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Antimicrobial Strategies
Inhibition of Viral Polymerases by 3′-Hydroxyl Nucleosides

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Abstract

Over the past 20 years, nucleoside analogues have constituted an arsenal of choice in the fight against HIV, hepatitis B and C viruses, and herpesviruses. Classical antiviral nucleosides such as zidovudine act as obligate chain terminators. Once incorporated as monophosphates into the viral nucleic acid, they immediately block the progression of the polymerase as a result of their lack of a reactive 3′-hydroxyl (3′-OH) group. This review explores beyond the paradigm of obligate chain termination, from a structural and a mechanistic perspective, the strategy of inhibiting viral polymerases (RNA- and DNA-dependant) with nucleoside analogues containing a 3′-OH group. Depending on their mechanism of action, these molecules typically fall into the following three categories: (i) delayed chain terminators; (ii) pseudo-obligate chain terminators; or (iii) mutagenic nucleosides. Delayed chain terminators (i.e. penciclovir, cidofovir and entecavir) block the polymerase at an internal position within the viral nucleic acid, whereas R7128 and the 4′C substituted nucleosides do not permit subsequent incorporation events. Ribavirin, 5-hydroxydeoxycytidine and KP1461 are not chain terminators. Instead, they inhibit viral replication after mispairing with the template base, resulting in random mutations that are often lethal. Finally, brivudine, clevudine and other L-nucleosides have unique or yet to be defined mechanisms of inhibition.

1. Beyond the Paradigm of Obligate Chain Termination

Sixteen commercially approved antiviral drugs are nucleoside analogues. For HIV alone, eight nucleos(/t)ide analogues have been successfully developed. These nucleoside analogue reverse transcriptase inhibitors (NRTIs) represent the backbone in the most frequently used drug regimens. Two NRTIs are often administered in combination with a non-nucleoside reverse transcriptase inhibitor or one to two protease inhibitors that, together, can suppress viral replication to levels below the limit of detection and reduce the risk of resistance development. In 1987, zidovudine (3′-azido-3′-deoxythymidine [AZT]) was the first commercialized anti-HIV drug and remains to this day an important component in frequently used drug regimens. Like all other polymerase inhibitors of this class, zidovudine requires activation by intracellular phosphorylation (figure 1). With the exception of the phosphonate prodrug tenofovir disoproxil fumarate, it is only under their triphosphate form that chain terminators such as zidovudine can compete against natural nucleotides for polymerase binding and catalysis. In the case of zidovudine, the second phosphorylation step performed by

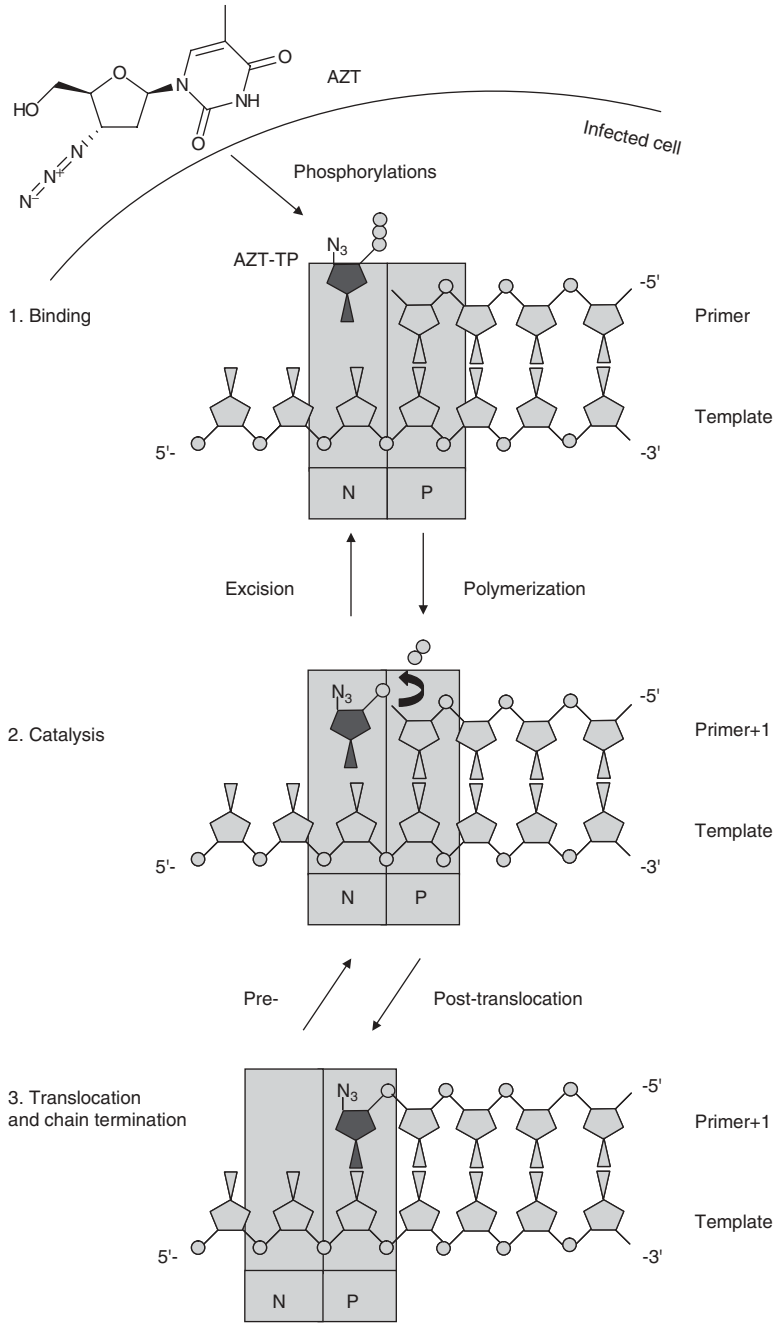

Fig. 1. Mechanism of action of zidovudine (AZT). Once entered into the cytoplasm of a HIV-infected cell, AZT is phosphorylated by cellular kinases. AZT triphosphate (AZT-TP) [dark grey] binds to the nucleotide site (N) within the active site of the reverse transcriptase of HIV and is incorporated into the nascent viral DNA (light grey) by in-line nucleophilic attack of the α-phosphate (solid arrow). After catalysis, the terminal nucleotide of the primer strand is usually translocated to the primer site (P). In the case of AZT monophosphate, the substitution of the 3′-hydroxyl group by an azido cannot support further DNA elongation. AZT is therefore classified as an obligate chain terminator.

