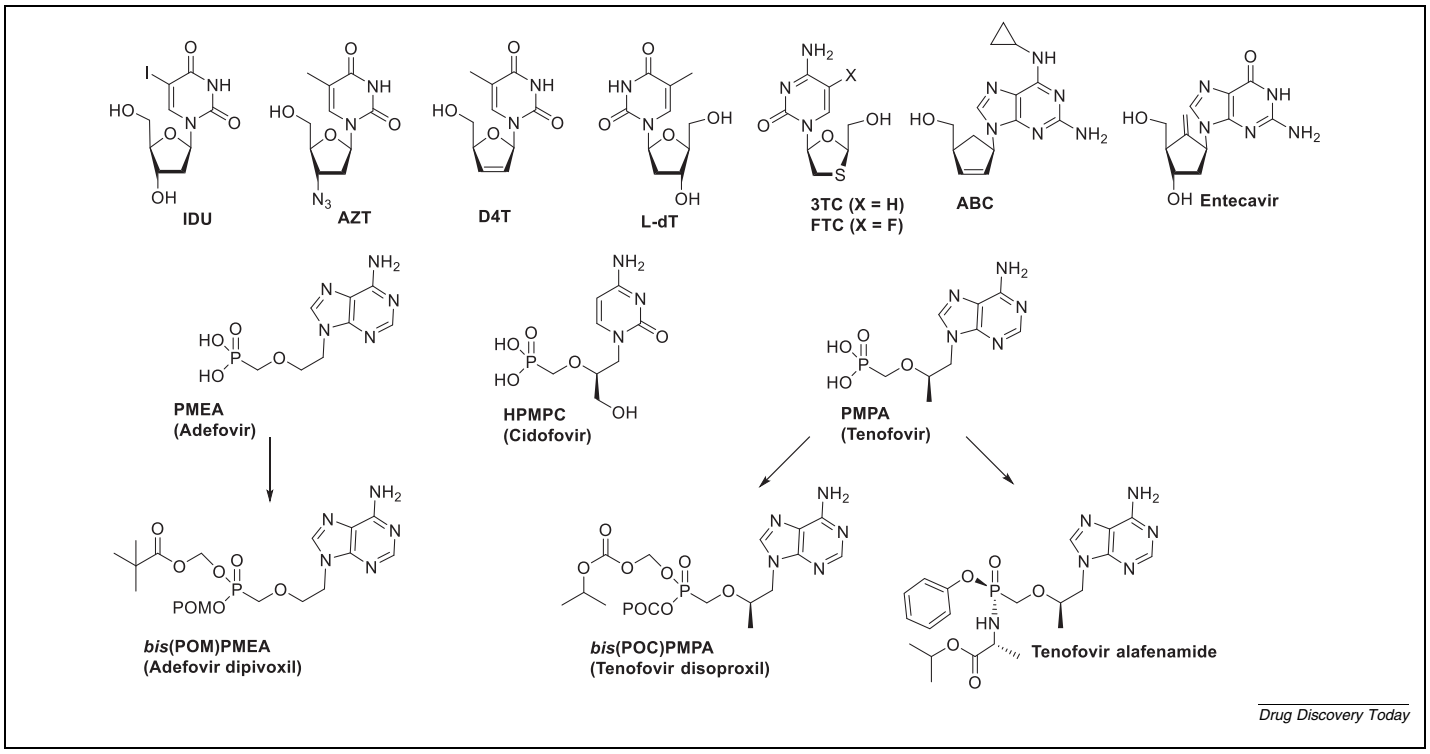

FIGURE 1

Chemical structures of antiviral nucleosides. This includes nucleoside analogs (IDU, AZT, D4T, L-dT, 3TC, FTC), carbocyclic nucleoside analogs (ABC and entecavir) and acyclic nucleoside phosphonates (PMEA, HPMPC, and PMPA) and their prodrugs [*bis*(POM)PMEA, *bis*(POC)PMPA, and tenofovir alafenamide].

catalyzed by various nucleoside and nucleotide kinases in the host cell or from some viruses (Fig. 2).[8]

However, both the first phosphorylation step and the penetration of Nu or NuMP into the cell remain the limiting steps; as a result, some nucleosides might appear inactive whereas their triphosphates inhibit the viral polymerase. To address these limitations, kinase bypass strategies have been developed that involve the direct delivery of phosphorylated nucleosides into cells.[9–12] To mask the negative charges of the phosphate moiety and increase cellular penetration while maintaining a good solubility in physiological fluids, various biolabile phosphate protecting groups have been developed. Nucleoside monophosphate is then released by enzymatic or intracellular chemical degradation of the biolabile groups by various enzymes, such as reductases, carboxylesterases, and cytochrome P450, allowing targeting to specific organs. Many biolabile groups have been developed to date, such as cycloSal, Hept-direct, nitrofuranylmethyl amidate, *bis*dithioethanole (DTE), and *bis*(*S*-acyloylthioethyl) (SATE) (Fig. 3). They have their own characteristics (stability, mechanism of release, polarity, solubility, etc.) that guide their use. Although some of these nucleoside prodrugs have entered clinical trials, none have been approved to date.

