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Review

Nucleotide prodrugs for HCV therapy

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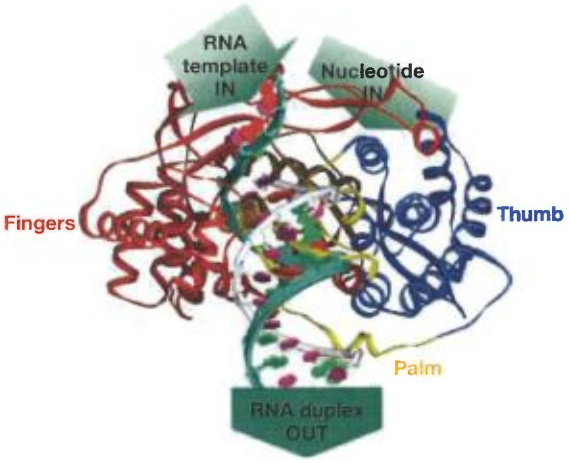
HCV infection is a significant worldwide health problem and is a major cause of hepatocellular carcinoma. The current standard of care, interferon and ribavirin, is only effective against a proportion of the patient population infected with HCV. To address the shortcomings of existing therapy, the development of direct acting antiviral agents is under investigation. The HCV RNA dependent RNA polymerase is an essential enzyme for viral replication and is therefore a logical target against which to develop novel anti-HCV agents. Nucleosides have been shown to be effective as antiviral agents for other viral diseases and therefore, have been investigated as inhibitors of HCV replication. The development of prodrugs of nucleoside 5′-monophosphates has been pursued to address limitations associated with poor nucleoside phosphorylation. This is required to produce the nucleoside 5′-triphosphate which is the anabolite that is the actual inhibitor of the polymerase enzyme. Prodrugs of nucleoside 5′-monophosphates have been developed that enable their delivery into cells and *in vivo* into the liver. The implementation of these prodrug strategies has ultimately led to the identification of several prodrugs of nucleoside 5′-monophosphates that are potent inhibitors of HCV replication *in vitro*. They have progressed into the clinic and the early data demonstrate greatly reduced viral load levels in HCV-infected patients. This review will survey the state of nucleotide prodrugs for the treatment of HCV.

Introduction

HCV is known to have infected approximately 180 million individuals worldwide [1]. It is estimated that of those infected with HCV approximately 80% will develop chronic liver disease and a significant proportion of those infected will eventually develop liver cirrhosis and subsequently hepatocellular carcinoma [2]. HCV is a single-stranded, positive sense RNA virus of the *Flaviviridae*. Six major viral genotypes with over 100 viral subtypes have been identified for HCV. Genotypes 1a and 1b are the most prevalent genotypes in the western world with genotypes 2 and 3 comprising 20–30% of this population. HCV genotypes 2–6 predominate in the developing world [3,4]. Because HCV replicates in the cytoplasm of infected cells by a membrane associated replication complex and the virus has an RNA genome with no DNA intermediate during replication, no genomic templates are stably integrated into the host genome. Therefore, virological cures are possible for HCV patients. This is in contrast to other viruses such as HIV and HBV where the viral genome is integrated into the host DNA and a virological cure is considered remote. However, for HCV-infected patients virological cures are made difficult due to the high rate of HCV viral replication and by the high spontaneous mutation rate of the virus. This high mutation rate is a result of poor replication fidelity exhibited by the HCV polymerase and an apparent lack of proof reading [4].

The current therapy for treating chronic HCV infection consists of regular injections of α-interferon (IFN) with daily oral administration of ribavirin (RBV). This standard of care (SOC) regimen does not act by directly attacking the virus but functions by boosting the host immune response. For genotype 1 patients regular IFN/RBV treatments for 48 weeks result in only 40–50% of patients achieving a sustained virological response (SVR) indicative of a cure [5,6]. However, for genotype 2 and 3 patients the SVR rates can be as high as 75%. It is also known that subpopulations which include individuals of African ancestry tend to respond less well to IFN/RBV treatments [7]. Recent genome-wide association studies have shown that a single nucleotide polymorphism 3kb upstream of the IL28B gene correlates with a significant difference in response to IFN therapy [8]. IL28B which encodes the type III interferon IFN-λ–3, is known to be upregulated by IFNs and by RNA viral infections. It has been shown that HCV patients who harbour a TT or TC allele in their IL28B gene tend to respond less well to IFN/RBV treatment than do those having the CC genotype.