the thymidylate kinase is rate limiting. As depicted in figure 1, binding of zidovudine triphosphate to the HIV reverse transcriptase viral target takes place at the nucleotide binding site. Unlike natural nucleotides, in zidovudine, the 3′-hydroxyl (3′-OH) group is substituted with a bulky azido (N_3). This neither decreases the binding of its triphosphate to the HIV reverse transcriptase nor does it affect the incorporation of its monophosphate form. Instead, the absence of the 3′-OH group in the newly incorporated zidovudine monophosphate immediately blocks further DNA synthesis after the polymerase slides to the post-translocation position with zidovudine monophosphate in the primer site. This is the reason why zidovudine is termed an obligate (or immediate) chain terminator. One of the limitations to the use of zidovudine as a chain terminator comes from the selection of mutations within the HIV reverse transcriptase sequence that induce a loss of sensitivity to the drug. In particular, the well characterized thymidine-associated mutations at positions 41, 67, 70, 210, 215 and 219 in HIV-1 reverse transcriptase increase the stability of the pre-translocation complex, with the nucleotide site (N) occupied by zidovudine monophosphate (for a complete review see Gotte[1]). This allows pyrophosphate-based excision of neo-incorporated zidovudine monophosphate by pyrophosphorolysis, the reverse reaction of polymerization. In physiological conditions, adenosine triphosphate and guanosine triphosphate are likely to be the relevant pyrophosphate-donors for excision. Once zidovudine monophosphate is excised, DNA polymerization by HIV reverse transcriptase can resume because the 3′-OH end of the primer strand becomes available again.

Beyond the paradigm of obligate chain termination, this review summarizes the progress made in designing novel nucleoside analogues across virus species, as well as understanding their mechanisms of actions. Special attention is given to nucleoside analogues that inhibit viral polymerases in spite of the presence of a 3′-OH group (figure 2a). These molecules contain a modified nucleobase, a modified or removed sugar, or an inversion of configuration (figure 2b). The resulting derivatives fall into one of the following three categories depending on their mechanism of action: (i) delayed chain terminators; (ii) pseudo-obligate chain terminators; and (iii) mutagenic nucleosides. The antiviral spectrum of nucleoside analogues bearing a 3′-OH group is listed in table I. Finally, this review also exemplifies how non-obligate chain terminators can provide attractive solutions to improve activation of nucleosides to their triphosphate form and to prevent the loss of potency as a result of resistance mutations acquired by the polymerase target.

2. Delayed Chain Terminators

2.1 Penciclovir and Ganciclovir

The concept of a delayed chain terminator is not new. It was pioneered with penciclovir and ganciclovir, which were developed against herpesviruses in the 1980s. Their predecessor, the acyclic guanosine analogue aciclovir (acyclovir), is an obligate and therefore, immediate chain terminator that was first described as an inhibitor of herpes simplex virus (HSV) in 1978.[2] Penciclovir and ganciclovir are structurally related to aciclovir (figure 3). As a result of the addition of a hydroxymethyl group, both molecules have an enhanced binding affinity for the HSV thymidine kinase compared with aciclovir.[3] As a consequence, intracellular concentrations of their triphosphate forms can reach higher levels compared with aciclovir triphosphate.[4] Ganciclovir monophosphate considerably reduces the efficiency of the next event of phosphoryl transfer once incorporated into the DNA chain.[5] However, it is the efficiency of accommodation of the following nucleotide that is most significantly reduced (by 500-fold) once ganciclovir is incorporated at the penultimate position on the primer strand.[5,6] Non-immediate chain termination following penciclovir monophosphate incorporation by HSV-2 polymerase has also been documented.[7] For this reason, ganciclovir and penciclovir can be considered delayed chain terminators.

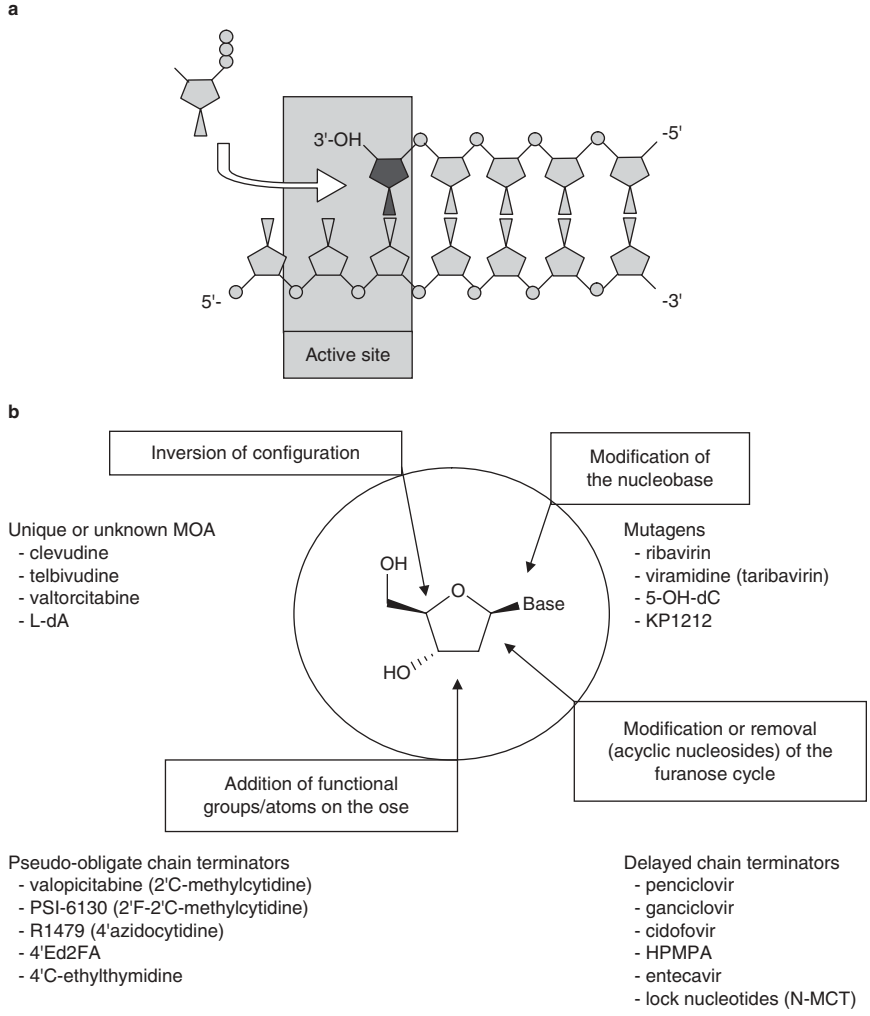

Fig. 2. Concept and mechanism of action (MOA) of non-obligate chain terminators. (**a**) Non-obligate chain terminators are nucleotide analogues that contain a reactive 3′-hydroxyl (3′-OH) group (dark grey). Once incorporated, these molecules can therefore theoretically support further nucleic acid synthesis. (**b**) Types of modifications leading to non-obligate chain terminators. Changes can be found either on the nucleobase or within the sugar moiety. Corresponding MOAs and examples of molecules are also listed, and the antiviral spectrum can be found in table I. **4′Ed2FA** = 2′-deoxy-4′C-ethynyl-2-fluoroadenosine; **5-OH-dC** = 5-hydroxydeoxycytidine; **HPMPA** = 3-hydroxy-(2-phosphonomethoxy)propyl]-adenosine; **L-dA** = L-deoxyadenosine; **N-MCT** = North-Methanocarbathymidine.