Other prodrugs, such as *bis*(POM) and *bis*(POC), which were mainly applied to ANP, led to marketed antiviral nucleosides (Fig. 4). Furthermore, the ProTide technology, based on triester aryloxy phosphoramidate prodrugs, invented by C. McGuigan in 1990,[13–16] has been successfully applied to various nucleoside analogs, including the marketed antiviral drugs sofosbuvir, tenofovir alafenamide, and remdesivir, with generally increased antiviral activity compared with the parent nucleoside. This is a versatile method because variations can be made at the ester (R), amino acid (R′) and aryl moieties. In addition, the chirality at the phosphorus (*R*p or *S*p) is also important for the antiviral activity.

Once incorporated into the growing viral nucleic acid, if a nucleoside lacks the 3′-OH group (such as AZT, D4T, 3TC, PMEA, or PMPA), they inhibit chain elongation and act as (obligate) chain terminators. However, some nucleosides that still have a 3′-OH group (such as entecavir, L-dT, IDU, and HPMPC) can also inhibit chain elongation, but only after several incorporations that result in changes in the structure of viral nucleic acid and a pause in its synthesis; these compounds are referred to as 'delayed chain terminators'.

(Re)emerging RNA viruses: A new threat

The continuous growth of the human population, as well as human interactions with wild environments, have resulted in several emerging and re-emerging RNA viruses responsible for highly lethal viral diseases and pandemics.[17–20] This includes not only SARS-CoV (2002, global), but also DENV (2002, 2010, 2019 Americas and 2013, Southeast Asia), Chikungunya (2005, India and 2014, Americas), Rift valley fever (2007, East Africa), H1N1 influenza (2009, global), Middle East respiratory syndrome coronavirus (MERS-CoV, 2012, Middle East), Ebola virus (2013, West Africa and 2018, Africa), Zika virus (2015, pan-Americas), Yellow fever (2014, Africa), Nipah virus (2018, India), and, more recently, the SARS-CoV-2 virus (2019, worldwide).[21–23] Most RNA viruses are often zoonotic or vector-borne infectious agents