Figure 1. Model of HCV NS5B RNA complex



Ribbon structure of the HCV NS5B RNA dependent RNA polymerase showing the Palm, Finger and Thumb domains typical of a RNA polymerase. Also depicted is a bound template-primer strand of RNA and the nucleotide binding site.

Patients who choose to undergo IFN/RBV therapy face not only the possibility of not responding to treatment but also must contend with the potential for multiple and sometimes serious side-effects that include influenza-like symptoms, fatigue, hemolytic anaemia and depression. The intolerable side effects can result in a high rate of drug discontinuations. Consequently, the modest cure rates and subpopulation differences combined with the side-effect profile for SOC have prompted an urgency to develop alternative novel, safe and effective therapies. As in the case of other viral diseases, the development of direct acting antivirals (DAAs) has become a focus. Development of small molecule agents to attack essential viral proteins has the potential benefit of reducing toxicities and side effects associated with manipulating host functions and hopefully such DAA therapies will not have the intolerable side effects exhibited by current SOC.

The push to identify small molecule DAAs has also prompted the discussion around the possibility of eliminating or at least reducing the use of IFN/RBV from treatment regimens. Although clinical development of first generation DAAs has focused on combinations with SOC in the hope of both shortening duration of treatment and increasing the cure rate, the long-term desire is to completely eliminate the use of IFN/RBV from treatment regimens. Clearly this is an aspirational goal and a goal that can only be achieved if an immune component of therapy is not absolutely required to eliminate those vestiges of undetectable virus [9]. In addition, such a lofty goal can be realised if small molecule DAAs either alone or more probably in combination can drive viral loads to undetectable limits and maintain those undetectable levels over the necessary course of therapy and after cessation of treatment without having viral breakthrough resulting from the emergence of resistant virus. As has been shown with HIV highly active antiretroviral therapy (HAART), the HCV treatment paradigm will likely require combinations of anti-HCV agents [7]. The desire is to suppress virus as rapidly and completely as possible in order to give the body's natural immune system the opportunity to clear residual virus and to hold back emergence of resistant virus. However, what will comprise those ideal combinations of DAAs is yet to be determined and studies to clarify this question are under active discussion and investigation.

HCV has a 9.6 kb genome of positive-stranded RNA. This genome encodes a precursor polyprotein that is processed into 10 functional proteins: three structural proteins and seven non-structural proteins [10]. Several of the non-structural proteins have been the focus of intensive efforts to identify small molecule DAA agents as inhibitors of HCV replication. Of the seven non-structural proteins, molecules that inhibit the functions of the NS3/4 protease, NS4A, NS4B, NS5A and NS5B RNA dependent RNA polymerase (RdRp) have advanced to the clinic [11–15]. The most advanced agents are the NS3/4 protease inhibitors telaprevir and boceprevir. Each of these compounds has completed Phase III clinical investigation and both have been shown to be efficacious in treating HCV infection when given in combination with SOC. However, each of these first generation protease inhibitors suffers from the lack of genotype coverage, undesired side effects, that may limit their usage, and the early emergence of resistant virus. It is therefore not surprising that even with the potential benefits of these first generation DAAs, warehousing of patients by physicians occurs in order to wait for approval of more effective and tolerable agents.

The HCV RNA dependent RNA polymerase is an ~68 kd protein that has the typical palm-finger-thumb structural motif found in many viral polymerases (Figure 1) [16,17]. HCV polymerase is an essential enzyme involved in RNA replication. Phylogenetic analysis shows a 65% homology of HCV RdRp across genotypes and an 80% homology within a particular genotype [3]. The HCV polymerase active site is located in the palm domain where the conserved aspartic acid residue-containing GDD motif is located [18]. This conserved GDD motif is common to viral polymerases in general [18]. Through a divalent metal ion (Mg^{++} or Mn^{++}) the GDD motif functions to coordinate the binding of the ribonucleoside triphosphate. The HCV polymerase catalyzes the addition of a single ribonucleoside triphosphate monomer to the 3′-end of the growing RNA chain by the

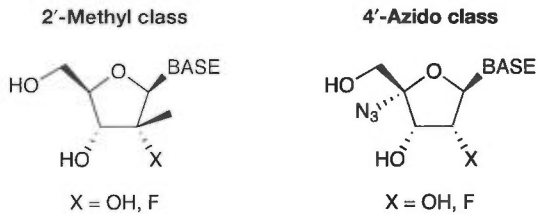
formation of a 3′,5′-phosphodiester linkage. To accomplish this process, the polymerase must simultaneously bind a template RNA strand, a primer RNA strand and a ribonucleoside triphosphate monomer [19,20]. Therefore, investigation of nucleoside analogues is a rational choice for the development of inhibitors of the HCV NS5B polymerase.