2.2 Cidofovir and Derivatives

The acyclonucleotide analogue 3-hydroxy-(2-phosphonomethoxy)propyl]-cytosine (HPMPC) displays a broad spectrum of anti-DNA virus polymerase inhibition. HPMPC contains one asymmetric centre on the 2′ carbon of the phosphonyl side chain. The S-enantiomer ([S]-HPMPC [cidofovir]) is the antipode showing the greatest antiviral activity, and it is currently used in cytomegalovirus (CMV) infection. Because cidofovir already contains a phosphonate group, its first metabolic activation step is not limited by the virus-encoded protein kinase UL97, and only two phosphorylations are required prior to its incorporation into viral DNA (figure 4). Cidofovir contains a hydroxyl group, located on the pseudo-sugar moiety. As a result, incorporation

Table I. Antiviral spectrum of 3′-hydroxyl nucleosides

Molecule	Antiviral spectrum
Penciclovir	HSV, VZV
Ganciclovir	CMV
Cidofovir	Herpesviruses, poxviruses
HPMPA	Adenoviruses
Entecavir	HBV, HIV
N-MCT	HIV
Valopicitabine	HCV
PSI-6130	HCV
R1479	HCV
4′Ed2FA	HIV
4′C-ethylthymidine	HIV
Ribavirin	Broad spectrum[a]
Viramidine	Similar to ribavirin
5-OH-dC	HIV
KP1212	HIV
Brivudine	VZV
Clevudine	HBV, EBV
Telbivudine	HBV
Valtorcitabine	HBV
L-dA	HBV

a Includes picornaviruses, respiratory syncytial virus, filoviruses, arenavirus, influenza virus, flaviviruses, vaccinia virus, HSV-1 and -2.

4′Ed2FA = 2′-deoxy-4′C-ethynyl-2-fluoroadenosine; **5-OH-dC** = 5-hydroxydeoxycytidine; **CMV** = cytomegalovirus; **EBV** = Epstein-Barr virus; **HBV** = hepatitis B virus; **HCV** = hepatitis C virus; **HPMPA** = 3-hydroxy-(2-phosphonomethoxy)propyl]-adenosine; **HSV** = herpes simplex virus; **L-dA** = L-deoxyadenosine; **N-MCT** = North-Methanocarbathymidine; **VZV** = varicella zoster virus.

of cidofovir does not readily block DNA synthesis by CMV polymerase. Instead, two consecutive events of cidofovir incorporation are required to inhibit enzyme progression by inducing a distortion of the primer end.[8] Moreover, it has been documented that DNA templates containing cidofovir do not support efficient DNA replication.[8] Similar observations have been made recently when the polymerase activity of vaccinia virus was measured in the presence of (S)-HPMPA, the adenosine counterpart of cidofovir.[9] When (S)-HPMPA diphosphate was added by the viral polymerase to a position N on a synthetic primer, it caused a very weak stop at the N+1 position. The strongest inhibition effect was observed when (S)-HPMPA was present instead in the template strand (figure 4). This implies that the mechanism of action of cidofovir and (S)-HPMPA against CMV and vaccinia virus, respectively, is mainly to inhibit secondary rounds of DNA synthesis. This is in sharp contrast with related acyclic phosphonates, such as tenofovir and adefovir, which are obligate chain terminators of DNA synthesis and are currently used against HIV and hepatitis B virus (HBV) infections.

2.3 Entecavir

Entecavir is a guanosine analogue bearing a cyclopentyl sugar ring (figure 5a). Entecavir was identified in 1997 as a selective inhibitor of HBV.[10] It has recently been discovered that entecavir can also inhibit HIV replication. This was demonstrated by the fact that the clinical use of entecavir in HBV/HIV co-infected individuals can cause the selection of HIV-1 variants containing the lamivudine signature mutation (M184V) in the HIV-1 reverse transcriptase enzyme.[11] Interestingly, the *in vitro* inhibition profile of entecavir reported by McMahon et al.[11] is atypical for an NRTI (saturating concentrations of entecavir can only inhibit 90% of HIV replication). Although it is not yet well understood, this partial HIV inhibition suggested that entecavir might have a mechanism of action that is different to other NRTIs. Convincing evidence for a distinct mechanism intrinsic to entecavir has been provided by an endogenous reverse transcriptase assay on purified HBV nucleocapsids, showing that DNA synthesis continues for at least three nucleotides following the incorporation of entecavir monophosphate.[12] Entecavir has the same effect on the reverse transcriptase activity of HIV – it reduces the efficiency of incorporation of the fourth downstream nucleotide by over 1000-fold (figure 5a).[13]

2.4 Lock Nucleotides

It is known from the crystal structures of HIV reverse transcriptase in complex with DNA that nucleotides near the active site adopt a N-conformation, with an equatorial 3′-OH group.[14,15] However, nucleotides progressively transition to the S-conformation near the thumb

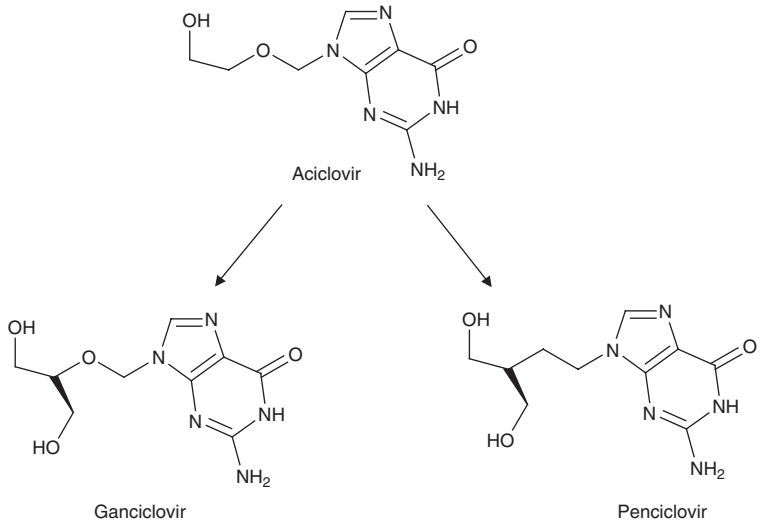

Fig. 3. Delayed chain terminators used against herpesviruses.