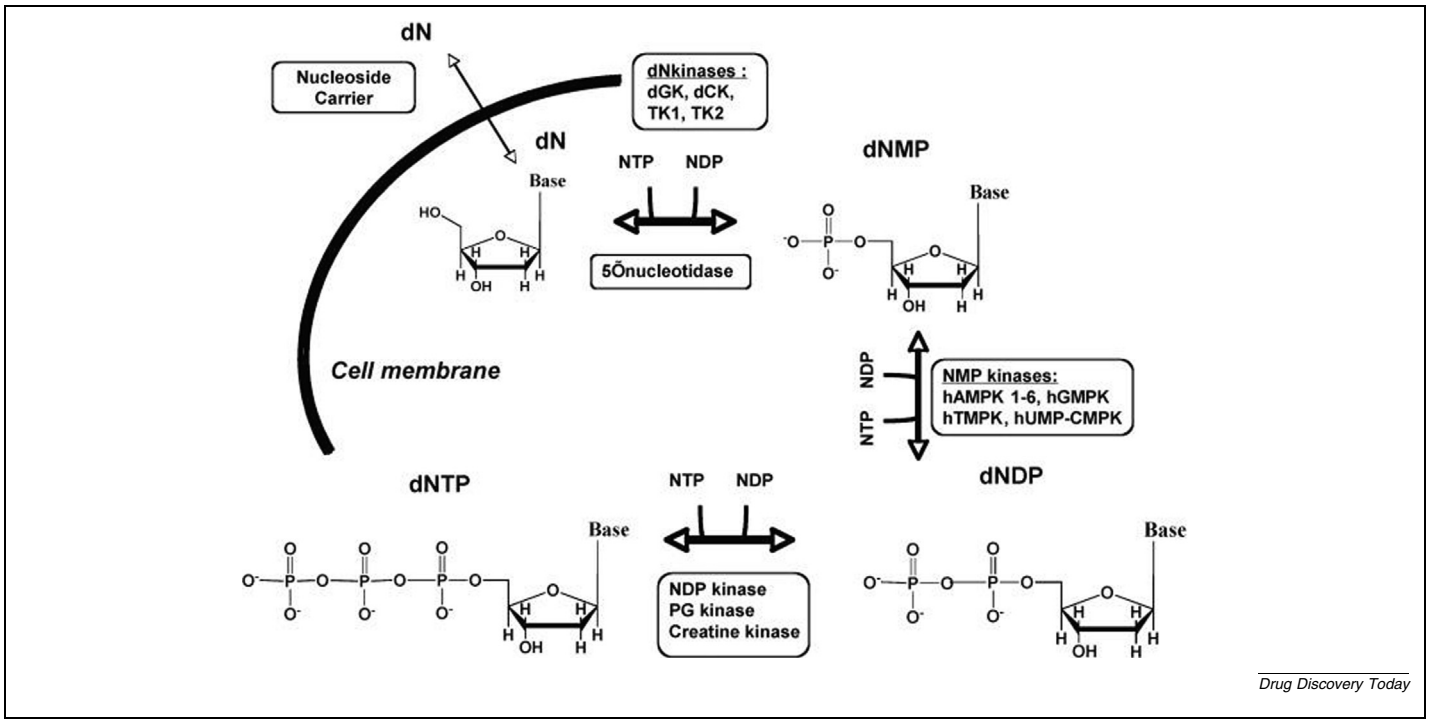

FIGURE 2

Main mechanism of activation of antiviral nucleosides by various nucleoside and nucleotide kinases. After cell penetration, the nucleoside is converted by nucleoside kinases (dN kinases) to its monophosphate, then to the diphosphate by nucleoside monophosphate kinases (NMP kinases) and finally to the triphosphate by nucleotide diphosphate kinase (NDP kinases). Adapted from.[8]

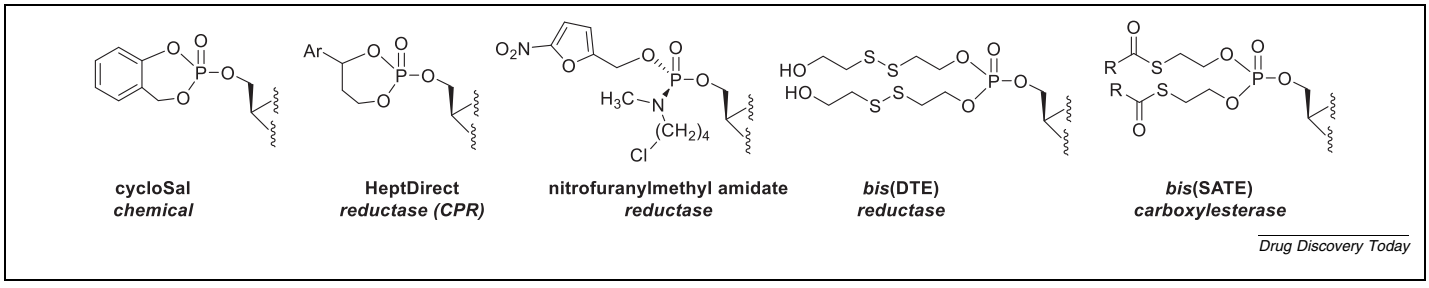

FIGURE 3

Examples of kinase bypass strategies applied to deliver antiviral nucleosides as their monophosphate prodrug analogs.

with natural reservoirs, such as chimpanzees for HIV, bats for MERS-CoV and SARS-CoV, fruit bats and primates for Ebola, human H1N1, and swine flu.[24–26] Approximatively two or three new RNA viruses are discovered each year, which can be a major public health challenge because their rates of spread and mutation are often higher than those of DNA viruses.[27–29] With the exception of SARS-CoV-2, for which vaccines are now available, there are no vaccines or antivirals for most other RNA viruses.

Nevertheless, several lessons learned from the fight against chronic DNA viral infections will help in designing novel antiviral nucleosides against RNA viruses: first, long-term antiviral therapy produces dominant strains of resistant mutants (even though nucleoside analogs have a high barrier to drug resistance).[30] Additionally, inhibition of virus metabolism has a direct impact on host cells. From a chemical point of view, research on antiviral nucleosides has benefited from a better understanding of their mode of action (either by acting directly on viral polymerases or based on interference of cellular enzymes), from their structure *per se* (conformation, stereoselectivity, and phosphonate and phosphate prodrugs), and their metabolisms and interactions with target viral proteins; furthermore, some structural requirements for the antiviral activity of nucleosides have been established.[31–33] Nucleobase modifications have also been extensively explored,[34] and it appears that, for enzymatic incorporation of nucleosides, modifications at C5 of pyrimidine, and at C7 of purines are tolerated[35] as long as they maintain Watson–Crick base pairing. Other sugar or nucleobase modifications have also been correlated with antiviral activities,[36] and docking studies have helped to understand the interaction of some ribonucleosides with the RdRp of RNA viruses.[37]

