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Inhibition of viral RNA polymerases by nucleoside and nucleotide analogs: therapeutic applications against positive-strand RNA viruses beyond hepatitis C virus
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A number of important human infections are caused by positive-strand RNA viruses, yet almost none can be treated with small molecule antiviral therapeutics. One exception is the chronic infection caused by hepatitis C virus (HCV), against which new generations of potent inhibitors are being developed. One of the main molecular targets for anti-HCV drugs is the viral RNA-dependent RNA polymerase, NS5B. This review summarizes the search for nucleoside and nucleotide analogs that inhibit HCV NS5B, which led to the FDA approval of sofosbuvir in 2013. Advances in anti-HCV therapeutics have also stimulated efforts to develop nucleoside analogs against other positive-strand RNA viruses. Although it remains to be validated in the clinic, the prospect of using nucleoside analogs to treat acute infections caused by RNA viruses represents an important paradigm shift and a new frontier for future antiviral therapies.

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Introduction: the RNA polymerase of HCV as the target for nucleoside analogs
Hepatitis C virus (HCV) is a member of the Flaviviridae family. Viruses from this family contain a single-strand, positive-sense RNA genome of about 9.5 kb. The viral genome encodes only one open-reading frame translated into a polyprotein of approximately 3000 amino acids. HCV is estimated to have infected approximately 175 million individuals worldwide, with 2–4 million new infections occurring each year [1]. Until recently, treatment options for chronic HCV infections were largely suboptimal due to limited efficacy and substantial toxicity. The standard of care (SOC) was a 24-week or 48-week course of pegylated interferon alpha (PEG-IFN-α) in combination with ribavirin. Effective clearance or sustained virologic response (SVR) rate of the virus was achieved in less than 50% cases of genotype-1 infection, the most prevalent strain of HCV in the United States and Europe. Since 2011, two inhibitors of the viral serine protease, NS3/4A, boceprevir and telaprevir, were approved for use in combination with PEG-IFN-α and ribavirin. These molecules are called direct-acting antivirals (DAAs) because they specifically bind to, and inhibit, a viral protein required for virus replication. Although the toxicity burden of these newer treatment options remains high, the SVR rate in the presence of protease inhibitors has improved to 70–80% in difficult-to-treat genotype-1 infections [2,3]. Other DAAs that specifically block HCV enzymatic functions have been intensely studied over the last decade, and the polymerase function of NS5B has emerged as one of the most attractive targets for the next generation of anti-HCV therapy.

The HCV NS5B protein is an RNA-dependent RNA polymerase (RdRp). NS5B is required both for replication of the viral genome by synthesis of the minus-strand intermediate and at the transcription level for synthesis of viral mRNA. The RdRp enzymatic activity of NS5B is unique to viruses and not found in human cells, which makes NS5B an attractive target for antiviral drug development (see [4] for a more detailed review on the structure and functions of NS5B). The NS5B protein is composed of 591 amino acids. Similar to other known RdRps, the HCV NS5B contains six conserved motifs designated A–F. The amino acids involved in the catalytic activity of NS5B are located within motif A (aspartate at position 220) and the catalytic triad GDD at position 318–320 in motif C [5**]. The orientation of these residues in the active site of NS5B and their contribution to the catalytic activity are supported by the crystal structure of the protein [5**,6,7**]. Using the polymerase right-hand analogy model, the HCV NS5B protein features the fingers, palm, and thumb subdomains (Figure 1a). Unlike the traditional open-hand conformation shared by many DNA polymerases, the HCV NS5B features an encircled active site due to extensive interactions between the fingers and thumb subdomains. These contacts restrict the flexibility of the subdomains and favor the first steps — or initiation — of RNA synthesis leading to the formation of the primer strand. Therefore, important structural changes involving an opening of the thumb and the fingers are required for
Another unique feature of NS5B is its β-hairpin loop that protrudes into the active site located at the base of the palm subdomain (Figure 1a). This 12 amino acid loop located within the thumb (residues 443–453) was suggested to interfere with binding to double-stranded RNA due to steric hindrance. Its deletion allows the enzyme to favor primer-dependent RNA synthesis [11,12,13], and the resulting truncated protein was co-crystallized in the elongation mode with double-stranded RNA [14]. Primer extension also requires the C-terminal part of NS5B to move away from the catalytic site, a structural feature shared with other RNA polymerases [15]. Once these important conformational changes take place, the enzyme becomes processive and the efficiency of RNA synthesis increases considerably [16,17]. It is precisely during the elongation phase of RNA synthesis that HCV NS5B is inhibited by nucleotide analogs acting as chain terminators (Figure 1b).