subdomain. In theory, blocking the transition from the N- to the S-conformation would provide a novel mechanism of inhibition of polymerization. To achieve this goal, nucleoside analogues with fixed N-conformation as a result of a pseudosugar moiety have also been tested *in vitro* against purified HIV reverse transcriptase.[16] One of them, North-Methanocarbathymidine (N-MCT), does not readily block DNA synthesis at the point of incorporation (figure 5b). Instead, the primer strand is further extended by two or three more bases before DNA synthesis slows down, but is not completely blocked.[16] As a proof-of-principle, these 'locked' nucleotides would potentially be beneficial to inhibit HIV particles containing NRTI resistance mutations. Unfortunately, the corresponding nucleosides cannot be efficiently activated by phosphorylation in mammalian cells, compromising the therapeutic potential of these particular derivatives.

3. Pseudo-Obligate Chain Terminators

3.1 2′C-Methyl Analogues

Although delayed chain terminators allow further polymerization steps, pseudo-obligate chain terminators instead prevent binding and/or catalysis of the next subsequent nucleotide, and by this definition behave essentially like 'classical' chain terminators. One example of a pseudo-obligate chain terminator is valopicitabine, the first specific nucleoside inhibitor of hepatitis C virus (HCV) polymerase to enter clinical trials (figure 6). Valopicitabine is the 3′-valine ester form of 2′C-methylcytidine. Its antiviral activity in the submicromolar range was first demonstrated in the HCV-like bovine viral diarrhoea virus (BVDV) model (for a recent review see Toniutto et al.[17]). Valopicitabine was later tested in HCV-infected chimpanzees, where it induced a single log reduction in HCV viral RNA.[17] It was also in the BVDV model that the selection of a resistance mutation of a conserved serine within the viral polymerase was first reported. This equivalent S282T mutation in HCV polymerase also induced a loss of sensitivity towards 2′C-methyl analogues.[18] The mechanism of resistance is likely to involve a steric hindrance between the 2′C methyl group and the neighbouring threonine (figure 7). Valopicitabine also demonstrated efficacy in humans, but its unfavourable gastrointestinal toxicity profile resulted in the discontinuation of clinical trials after phase II.

It is also known that 2′-deoxy-2′-fluorocytidine triphosphate is a potent inhibitor of HCV

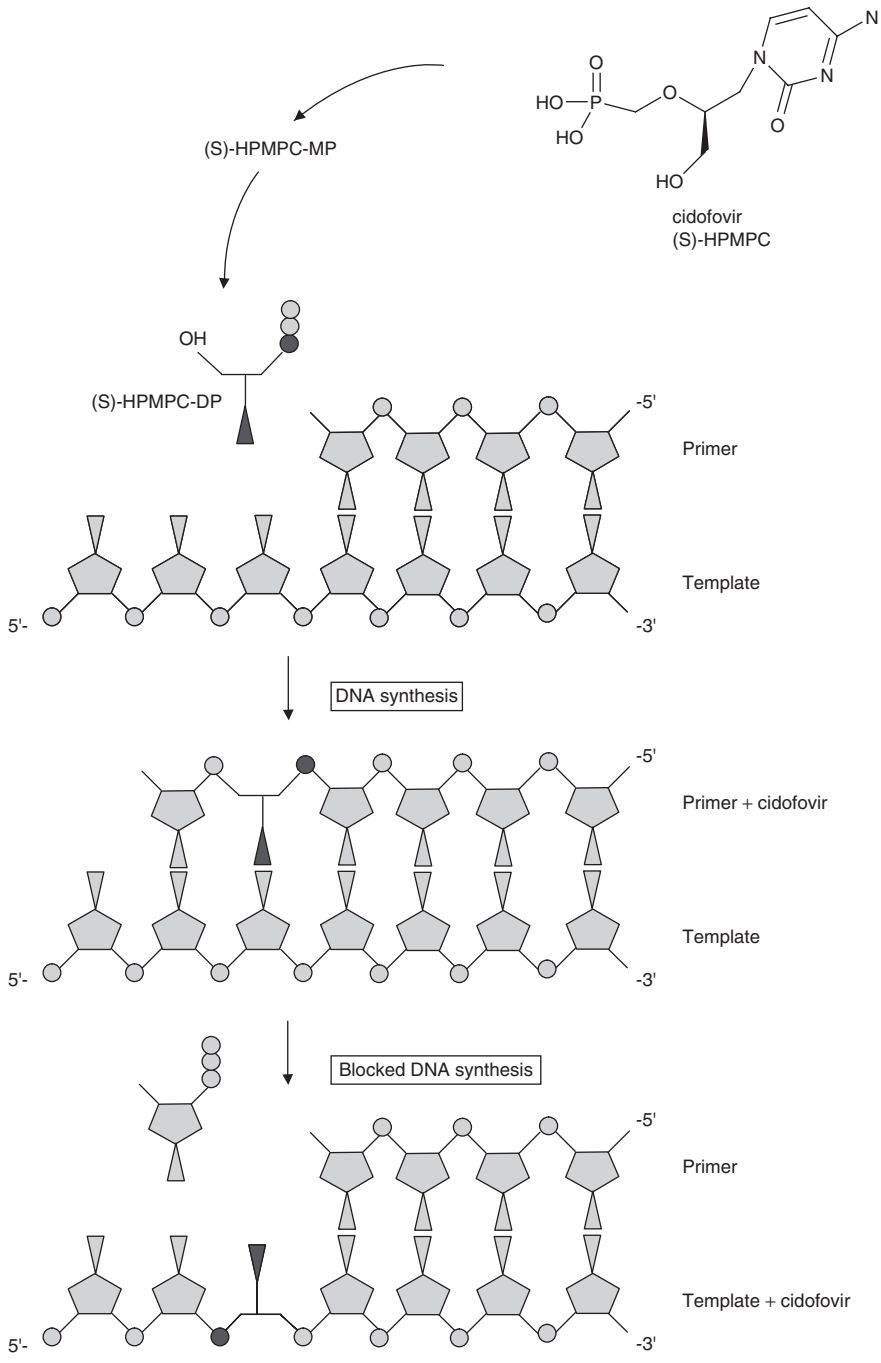

Fig. 4. Mechanism of inhibition of cidofovir [(S)-HPMPC]. As the molecule contains a phosphonate group, (S)-HPMPC only requires two steps of phosphorylation before reaching its active metabolic form of 3-hydroxy-(2-phosphonomethoxy)propyl]-cytidine (HPMPC) diphosphate (DP). Incorporation of HPMPC to the viral genome does not readily block further DNA synthesis. However, HPMPC is believed to inhibit second round synthesis of DNA when it is present in the template strand. **MP** = monophosphate.