The search for broad-spectrum antivirals is the preferred strategy for inhibiting viral replication of RNA viruses. Indeed, they

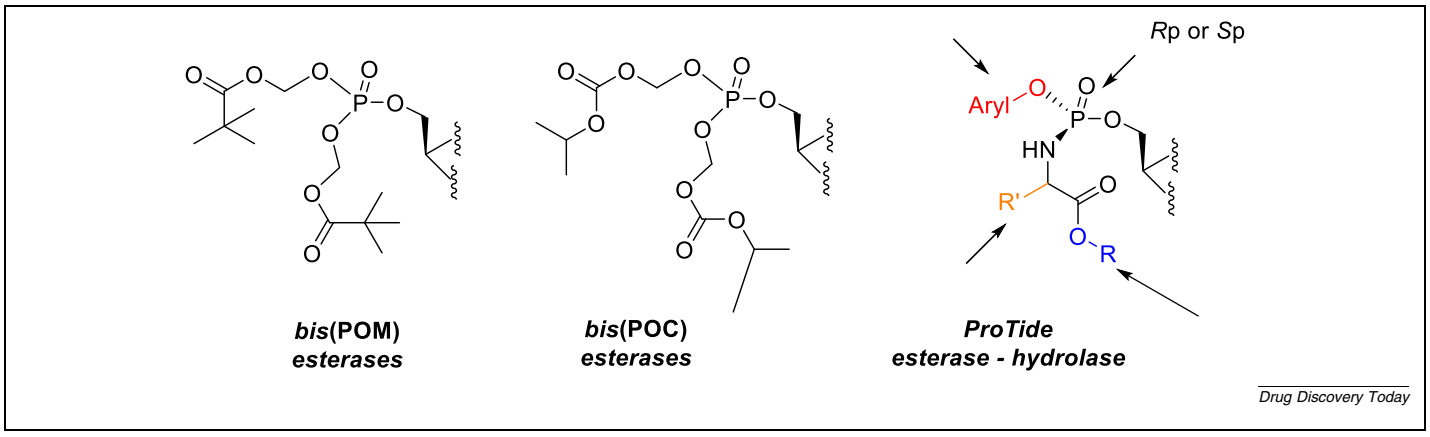

FIGURE 4
Relevant prodrugs, including ProTide technology, applied to marketed antiviral nucleosides.

all have a relatively conserved RdRp,[38–40] which is a key viral enzyme and, thus, represents the main therapeutic target (Fig. 5).[41–44] However, most viral RNA replicases lack proofreading activity (via an exonuclease), except coronaviruses,[45] leading to many errors during the replication process.[46,47] Thus, RNA viruses can not only become resistant, but also escape vaccine-induced immunity. [48]

Therefore, viral infections caused by RNA viruses can be treated with inhibitors of nucleic acid synthesis or those that induce lethal mutagenesis by a high rate of viral mutations.[49,50] Given that no homolog of RdRp has been found in human cells and the extensive knowledge of its function, it is an important target for the discovery of new nucleoside analogs against RNA viruses.[51–53]

Nucleoside and nucleotides analogs against RNA viruses

Sofosbuvir, a uridine nucleotide prodrug, is one of the most successful discoveries of an RdRp inhibitor, now used in the treatment of HCV. However, compared with the antiviral nucleoside analogs approved against DNA viruses, only a few nucleoside analogs and nucleobases have been developed against RNA viruses so far. Therefore, we discuss the structural features and mechanisms of action of selected antiviral nucleoside analogs acting against RNA viruses (Fig. 6).

Ribavirin

Ribavirin (RBV) is a unique ribonucleoside, first synthesized in 1970 at ICN pharmaceuticals, which bears a 1H-1,2,4-triazole-3-carboxamide moiety as nucleobase. This 'old' antiviral compound is a broad-spectrum agent, active against various DNA and RNA viruses.[54–58] It has been clinically approved together with IFN-α not only as an HCV treatment, but also for treating infections caused by respiratory syncytial virus, adenovirus, hantavirus and some hemorrhagic fever viruses (Lassa, Congo). RBV has several mechanisms of action; RBV monophosphate (RBV-MP) can first inhibit the inosine monophosphate dehydrogenase, which is involved in the *de novo* synthesis of purine nucleotides (IMP and GTP). This results in the depletion of intracellular