The evolution of HCV RNA polymerase inhibitors leading to the discovery of sofosbuvir

The initial major class of nucleoside analogs of therapeutic potential to demonstrate potent inhibition of HCV RNA polymerase activity were 2′C-methyl-ribonucleosides. The first 2′C-methyl ribonucleosides were originally synthesized in the 1960s [18]. Later, 2′C-methyl-uridine triphosphate was found to act as a chain terminator of Escherichia coli RNA polymerase [19,20]. In antiviral assays, 2′C-methyl-cytidine was originally described as an inhibitor of bovine diarrhea virus (BVDV), a virus closely related to HCV and used as a surrogate [21–24]. Although the compound was highly potent and selective in tissue culture, its low bioavailability made it unsuitable for oral dosing. This limitation was overcome by adding an L-valine ester group at the 3′-OH position on the sugar (Figure 2a). The resulting drug, valopicitabine (NM283), was efficacious when dosed orally in HCV-infected chimpanzees [25]. Although this nucleoside significantly reduced HCV viral load in patients, its development was discontinued in phase II clinical trials due to dose-limiting gastrointestinal (GI) toxicity [26]. Other 2′C-methyl nucleosides such as 2′C-methyl-adenosine or 2′C-methyl-7-deaza-adenosine have been reported to inhibit HCV replication [27,28], but none were evaluated in clinical trials presumably due to tissue retention issues in preclinical species [29]. In vitro, prolonged culture of HCV replicon-containing hepatocytes with 2′C-methyl-nucleosides results in the selection of a single S282T mutation within NS5B, and the resulting polymerase is resistant to this class of nucleosides [30–32].

The second class of anti-HCV nucleosides is the 4′-azido-nucleoside scaffold. Molecules in this class resemble 3′-azido-thymidine (AZT) and were originally synthesized for testing against human immunodeficiency virus [33]. During compound library screening in the sub-genomic replicon assay, 4′azido-cytidine was later identified as a potent inhibitor of HCV [34]. In its 5′-triphosphate form, the inhibitor was recognized as a substrate for HCV NS5B, and its incorporation to the growing RNA strand resulted in immediate chain termination. One advantage of 4′-azido-cytidine over the 2′C-methyl-nucleosides was the lack of cross-resistance associated with the presence of the S282T mutation [34,35]. The uridine analog counterpart was inactive in the replicon assay due to lack of intracellular phosphorylation. However, the 5′-triphosphate forms of both cytidine and uridine analogs were equally potent as chain terminators against HCV NS5B. The pharmacokinetic properties of 4′-azido-cytidine were further improved with the triester prodrug balapiravir (Figure 2a), which achieved a 3.7 log10 reduction in viral RNA at the highest dose in a 14-day phase 1b monotherapy clinical trial [36]. Four weeks of treatment with balapiravir in combination...
Nucleoside and nucleotide analogs as inhibitors of HCV. (a) Representative molecules of the three main scaffolds of nucleoside analogs, with valopicitabine for the 2′C-methyl scaffold, balapiravir for the 4′azido scaffold, and sofosbuvir for the 2′-fluoro-2′C-methyl scaffold. The nucleoside backbones are shown in black, the sugar modification in red, and the prodrug moieties are in blue. (b) Efficiency of chain termination of 2′-fluoro and 2′-fluoro-2′C-methyl UMP. Principle of the reaction: elongation by HCV polymerase of RNA containing at the 3′-end a modified UMP (U*MP), in the presence of GTP as the next correct nucleotide. In the case of 2′-fluoro-UMP (left), the RNA is further extended with GTP from the 10-mer to the 11-mer and 12-mer positions. In contrast, the addition of the 2′C-methyl moiety to 2′-fluoro-UMP (right) completely blocks the ability of the enzyme to further extend the RNA with GTP [49]. GTP, guanosine triphosphate; UMP, uridine monophosphate.

with SOC resulted in a further decrease in viral load, but also in hematologic adverse events such as lymphopenia, which led to the discontinuation of development of balapiravir for HCV infection [37]. Analogs of balapiravir with similar 4′-modification scaffolds have also been reported, but none have progressed into further development [38–41]. Recently it was shown that 4′-azido-CTP is a good substrate for human mitochondrial RNA polymerase, one of the proteins considered to be responsible for the mitochondrial toxicity of several other ribonucleosides [42**].