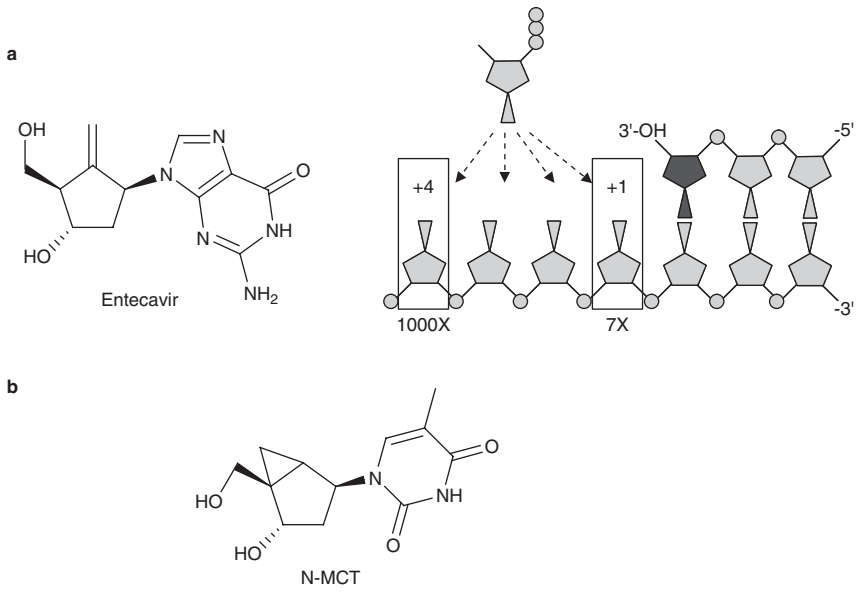

Fig. 5. Other delayed chain terminators. (**a**) Entecavir. Incorporation of its monophosphate form by HIV reverse transcriptase reduces the efficiency of the next nucleotide incorporation by 7-fold and by 1000-fold at the +4 position. (**b**) Lock nucleotide North-Methanocarbathymidine (N-MCT).

polymerase.[19] However, this and other related nucleosides have limited selectivity because they can be recognized as substrates for human polymerases, which causes cellular toxicity.[19,20] A breakthrough came with the discovery of PSI-6130 (2′-deoxy-2′-fluoro-2′C-methylcytidine), a non-toxic molecule that combines the features of valopicitabine with the 2′-deoxy-2′-fluorocytidine (figure 6).[21] PSI-6130, which is being co-developed by Pharmasset and Roche, is efficiently converted to its triphosphate form to inhibit the polymerase of HCV. Interestingly, the presence of the fluoro group reduces the resistance of the S282T mutant towards PSI-6130, compared with 2′C-methylcytidine.[22] Recently, it was also discovered that PSI-6130 monophosphate could be deaminated in hepatocyte cultures to its uridine form by the cellular deoxycytidine monophosphate deaminase, which is later converted to the corresponding triphosphate. This led to the formation of another pharmacologically active molecule capable of inhibiting HCV replication.[23,24] PSI-6130 was shown to be non-toxic when tested against various cell lines and bone marrow precursor cells.[25] Its clinical safety and efficacy is currently under investigation.

3.2 4′C Substituted Nucleosides

In addition to the 2′C-methyl substitution, modifications at the 4′ position have also been explored for anti-HCV indications and a structure-activity relationship study led to the identification of 4′-azidocytidine (R1479).[26] Although R1479 also contains a reactive 3′-OH group, it is a potent inhibitor of HCV polymerase (figure 6). One possible explanation is that the 4′-azido forces the hydroxyl into a pseudo-equatorial orientation that is incompatible with the in-line nucleophilic attack.[26] The S282T signature mutation of the 2′C-methyl derivatives does not confer cross-resistance to R1479.[27] Instead, R1479 selects its own pattern of mutations at positions 96 and 142 in NS5B. R1626, the triisobutyl ester prodrug of R1479, is currently under evaluation in clinical trials. In a phase IIa

study, the presence of R1626 added to the standard of care (pegylated interferon [IFN]-α 2a plus ribavirin) resulted in undetectable levels of HCV RNA in up to 74% of patients infected with genotype 1 virus at 4 weeks of treatment compared with 5% of patients whose treatment did not include R1626.[28] The dose administration of R1626 was limited by adverse effects, essentially mild to moderate neutropenia.[28]

Independently, increasing attention has been given to anti-HIV deoxynucleosides with a 4′C substitution (figure 8). The first reported molecules are the 4′ethynyl derivatives, such as 2′-deoxy-4′C-ethynyl-2-fluoroadenosine (4′Ed2FA) [for a review see Kodama et al.[29]]. This molecule, which is currently under preclinical evaluation, confers the advantage of inhibiting virtually all variants of reverse transcriptase containing the most relevant drug-resistance mutations.[29,30] Removing the 3′-OH group completely abrogates the antiviral activity of the molecule.[31] Two additional non-obligate chain terminators have recently been evaluated *in vitro* against HIV reverse transcriptase, 4′C-ethyl- and 4′C-methyl-thymidine.[32] Interestingly, the larger substitution causes immediate chain termination, while the 4′C-methyl acts as a delayed chain terminator. Interestingly, 4′C-methyl-thymidine is reminiscent of 4′-azidothymidine by its mode of action, the latter molecule inhibiting HIV reverse