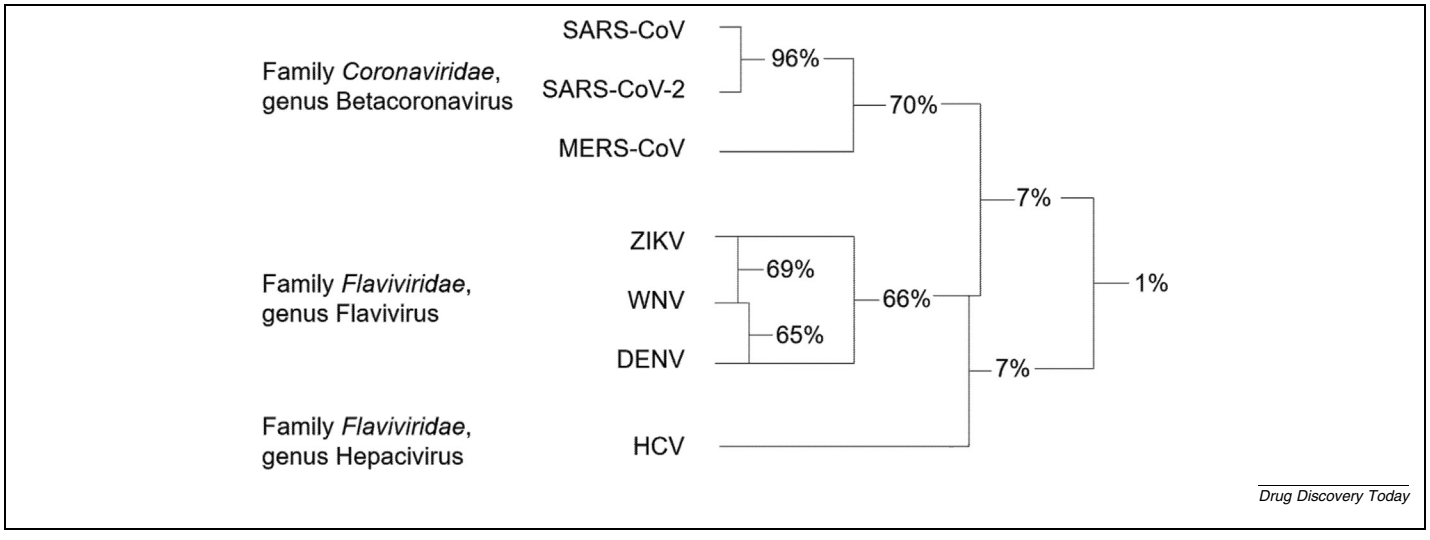

FIGURE 5
Percentage similarity of RNA-dependent RNA polymerases of various RNA viruses. Adapted from.[51] For definitions of abbreviations, please see the main text.