The third major class of nucleoside analogs is the 2′-fluoro-2′C-methyl modification, which includes sofosbuvir. The double substitution at the 2′-position on the ribose evolved from the earlier 2′C-methyl scaffold, combined with further change resulting from the observation that 2′-deoxy-2′-fluoro-2′C-methyl cytidine was weakly active in the HCV replicon [43]. Compared with its 2′-fluoro mono-substituted counterpart, 2′-fluoro-2′C-methyl cytidine (PSI-6130) was significantly more potent in the HCV replicon assay and less toxic to the Huh-7 hepatocarcinoma cells in vitro [44]. The parent 2′-fluoro-2′C-methyl-cytidine
nucleoside was also developed as the orally bioavailable diisobutyrate ester prodrug, mericitabine, which is currently under phase II clinical development. In an important series of experiments, it was found that 2′-fluoro-2′-C-methyl-cytidine is metabolized also to its uridine 5′-triphosphate form as a result of intracellular deamination [45*,46]. As the parent uridine analog was not readily converted to its monophosphate form by intracellular kinases, a series of monophosphate forms of 2′-fluoro-2′-C-methyl-uridine were designed to bypass the first and most limiting kinase step [47]. Phosphoramidate prodrugs were made to mask the charges of the alpha-phosphate with an amino acid ester and an aryl group, both protecting groups being removed in the cytoplasm of hepatocytes after cell penetration [47]. Optimization of the leaving groups of the prodrug and separation of stereoisomers led to the selection of sofosbuvir (PSI-7977), as one of the most potent and selective inhibitors in this series (Figure 2a) [48]. In addition to forming high levels of the nucleoside 5′-triphosphate (NTP), the exquisitely potency of sofosbuvir can be explained in vitro by the fact that its active form 2′-fluoro-2′-C-methyl-uridine 5′-triphosphate is a very efficient substrate and chain terminator for HCV NS5B (Figure 2b) [49]. This study also showed that the 2′-fluoro substitution contributes less to chain termination than the 2′-C-methyl moiety. The NTP derivative of sofosbuvir is a very poor substrate for human mitochondrial RNA polymerase, one of the proteins considered to be responsible for the mitochondrial toxicity of several other ribonucleosides [42**]. Similar to the two former classes of inhibitors, many other 2′-fluoro-2′-C-methyl-nucleosides have been evaluated, including the very potent monophosphate guanosine analog, PSI-353661, that progressed to phase II clinical trials, before being discontinued due to elevated alanine aminotransferase levels (see complete reviews of recent HCV nucleoside and nucleotide development in [4,50,51]).

The quest for structurally novel nucleoside inhibitors of HCV NS5B continues to be an active area of pharmaceutical research, and other chemical scaffolds have been recently discovered (e.g. [52,53]). To this date, none of the other classes of nucleoside analogs have advanced beyond phase II clinical trials.

**Repurposing anti-HCV nucleosides against other positive-strand RNA viruses**

Several important and sometimes severe human diseases are caused by RNA viruses in the *Flaviviridae*, *Picornaviridae*, *Caliciviridae*, and *Coronaviridae* families. All these viruses contain a positive-strand RNA genome, and their RNA-dependent RNA polymerases share significant amino acid similarities based on sequence alignment and phylogenetic analysis [54,55]. Since HCV belongs to the *Flaviviridae* family, some of the nucleoside analogs originally developed against HCV would also be expected to inhibit related pathogens within the same family or even viruses in other positive-strand RNA families. This prediction was confirmed by counter-screening anti-HCV molecules against representative panels of viruses from other families and sub-families (Table 1). In particular, 2′C-modified nucleosides are known to inhibit multiple positive-strand RNA virus families. In one of the first reported examples, 2′C-methyl-cytidine was found to be potent in cell-based in vitro assays against flaviviruses such as West Nile, yellow fever, and dengue virus [56]. This result is not entirely surprising since the same molecule was already known to inhibit BVDV, which also belongs to the *Flaviviridae* family, and was used as a surrogate for HCV antiviral screening. In an in vivo efficacy model, 2′C-methyl-cytidine protected hamsters challenged with a lethal dose of yellow fever virus even when administered up to 3 days post-infection [57]. The cytidine analog also inhibits the in vitro replication of tick-borne, hemorrhagic fever-associated flaviviruses [58]. In addition, 2′C-methyl-cytidine inhibits the replication of the Norwalk virus [59,60] and foot-and-mouth disease virus [61] from the *Caliciviridae* and *Picornaviridae* family, respectively. Although it has not been as extensively profiled, the purine analog 7-deaza-2′-C-methyl-adenosine similarly displays broad antiviral activity against positive-strand RNA viruses, while being inactive against single-strand negative-sense RNA viruses [27**,28]. This broad spectrum activity was further profiled with the chemically related 7-deaza-2′-C-ethylidyne-adenosine. Although it was found to also inhibit HCV, the molecule was potent enough to be developed specifically against dengue virus infection [62,63*]. To date, it is the only