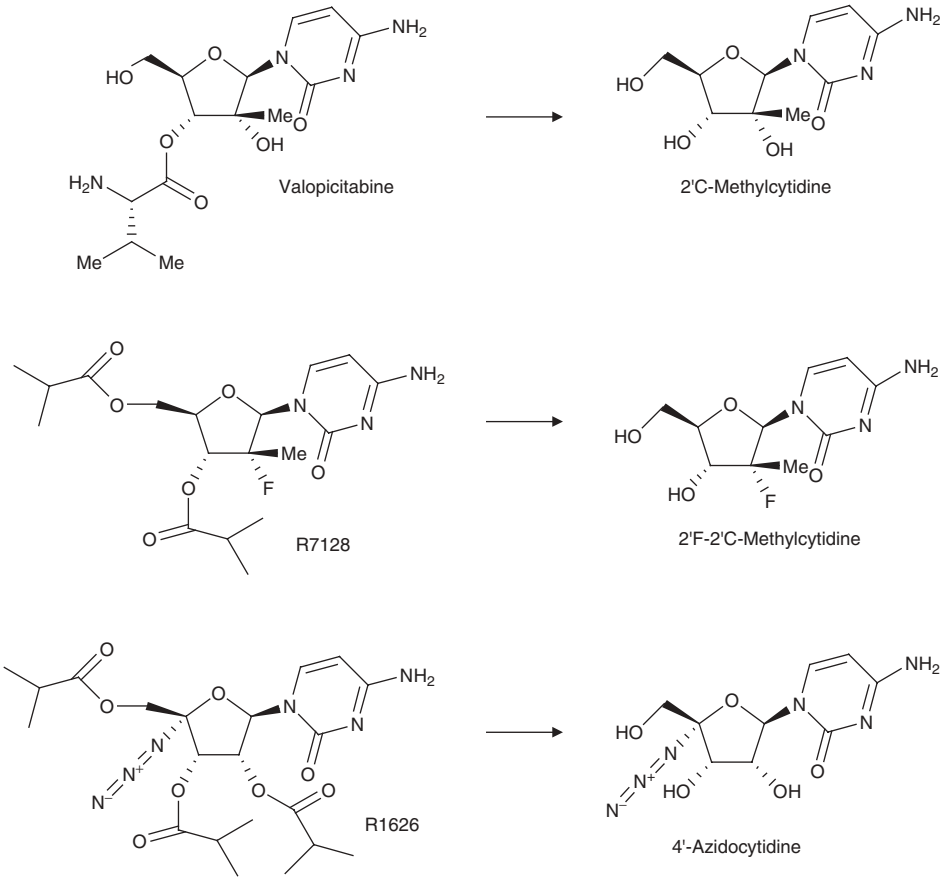

Fig. 6. Cytidine analogues used in clinical trials as pseudo-obligate chain terminators to inhibit hepatitis C virus replication. Molecules on the left represent the parent prodrugs.

transcriptase after two consecutive incorporation events into DNA.[33] These last three examples (4′C-ethyl, 4′C-methyl and 4′C-azido thymidine) illustrate the difficulty to predict the mechanism of action of nucleotide analogues containing a 3′-OH group.

4. Mutagenic Nucleosides

4.1 Ribavirin and Derivatives

Ribavirin is a guanosine analogue that has been commercialized for the treatment of chronic HCV infection in combination with IFNα and as monotherapy for severe respiratory syncytial virus infection (figure 9a). The broad-spectrum antiviral effect of ribavirin was discovered in 1972.[34] There are currently four non-exclusive mechanisms of action proposed to explain the antiviral activity of ribavirin (for a review see Lau et al.[35]): (i) the nucleoside form of ribavirin is thought to enhance the T-cell-mediated adaptive immunity by favouring the release of type-1 cytokines such as IFNγ and tumour necrosis factor-α; (ii) as a monophosphate, ribavirin also inhibits the host inosine monophosphate dehydrogenase (IMPDH), the enzyme that catalyses the rate-limiting step in guanosine monophosphate synthesis. This leads to the depletion of GTP pools, which reduces the replication of HCV *in vitro*. Moreover, ribavirin triphosphate is recognized by viral polymerases and integrated into the viral genome. This can result in (iii) the immediate inhibition of the polymerase; and, perhaps more significantly, (iv) the accumulation of lethal mutations in the viral genome. This last mechanism, known as error catastrophe, was revealed by the observation that poliovirus incorporates ribavirin into its RNA. Incorporated ribavirin can later base pair with cytidine or uridine with equal efficiency.[36] *In vitro*, a 4-fold increase in mutation frequency resulted in a 10-fold decrease of poliovirus infectivity.[37,38] Prolonged treatment of poliovirus with ribavirin can select for a mutation within the viral polymerase.[39] The phenotype of the mutated enzyme is unique in the sense that it increases its fidelity by discriminating against mispairing

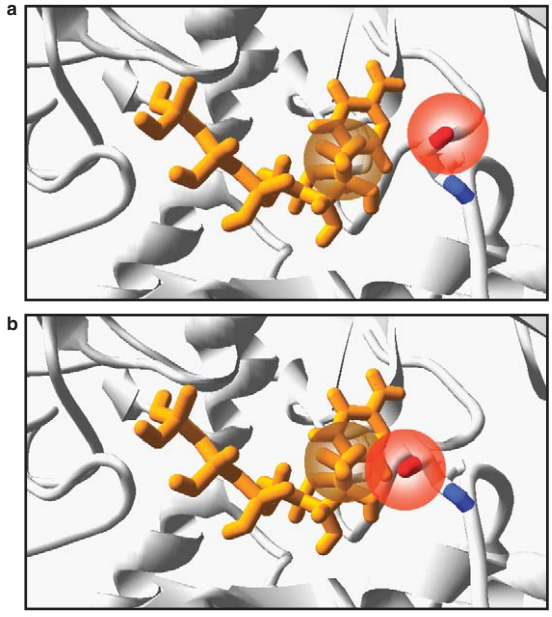

Fig. 7. Effect of the S282T mutation in HCV polymerase on the accommodation of 2′C-methylcytidine. (**a**) Binding of 2′C-methylcytidine (orange) to the active site of NS5B. The conserved serine is in colour. (**b**) Positioning of the threonine after mutation. The side chain (red) is directly pointing towards the 2′C-methyl group, which is likely to cause a steric hindrance (circles) and, in turn, decrease the binding affinity of the polymerase to 2′C-methylcytidine.

nucleotides, including ribavirin.[40] The pleiotropic mechanism of action of ribavirin makes it a broad-spectrum inhibitor, but it is likely that viruses express different susceptibility to each of the four described activities.