Of all the DAAs under clinical investigation, nucleoside/nucleotide NS5B polymerase inhibitors hold the promise of pan-genotype coverage and a high barrier to development of resistant virus. As in the case of HIV infection where nucleosides have become the backbone of therapy (for example, TRUVADA® and Combivir®), HCV nucleosides/nucleotides are positioned to assume a similar role. To date, only nucleosides/nucleotides have demonstrated broad genotype coverage both in the laboratory and in human clinical studies [21]. In addition, to date, no pre-emergent resistant virus has been detected in clinical studies [22]. It is for these reasons that nucleosides/nucleotides are positioned to play a prominent role in developing HCV treatment paradigms.

The HCV polymerase has been shown to be a uniquely selective polymerase as it relates to the development of nucleoside/nucleotide inhibitors. Over the last 10 years only two broad classes of nucleosides have emerged as inhibitors of this polymerase [23–25]. These include the 2′-methyl and the 4′-azido classes (Figure 2). However, these classes and subgroups within these classes have clearly differentiated themselves in preclinical and clinical studies. This differentiation is exemplified in their viral selectivity, viral resistance, overall safety and clinical efficacy profiles. Resistance associated with the 2′-methyl class of nucleosides is associated with the S282T amino acid alteration located in the finger domain of the HCV polymerase [26–29]. This mutation has been shown to be difficult to raise *in vitro* and has not been detected as a pre-existing mutation in clinical isolates [22]. Similarly, for the 4′-azido class, the S96T amino acid alteration has been identified *in vitro* but has not been observed in the clinic [27].

Implementation of a prodrug strategy has played a prominent role in the development of a number of nucleosides and nucleotides for the treatment of HCV infection [23,30]. These prodrugs have been developed to overcome, not only, bioavailability and stability issues but also to address key anabolism limitations important to nucleoside activation. Simple ester prodrugs of both the 2′-methylcytidine, 2′-α-fluoro-2′-β-C-methylcytidine and 4′-azidocytidine nucleosides were developed to overcome both bioavailability issues and to curb undesirable metabolism [21,31,32]. Prodrugs of the phosphate group of nucleoside 5′-monophosphates were developed to address not only bioavailability

Figure 2. Two major classes of nucleosides that are known to be inhibitors of HCV polymerase.

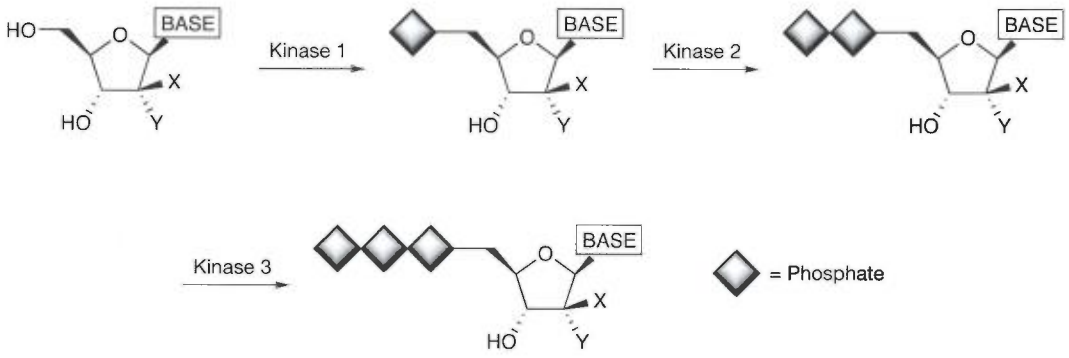


These classes include the 2′-methyl and the 4′-azido ribosides.

issues but also poor *in vitro* and *in vivo* conversion of the parent nucleoside to the active nucleoside 5′-triphosphate [33]. Because nucleosides must be converted to their 5′-triphospates to be active as inhibitors of the HCV polymerase, they need to undergo a series of phosphorylation steps catalyzed by three separate kinases (Figure 3). These kinases convert the nucleoside first to the monophosphate, then to the diphosphate and finally to the active triphosphate. However, it is not uncommon that in the phosphorylation cascade, a nucleoside or its corresponding mono- or diphosphate is a poor substrate for one of the kinases. In particular, it is the first kinase in the phosphorylation cascade that is generally the most substrate selective. Therefore, it is not unusual that bypassing the first kinase results in achieving high levels of the active triphosphate. Because nucleoside monophosphates are enzymatically dephosphorylated and negatively charged, they do not readily enter cells and therefore are not desirable as drug candidates. To overcome the limitations of administering a nucleoside monophosphate-containing agent, prodrugs of the 5′-monophosphate nucleoside have been employed. Prodrugs of nucleoside monophosphates have been known for many years and a number of phosphate prodrug strategies have been developed to address the need to deliver a 5′-monophosphate nucleoside into the cell [33–35]. However, there have been few examples where a nucleotide prodrug has been shown to deliver the corresponding 5′-monophosphate *in vivo* to the desired site of action [33]. Often the prodrug moiety decomposes prior to achieving its objective because of either chemical or enzymatic instability in the gastrointestinal tract and/or plasma.