**Table 1**

| Inhibitor                     | Virus                                      | Family          | References               |
|-------------------------------|--------------------------------------------|-----------------|--------------------------|
| 2′-C-Methyl-cytidine          | Yellow fever                               | Flaviviridae    | [56]                     |
|                               | Kyasanur Forest disease                    | Flaviviridae    | [58]                     |
|                               | Norwalk                                    | Caliciviridae   | [59,60]                  |
|                               | Foot-and-mouth disease                     | Picornaviridae  | [61]                     |
| 7-Deaza-2′-C-methyl-adenosine | Dengue, yellow fever, West Nile            | Flaviviridae    | [27**,28]                |
|                               | Rhinovirus types 2, 3, 14                  | Picornaviridae  | [27**,28]                |
| 7-Deaza-2′-ethynyl-adenosine  | Dengue, yellow fever, West Nile            | Flaviviridae    | [62,63*]                 |
| Balipiravir/4′-azido-cytidine | Dengue                                     | Flaviviridae    | [64]                     |
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Challenges for discovering novel nucleoside analogs targeting positive-strand RNA viruses other than HCV

Although many anti-HCV nucleoside analogs may potentially inhibit other positive-strand RNA viruses in vitro, there are currently no obvious drug candidates for direct repurposing from HCV infection to other disease indications. In particular, sofosbuvir, the only FDA-approved anti-HCV nucleoside analog, is a phosphoramidate prodrug that has been optimized to specifically deliver high levels of the nucleoside S′-triphosphate as the active species through release of the prodrug moiety by first-pass effect, the process by which drugs get metabolized into the liver before reaching systemic circulation [65]. Therefore, the active metabolite of sofosbuvir is likely not significantly distributed to organs and tissues other than the liver and targeted by most positive-strand RNA viruses. In comparison, 2′C-methyl-cytidine and 7-deaza-2′C-ethyladenosine are among the only known broad-spectrum nucleoside analogs with potential for systemic organ exposure of the S′-monophosphate and S′-triphosphate forms in levels sufficient to achieve in vivo efficacy. However, their relatively poor safety profiles and narrow dose margins make them poor candidates for further clinical development.

What are the main challenges to designing and optimizing new inhibitors of non-HCV positive-strand RNA viruses? The search for such molecules has been hampered by several factors, the first one being the need to achieve in vivo pharmacokinetic properties compatible with delivery of the NTP to the site of infection, which differs by virus and includes, for example, the GI tract (Norwalk virus), the lungs (rhinovirus, Middle East respiratory syndrome virus), the brain (West Nile virus), and lymphoid organs (dengue virus). As mentioned before, the only organ-specific prodrugs that have been successfully developed to date for nucleosides target the liver, and will likely not be useful for non-liver infections. The second important limitation to finding new nucleoside analogs is also related to S′-triphosphate formation and the choice of the immortalized cell lines used for in vitro infection experiments. The metabolic kinase activation pathways of many common laboratory strains and species of cell lines differ from natural human tissues or human primary cells. Although this is not generally a problem for small molecule drug testing, the metabolic activation of nucleoside analogs to NTPs entirely relies on the presence of specific nucleoside and nucleotide kinases that are sometimes deficient in common lab-adapted cell lines. Finally, it will be important to thoroughly assess the selectivity and toxicity of new nucleoside analogs to ensure that they do not interfere with cellular mechanisms at efficacious doses. In the case of acute infections, the safety requirements for short-term treatments may differ from those of anti-HCV nucleotides. Despite these challenges, the prospect of using nucleoside analogs to treat acute infections caused by RNA viruses represents an important paradigm shift and a new frontier for future antiviral therapies.

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