Although ribavirin itself does not reduce HCV RNA levels *in vivo*, its clinical benefit has been demonstrated in combination therapy with IFNα. However, clinical administration of ribavirin is limited because of its toxicity against red blood cells causing haemolytic anaemia. This toxicity appears to be caused by the intracellular accumulation of ribavirin triphosphate in erythrocytes. Two new derivatives of ribavirin have been more recently investigated for their potentially reduced adverse effects. As shown in figure 9a, levovirin (ICN-17261) is the L-enantiomer form of ribavirin (L-ribavirin) [for a review see Watson[41]]. As a result of its particular structure, levovirin is not phosphorylated and is excreted

unchanged in urine. Therefore, levovirin does not accumulate in erythrocytes, explaining its superior safety profile when administered in doses up to 1200 mg. Without being phosphorylated, levovirin cannot inhibit IMPDH or viral polymerases, which explains why levovirin has no antiviral activity *in vitro*. Indeed, only the immunomodulatory activity of levovirin is conserved when compared with ribavirin. Viramidine (taribavirin) is another derivative of ribavirin where a carboxamidine group has replaced the original carboxamide. Unlike ribavirin, viramidine shows limited accumulation in erythrocytes, combined with improved transport inside hepatocytes. Viramidine is converted to ribavirin inside hepatic cells and is then phosphorylated. Viramidine and ribavirin have comparable *in vitro* antiviral activity.[42] Viramidine has recently completed phase II clinical trials for the treatment of chronic hepatitis C under the name of taribavirin, conducted by Valeant Parmaceuticals.[43]

4.2 Other Mutagenic Agents

The concept of error catastrophe has been explored beyond the boundaries of HCV and poliovirus. Early on, 5-hydroxydeoxycytidine was identified as a potentially attractive inhibitor of HIV (figure 9b). When used at high concentrations, this molecule blocked viral replication in spite of the presence of its 3′-OH group. However, its somewhat limited potency prevented further development.[44]

KP1461 is the prodrug of 2′-deoxy-5-azacytidine (KP1212). Contrary to other NRTIs, KP1212 does not block DNA synthesis, but instead causes mutations as a result of its non-planar heterocycle base. The increased rate of mispairing following the incorporation of KP1212 monophosphate into the HIV genome compromises the infectivity of the virus progeny. In cell culture, the parent KP1461 retained complete potency when tested against HIV strains resistant to approved drugs. Additionally, the compound demonstrated a clean genotoxic profile, which implies that KP1461 is not recognized by cellular polymerases.[45,46] The development of KP1461 by Koronis Pharmaceuticals was recently interrupted in phase II clinical trials.

5. Nucleosides with Unique or Unknown Mechanisms of Inhibition

5.1 Brivudine

The discovery of brivudine as a selective antiherpesvirus molecule came shortly after that of aciclovir in the late 1970s[47] (figure 10). It is now marketed in Germany and parts of Europe for the treatment of herpes zoster (shingles)

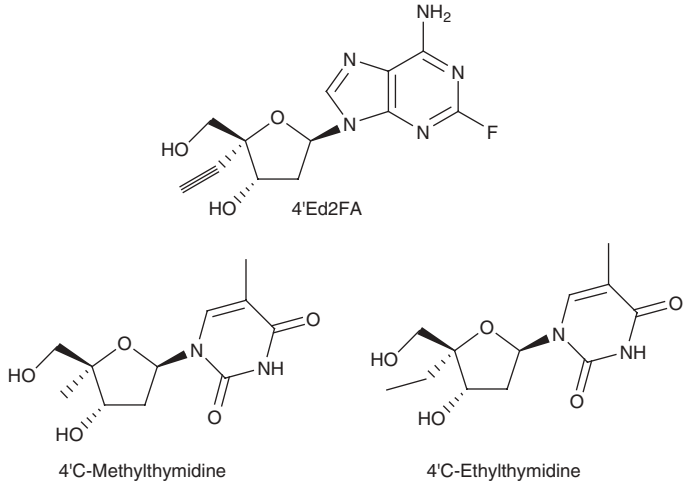

Fig. 8. Non-obligate chain terminators known to inhibit of HIV reverse transcriptase. **4′Ed2FA** = 2′-deoxy-4′C-ethynyl-2-fluoroadenosine.

caused by varicella zoster virus (VZV). Brivudine is metabolized to its monophosphate and diphosphate form by the viral thymidine kinase. However, it is only the triphosphate form of brivudine that inhibits herpesviruses, indicating that it targets the VZV polymerase. The incorporation of brivudine monophosphate into the growing DNA chain by the viral polymerase does not cause chain termination. Instead, it seems that viral DNA containing brivudine is more prone to single-stranded breaks.[48] This unique property conferred by the 2-bromovinyl moiety is believed to compromise the infectivity of the progeny population. Interestingly, the E isomer (or trans)