The development of a nucleoside phosphate prodrug useful for the treatment of HCV faces several challenges. The nucleoside phosphate prodrug must have sufficient chemical stability to be formulated for oral

Figure 3. Nucleoside kinase activation pathway



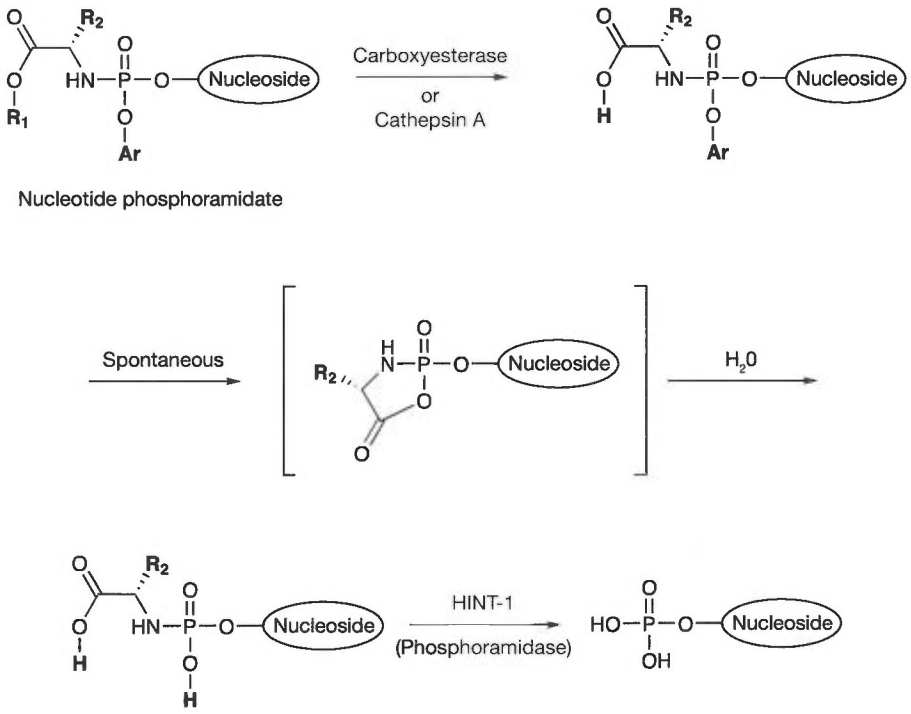
Nucleoside kinase activation pathway resulting in the nucleoside triphosphate which is the active substrate for a polymerase allowing incorporation of the nucleoside or nucleoside analogue into the growing RNA chain and thus resulting in inhibition of virus replication. Examples of kinases 1, 2, and 3 include deoxycytidine kinase (dCk), nucleoside monophosphate kinase (YMPK) and nucleoside disphosphate kinase (NDPK), respectively.

administration. It must be stable to conditions of the gastrointestinal tract such that the prodrug reaches the site of absorption intact. The prodrug must have good absorption properties and must not undergo appreciable enzymatic degradation during the absorption phase. Once absorbed, the prodrug needs to have sufficient stability in the blood in order to reach the target organ: for example the liver in the case of HCV. The prodrug must then be transported into hepatocytes and release the free 5′-monophosphate nucleoside which can subsequently be converted to the active triphosphate derivative. Since HCV is a disease of the liver, and the liver is the first organ the prodrug encounters after absorption, HCV is an ideal disease for which to develop a targeted nucleotide prodrug strategy. Consequently, several of these nucleoside monophosphate prodrugs have advanced to the clinic and have demonstrated proof of concept for treating HCV. Here, the application of phosphate prodrugs in the development of nucleotide inhibitors of HCV and the status of nucleotide prodrugs under investigation for the treatment of HCV infection will be reviewed.