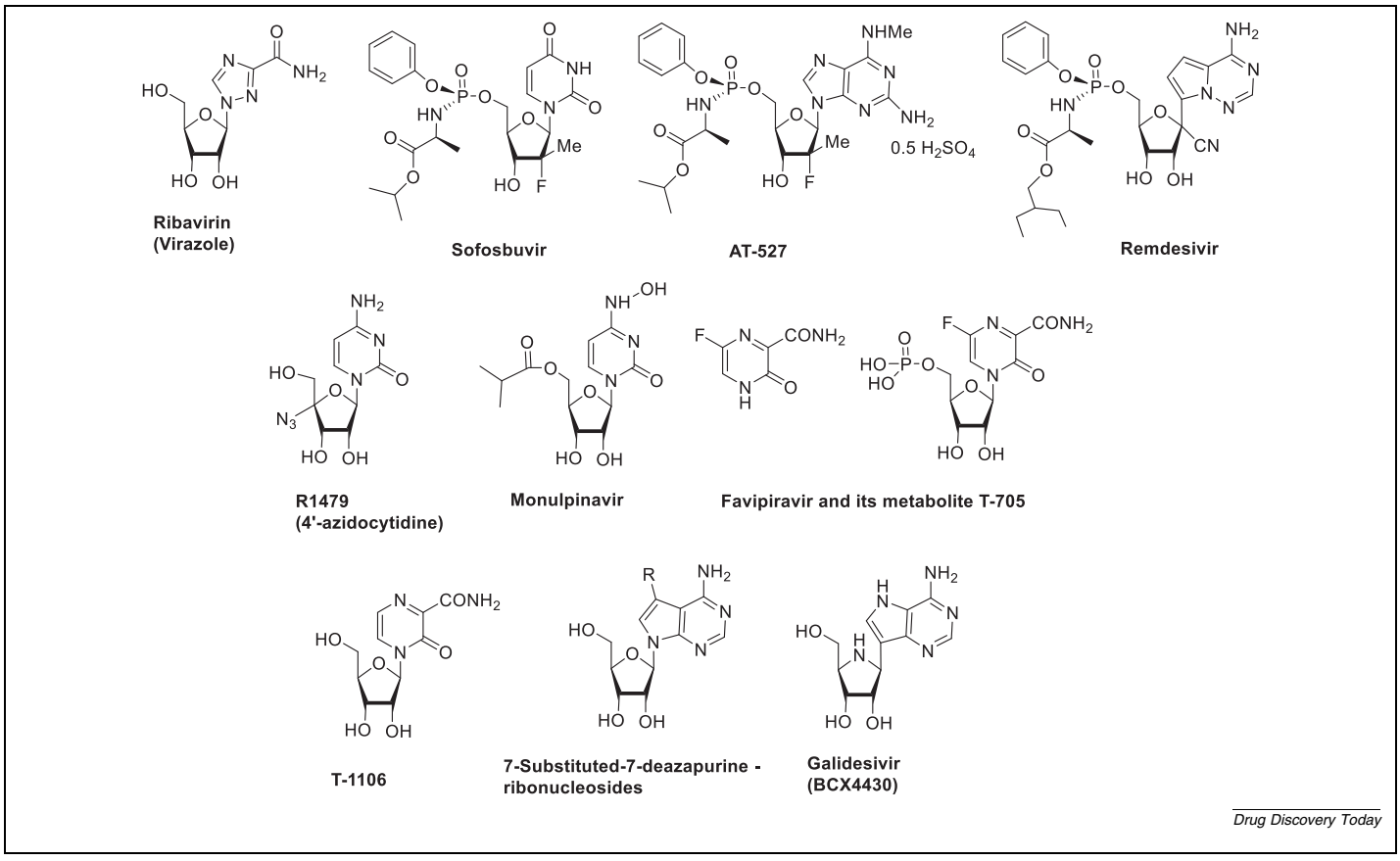

FIGURE 6
Structure of antiviral nucleosides inhibitors of RNA-dependent RNA polymerases of RNA viruses.

GTP and, thus, has a direct impact on both cell and viral replication. An immunomodulatory activity of RBV was also suggested (increase in T helper lymphocyte activity). The 5′-triphosphate form of RBV (RBV-TP) can directly inhibit the RdRp of RNA viruses. In addition, ribavirin can interfere with the formation of the 5′ cap structure of viral mRNA, probably by inhibiting guanyl transferase and methyltransferase. Finally, ribavirin could enhance viral mutagenesis by substitution of RTP for GTP, because most RdRps lack proofreading abilities, which could explain why RBV is not used in the clinic as widely as one might expect. However, overall, RBV remains a potential important drug for the treatment of (re)emerging viruses.

Sofosbuvir discovery

Sofosbuvir was discovered in 2007 by M. Sofia at Pharmasset through in-depth investigation of the impact of structural modifications at the C2′ position of ribofuranose [e.g., by 2′-methyl and 2′-fluoro modifications (direct impact on the 3′-endo conformation)] on antiviral activity. It was approved by FDA in 2013 for the treatment of chronic HCV infection.[59] HCV belongs to the large *Flaviviridae* family, which includes hepaciviruses (e.g., HCV) and flaviviruses [e.g., Yellow fever, Dengue (DENV), West Nile (WNV), and Zika viruses), all of which are important threats to human health.[60] Sofosbuvir is the 2′-β-methyl analog of the 2′-α-fluorouridine 5′-monophosphate containing a phosphoroamidate moiety where R′ = *L*-alanine, R = *iso*propyl ester, and aryl = -phenyl (Fig. 4).[61,62] Sofia determined that the *S*p isomer (EC_{90} = 0.42 μM) was tenfold more active than the *R*p isomer (EC_{90} = 7.5 mM), with no cytotoxicity (up to 100 μM). After cell penetration, the prodrug is cleaved by host enzymes and chemical hydrolysis, releasing sofosbuvir-5′-monophosphate, which is then converted by various host kinases to its active metabolite, resulting in high levels of the triphosphate analog in the liver. Sofosbuvir is a chain terminator.