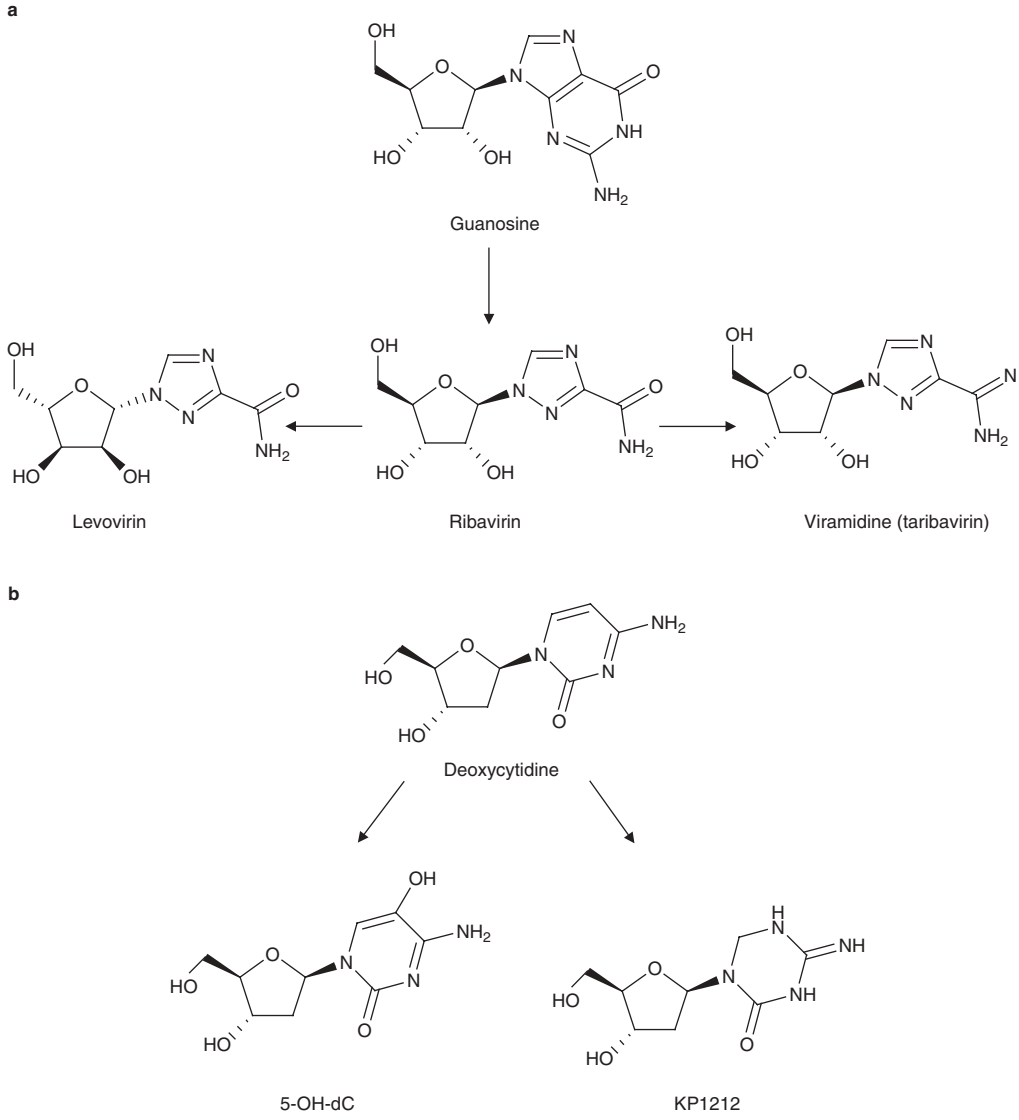

Fig. 9. List of mutagenic agents. (**a**) Guanosine analogues and ribavirin derivatives. (**b**) Deoxycytidine analogues tested against HIV. **5-OH-dC** = 5-hydroxydeoxycytidine.

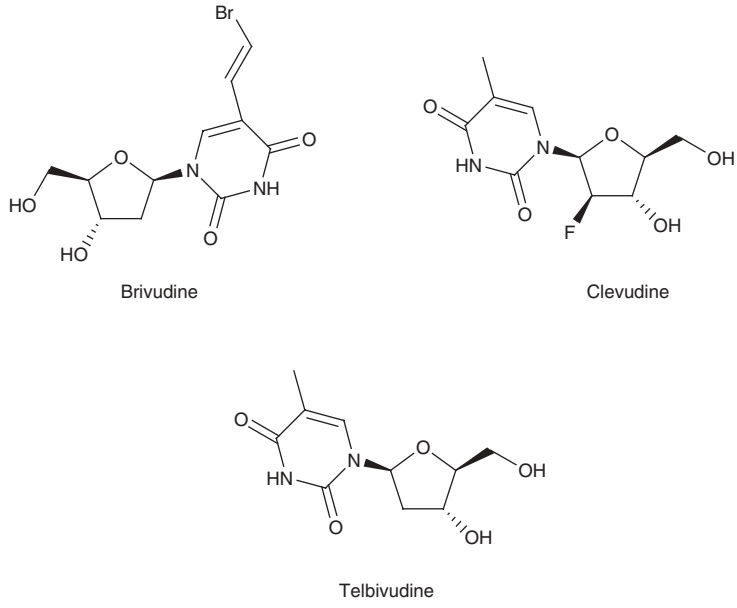

Fig. 10. Non-obligate chain terminators with unique or unknown mechanisms of action.

of brivudine is more active than the Z (cis) form. Several bromovinyl derivatives have also been synthesized, but their *in vivo* efficacy was never greater than the parent brivudine compound.[47]

5.2 Clevudine and Other L-Nucleosides

Clevudine is a pyrimidine analogue currently in phase III for the treatment of chronic hepatitis B (figure 10). Clevudine is also known to be a potent inhibitor of Epstein-Barr virus (EBV).[49] Surprisingly, it was found that clevudine triphosphate is not used as a substrate for EBV polymerase because it is not incorporated into viral DNA, which suggests that clevudine has a distinct mechanism of action.[50] Although this mechanism remains to be completely elucidated, it is now clear that DNA polymerization is the target step for clevudine. The main evidence comes from HBV-infected patients, for which treatment with clevudine can select the A181T polymerase mutation, which is also responsible for lamivudine and adefovir resistance.[51] One hypothesis for its unique mechanism of action is that clevudine triphosphate could bind to the active site of HBV polymerase in a conformation that, while preventing its own incorporation, competes against the binding of natural nucleotides.[52]

Other L-nucleosides have been developed for chronic hepatitis B, including telbivudine (L-deoxythymidine [L-dT]), which has been commercialized since 2006 in the US by Novartis (figure 10). Together with valtorcitabine (L-deoxycytidine [L-dC]) and L-deoxyadenosine (L-dA), telbivudine has been shown to be a potent and selective inhibitor of hepadenovirus replication *in vitro*, with a clean toxicity profile in the woodchuck model.[53] Removing the 3′-OH group resulted in either a loss of activity (L-dA and telbivudine) or in a loss of specificity (valtorcitabine). How these molecules inhibit viral replication in spite of their 3′-OH group remains unclear. They might behave like obligate chain-terminating L-nucleosides, such as the HIV inhibitor lamivudine, or may not be incorporated into the DNA like clevudine.

6. Conclusion and Perspectives

The strategies reviewed here all focus on blocking virus replication by targeting the DNA/RNA polymerization steps. Investigation of nucleoside

analogues bearing a 3′-OH group is gaining momentum, especially for the development of novel chronic hepatitis B and C therapies, where they appear to be more potent than obligate chain terminators. For many of these molecules, the mechanistic reason behind why the 3′-OH group cannot react in the nucleophilic attack is still poorly understood. What has been observed is that chain termination with these molecules can be pseudo-immediate or delayed, depending on the local orientation of the 3′-terminal sugar or pseudo-sugar moiety. In the case of mutagenic nucleosides, chain termination does not even occur.

Exemplified by HCV inhibitors, obligate chain terminators have major limitations. Canonical 3′-deoxyribonucleoside triphosphates are potent inhibitors of HCV polymerase *in vitro*, but the parent nucleosides are inactive in cell culture probably because of a lack of intracellular phosphorylation.[27,54] Instead, 2′C- and 4′C-derivatives containing a 3′-OH group can surmount this shortcoming, and be more efficiently activated by human nucleoside kinases. Unfortunately, this improved host activation is often at the cost of a reduced selectivity towards the viral polymerase, leading to unexpected off-target toxicity. The key to successfully develop new non-obligate chain terminators will be to obtain a balance between the greatest antiviral efficacy, while maintaining the best selectivity and safety profile.

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