Nucleotide phosphoramidates

Nucleotide phosphoramidates were first disclosed by McGuigan *et al.* [36] as a prodrug strategy to deliver a nucleoside 5′-monophosphate for the treatment of HIV and cancer. The structure of a nucleotide phosphoramidate typically consists of a nucleoside 5′-monophosphate where the phosphate group is masked by appending an aryloxy group (usually a phenol) and an α-amino acid ester (Figure 4); however, other related constructs have also appeared. The phosphate group is ultimately revealed by a sequence of enzymatic and chemical steps that requires either carboxyesterase or cathepsin A to cleave the terminal amino acid ester, intramolecular displacement of the phosphate phenol and then enyzmatic cleavage of the amino acid moiety by a phosphoramidase or histidine triad nucleotide-binding protein 1 (HINT 1) [37–39]. It is believed that the phosphoramidate prodrug construct increases lipophilicity of the nucleoside 5′-monophosphate and therefore increases cellular permeability and ultimately intracellular nucleotide concentrations. Since the phosphoramidate prodrug moiety contains a chiral phosphorus centre, issues arise with regard to development of a compound that consists of a mixture of isomers with implications arising from differential activity of each of the isomers, pharmacokinetics and manufacturing optimization, etc. In addition, the typical phosphoramidate contains a phenolic substituent that is released during metabolism to the free monophosphate. Successful development also considers the metabolic release of this phenolic substituent. Selective examples employing the phosphoramidate strategy to achieve kinase bypass for nucleoside inhibitors of HIV reverse transcriptase (RT) inhibitors, for example, ddA, d4T and d4A, showed that *in vitro* whole cell enhancement in potency could be achieved [40–42]. Although the phosphoramidate strategy was explored extensively to deliver nucleotides for the treatment of HIV and colon cancer [33,36], proof of concept in the clinic has yet to be reported. However, the phosphoramidate prodrug approach has proven to be a valuable strategy in the development of HCV nucleotide therapy.

Figure 4. The phosphoramidate prodrug decomposition pathway that results in the release of the nucleoside 5'-monophosphate



2′-C-Methyl ribonucleotide phosphoramidates

The 2′-C-methylcytidine nucleoside, NM107 (**1**; Figure 5), was shown to be an inhibitor of HCV in cell culture (50% effective concentration [EC_{50}] =1.23 μM) and its triphosphate (**3**) was demonstrated to be a potent inhibitor of the HCV polymerase enzyme (50% inhibitory concentration [IC_{50}] =0.09–0.18 μM) acting as a nonobligate chain terminator [43]. NM107 also showed broad antiviral activity against not only HCV but also bovine virus diarrhoea virus (BVDV), yellow fever virus, dengue virus and West Nile virus [31]. To overcome bioavailability issues the 3′-valinate ester prodrug, NM283 (valopicitabine; **2**) [31,44], was taken into clinical development. In a Phase I monotherapy study, NM283 demonstrated proof of concept delivering an ~1.2 $\log_{10}$ IU/ml reduction in viral load at a dose of 800 mg twice daily given over 14 days. Unfortunately, NM283 was discontinued because of significant gastrointestinal toxicity in Phase II studies [24,30].

Subsequent work on the development of the 2′-C-methyl class of nucleosides focused on 5′-phosphate nucleotide prodrugs. It was observed that the 2′-C-methylcytidine triphosphate (**3**) was highly active as an inhibitor of the NS5B polymerase, yet the parent nucleoside NM107 (**1**) was only modestly active in the whole cell based replicon assay. Studies had shown that NM107-triphosphate (**3**) formation was inefficient, particularly because of poor conversion of the nucleoside to its monophosphate by 2′-deoxycytidine kinase [45]. Consequently, to circumvent this phosphorylation problem and potentially improve the therapeutic index by increasing nucleoside triphosphate levels in the liver, a phosphoramidate prodrug approach was investigated [45]. This effort lead to the identification of phosphoramidate derivative **4** (Figure 5; $EC_{50} \leq 0.5$ μM) showing substantial increases in potency relative to NM283 [45]. The activity of compound **4** correlated with the levels of triphosphate produced in human hepatocytes and these levels were shown to be much higher than that seen with NM283 (**2**). *In vivo* studies assessing liver nucleoside triphosphate levels after oral administration in hamsters showed low triphosphate concentrations only twofold higher than obtained with NM283. Since substantial liver triphosphate levels were seen after subcutaneous administration *in vivo* and the compounds were shown to be stable in simulated gastric fluid, it was concluded that low oral bioavailability or metabolic degradation

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