Other 2′-alkylated ribonucleoside analogs

While working on the discovery of sofosbuvir, Sofia and others explored important structural features of this molecule.[63] For instance, the presence of 3′-OH in the α-orientation is required, whereas some modifications at the 2′ position by either a α-fluorine or a α-OCH_3 are tolerated. Thus, other 2′-methyl-ribonucleosides were developed either as nucleosides or as prodrugs. For instance, 2′-methylcytidine, 7-deaza-2′-methyladenosine exhibited antiviral potency against various other (+) single-stranded (ss)RNA viruses. In general, 2′-methyl ribose-modified nucleosides and their prodrugs are more potent against (+)ssRNA viruses than against (−)ssRNA viruses. AT-527 is a purine nucleotide prodrug, developed by Atea Pharmaceuticals against Coronavirus 2019 (COVID-19).[64] It is a salt formed at the nucleobase moiety (0.5 H_2SO_4), which, after dissolution, allows the release of AT-551 (free base form of AT-527). AT-551 then acts as a substrate for cathepsin A, carboxylesterase 1 (CES1), and several other enzymes, and is finally converted to

AT-9010, the active triphosphate metabolite.[65] Unfortunately, it recently failed in a Phase II COVID-19 clinical trial.

1′-Cyano and 4′-azido-substituted nucleosides, including remdesivir and R1479

Other modifications were explored at the 1′- and 4′-positions of the ribose moiety. For instance, 1′-methyl- and 1′-fluoromethyl-led to compounds with little or no activity.[66,67] Other analogs were designed in which the *N* in the glycosidic bond was replaced by a carbon, leading to the development of 1′-substituted *C*-nucleosides.[68] These compounds are not substrates of *N*-glycoside hydrolases and phosphorylases, which cleave parent nucleosides. GS-441524, discovered by Gilead from screening libraries of nucleoside analogs, is a *C*-nucleoside adenosine analog bearing a 1′-CN group.[69] Its phosphoramidate pronucleotide analog (remdesivir), originally developed against Ebola virus, has broad-spectrum antiviral activity against various RNA viruses, including Lassa fever virus, Nipah virus, and coronaviruses. It is an inhibitor of RdRp, which evades proofreading by viral exoribonuclease (ExoN), and acts as a RNA chain terminator (delayed chain terminator). Remdesivir is approved by the FDA for the treatment of COVID-19 in selected patients. It was found from various 1′-modifications (methyl, vinyl, and ethynyl) that the 1′-CN modification led to more a potent antiviral. Docking studies of the 5′-triphosphate form of remdesivir into viral RdRp revealed a unique pocket in the protein where the 1′-cyano group binds (with Asp865-Lys593); this might explain why 1′-cyano analogs are selective for viral polymerases and stable to the viral exonuclease.[70,71]

Similar small modifications were also introduced at the C4′ position of the ribosyl to modify the sugar pucker from the northern conformation 'C3′-endo/C2′-exo' to the southern 'C2′-endo/C3′-exo' one. Several 4′-fluorine and 4′-methyl analogs in a series of riboses and 2′-deoxyriboses, as well as their prodrugs with little or no antiviral activity, were designed. The 4′-azidocytidine (R1479 developed by Roche) is an inhibitor of RdRp from HCV ($IC_{50} = 1.28\ \mu M$), but is also active against DENV, henipaviruses, and respiratory syncytial virus.[72] Balapiravir, its *O*-acylated prodrug, was effective against HCV, but was less potent than sofosbuvir and, thus, its development was halted. Interestingly, 4′-azido-*ara*cytidine (RO-9187) was not only a potent anti-HCV analog ($IC_{50} = 0.171\ \mu M$), but also an effective inhibitor of tick-borne encephalitis virus (EC_{50} 0.3 μM). [73]

Heterocyclic base-modified nucleosides, including molnupiravir and favipavir

Among current efforts to develop antivirals agents against RNA viruses, nucleosides analogs bearing modifications at the base moiety represent an important class of drug candidates. Favipiravir (T-705), a pyrazine analog (6-fluoro-3-hydroxy-2-pyrazine carboxamide), developed by the Toyama Chemical Company, is a potent RNA polymerase inhibitor. It is used in Japan to treat influenza viruses [A(H1N1)pdm09, A(H5N1), and A(H7N1)] and has shown excellent results against oseltamivir-resistant viruses.[1,74] Favipiravir also exhibits efficient antiviral effects against other (+)ssRNA and (−)ssRNA strand viruses, such as filoviruses (Ebola), arenaviruses, noroviruses, bunyaviruses, togaviruses, hantaviruses, and flaviviruses.[75] The activation mechanism whereby it exert its antiviral activity requires its conversion to favipiravir ribofuranosyl 5′-triphosphate from (T-705 RTP) by cellular enzymes in host cells. Favipiravir-RTP is recognized as a purine analog and is incorporated selectively into RNA extensions by viral polymerase (not human DNA polymerase), acting as antiviral lethal mutagen.[76] Favipiravir could be repurposed for the treatment of moderate COVID-19 (Phase III clinical trials), although adverse events have been reported.[77–79]

Molnupiravir (MK-4482), a β-D-N^4-hydroxycytidine 5′-*iso*propylester prodrug, developed by Merck, is active against a broad spectrum of RNA viruses. During RNA synthesis through RdRp, molnupiravir is incorporated in place of cytidine or uracil, leading to mutated RNA products. Thus, this molecule inhibits viral replication via a lethal mutagenesis mechanism, resulting in the accumulation of mutations beyond the replication fidelity required for viability.[80,81] Molnupiravir was approved in the UK in November 2021 for the treatment of COVID-19 by oral administration and received FDA emergency use authorization in December 2021.[82,83] Molnupiravir also inhibits the replication of influenza viruses and respiratory syncytial viruses,[84] Venezuelan equine encephalitis virus,[85] Chikungunya virus,[86] and Ebola virus,[87] and confers minimal cytotoxicity with genetic barriers to resistance.

Among the nucleosides modified at the base moiety, 7-deazaadenosine analogs represent an emerging class for the development of new antivirals. Modifications of the ribose moiety by methyl at 2′-position (7-deaza-2′-C-methyladenosine, MK-608) and its fluorine derivative exhibit anti-HCV activity at submicromolar concentrations.[88,89] Since 2011, Hocek's group has reported various 7-substituted 7-deazaadenine ribonucleosides,[90] which were converted in their 5′-*O*-triphosphate form. They both inhibit the RdRp of Zika virus, Japanese encephalitis virus, and West-Nile virus. The nucleosides were then transformed into their prodrug forms [such as phosphoramidates, mono-SATE, and bis(SATE)], with micromolar or submicromolar antiviral activities. The bulkier aryl substituents were found to be less active with micromolar activities, but lower cytotoxicity. Surprisingly, the conversion in the prodrug forms of the corresponding nucleosides did not increase the antiviral activities or decrease the cytotoxicity, which might suggest unconventional nucleoside activation. With the emergence of RNA viruses and the lack of approved drugs, 7-substituted-7-deazapurines represent an important class of nucleoside analogs.

Imino-C-nucleosides, including galidesivir (BCX4430)

Immucilins are chemically stable C-nucleoside analogs in which the *O* of the sugar ring is replaced by an NH, and have attracted increased attention for drug discovery.[91] They target the inhibition of purine nucleoside phosphorylase (PNP), a key enzyme involved in purine metabolism. They mimic the transition state of PNP (e.g., the ribooxocarbenium intermediate). Immucilin-A (BCX4430, galidesivir) an adenosine nucleoside analog developed by BioCryst Pharmaceuticals, is broadly active against filoviruses and flaviviruses.[92] After incorporation into the growing viral RNA strand, it inhibits viral RdRp as chain terminator. Galidesivir is in development for the treatment of RNA viruses, such

as SARS-CoV-2, Ebola virus, Marburg virus, and Yellow Fever virus.[93]

Concluding remarks

RNA viruses are the causative agents of various pandemics, including COVID-19. Only a few ribonucleoside analogs (sofosbuvir, remdesivir, and molnupiravir) have been approved for the treatment of RNA viruses as direct-acting antivirals. It is expected that new structural details of RdRps from these RNA viruses, as well as ligand-bound analyses, will help to design new therapeutics. Given that RNA replication depends on a large supply of NTP from the host cell, ribonucleoside analogs that inhibit the *de novo* pathway might also lead to new antivirals. Other important ribonucleoside analogs are currently being explored, such as rigid amphipathic nucleosides, *N*6-aryl-substituted purine analogs and new prodrugs, *L*-analogs, 5′-modified nucleosides, and fleximer analogs. Lessons learned from DNA viruses as well as recent structural findings regarding RdRp in RNA viruses could help design new broad-spectrum nucleosides analogs through practical guidelines and facilitate their clinical development. However, small modifications of the nucleoside scaffold (sugar, nucleobase, or prodrug) can impact their biological activities (gain and loss) in terms of biological and pharmacokinetics parameters, cellular uptake, and so on. Thus, synthetic platforms should be used to develop new and more complex ribonucleoside analogs and their prodrugs. In general, for both academic laboratories and pharmaceutical industries, flow chemistry coupled with techniques used in artificial intelligence will maximize the chances of discovering new and potent antiviral nucleosides.

As recently stated by great discoverers and developers of antiviral nucleosides: 'Nucleosides: the best is still to come!!'

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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