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.
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 (Mg²⁺ or Mn²⁺) 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'-$\alpha$-fluoro-2'-$\beta$-C-methylcytidine and 4'-azidoctydine 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 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'-triphosphates 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

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

**2'-Methyl class**

![Diagram showing 2'-Methyl class](Image)

**4'-Azido class**

![Diagram showing 4'-Azido class](Image)

These classes include the 2'-methyl and the 4'-azido ribosides.
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 carboxysterase or cathepsin A to cleave the terminal amino acid ester, intramolecular displacement of the phosphate phenol and then enzymatic 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-binding 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.
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 \([EC_{50}] = 1.23 \mu M\)) and its triphosphate (3) was demonstrated to be a potent inhibitor of the HCV polymerase enzyme (50% inhibitory concentration \([IC_{50}] = 0.09–0.18 \mu M\) 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; \(EC_{50} \leq 0.5 \mu M\)) 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
Figure 5. 2′-C-Methylcytidine nucleosides and nucleotide phosphoramidate prodrug inhibitors of HCV replication

NM107
EC_{50}=1.23 \mu M

NM283

EC_{50}=0.09-0.18 \mu M

EC_{50}=0.22 \mu M, CC_{50}=7 \mu M

EC_{50}=0.24 \mu M

EC_{50}=8.2 \mu M, CC_{50}=>100 \mu M

NTP (human hepatocytes) AUC_{(0-4h)}=1,720 \mu M•h

NTP (human hepatocytes) AUC_{(0-4h)}=190 \mu M•h

AUC, area under the curve; CC_{50}, 50% cytotoxic concentration; EC_{50}, 50% effective concentration; IC_{50}, 50% inhibitory concentration.
in the intestine was the reason for the lack of oral efficacy [45].

Another series of 2′-C-methylcytidine phosphoramide prodrugs having an acyloxyethylamino phosphoramidate promoiety was studied (Figure 5) [46]. The phosphoramidate prodrug 5 provided up to a 30-fold improvement in HCV replicon potency over NM283 (2), and it was also shown that this activity correlated to levels of nucleoside triphosphate in rat and human hepatocytes. However, when administered orally to rats these phosphoramidates demonstrated no improvement in production of triphosphate in rat liver relative to NM283 (2). These results put into question the oral bioavailability and conversion of these prodrugs to the nucleoside triphosphate in the liver.

Phosphoramidate monoesters of 2′-C-methylcytidine were also explored (Figure 5) [45]. In this case the amide moiety was either an α-amino acid (6) or an acyloxyethylamino substituent (7). Although phosphoramidate monoester 6 was shown to have inferior replicon potency relative to its phenolic ester counterpart (<200-fold) it showed higher levels of triphosphate formation in human hepatocytes. Formation of nucleoside triphosphate levels were observed in hepatocytes of various species for both 6 and 7 but the phosphoramidate monoester 6 was shown to be superior to 7. In both cases, liver levels of nucleoside triphosphate 3 were achieved in vivo after subcutaneous administration but not after oral administration suggesting a lack of oral bioavailability.

Other 2′-C-methyl nucleosides containing purine bases were also investigated as inhibitors of HCV, including the 7-deaza-2′-C-methyladenosine derivative MK0608 (8) which showed potent inhibition of HCV replication in vitro (EC\textsubscript{50}=0.25 µM) and demonstrated SVR in an HCV-infected chimpanzee animal model [47,48]. Although MK0608 (8) was never progressed into clinical development, it did demonstrate the potential of purine nucleosides as inhibitors of HCV. Subsequently, phosphoramidate prodrugs of 2′-C-methyl purine analogues were studied to determine if improvement in potency could be achieved by kinase bypass. Although application of the phosphoramidate prodrug strategy was not successful for the adenosine derivative 11 (Figure 6), its application to the guanosine analogue 12 resulted in an 84-fold increase in activity in the replicon assay relative to the parent nucleoside (Figure 7) [49]. This result was rationalized by the observation that the guanosine triphosphate (13; IC\textsubscript{50}=0.13 µM) was more potent as an inhibitor of the HCV polymerase than the adenosine triphosphate (10; IC\textsubscript{50}=1.9 µM).
yet in the whole cell replicon assay the adenosine analogue (9; $EC_{50}=0.3 \mu M$; Figure 6) was more potent than the guanosine analogue (12; $EC_{50}=3.5 \mu M$; Figure 7). Therefore, this finding coupled with the observation that low levels of triphosphate were detected in cells for the guanosine nucleoside relative to that for the adenosine nucleoside hinted at poor phosphorylation in the case of the guanosine nucleoside. The development of the 2'-C-methylguanosine phosphoramidate derivative was further explored by systematically evaluating structural modifications to the phosphoramidate moiety in an effort to achieve increased HCV replicon potency, plasma stability across multiple species, and appropriate relative stability in intestinal and liver S9 preparations. Ultimately, these studies were able to identify 2'-C-methylguanosine phosphoramidate prodrugs 14 and 15 (Figure 7) [50]. These prodrugs contained benzyl or alkyl l-alanine and l-valine amino acid ester moieties and a naphthyl phosphate ester and exhibited a 10–30-fold enhancement in HCV replicon potency with acceptable plasma and intestinal S9 stability suitable for progression into in vivo studies. However, when mice were dosed orally in order to assess liver levels of the nucleoside triphosphate 13, the 2'-C-methylguanosine phosphoramidates 14 and 15 (Figure 7) produced substantial liver levels of triphosphate but these levels were not significantly improved over that observed when mice were dosed with the parent guanosine nucleoside 12.

Further investigation of the 2'-C-methylguanosine phosphoramidate series led to the evaluation of substitution at the C-6-position of the guanosine base with the

**Figure 7. 2'-C-Methylguanosine nucleoside and nucleotide phosphoramidate prodrug inhibitors of HCV replication**
intention of increasing lipophilicity and thus improving cellular uptake relative to the natural guanosine derivative 12. This led to the identification of the C-6-O-methyl derivative INX-08189 (16; Figure 7) containing both a neopentyl ester on the l-alanyl amino acid moiety and a naphthyl ester on phosphorus of the phosphoramidate [51]. In INX-08189 (16), in addition to metabolic conversion of the phosphoramidate pro-moiety to the 5'-monophosphate, the 6-O-methyl group of the purine base is metabolized to the guanine base. INX-08189 (16) demonstrated exceptional potency in the HCV 1b replicon assay (EC_{50}=0.01 µM; CC_{50}=7 µM), was active against genotype 1a and 2 replicons and produced substantial human hepatocytes over 48 h. The known 2'-HCV RNA reduction of -0.71 and -1.03 log_{10} IU/ml, or 25 mg. Antiviral activity was observed with a mean type 1 HCV patients dosed once daily at either 9 mg once daily oral dosing [53,54]. INX-08189 was progressed into the clinic and as a mixture of isomers at the phosphorus centre of the prodrug.

In a single-ascending-dose Phase Ia study in healthy volunteers administered doses ranging from 3 mg to 100 mg, INX-08189 (16) was shown to be generally well tolerated at all doses with no drug-related serious adverse events and pharmacokinetics (PK) supporting once daily oral dosing [53,54]. INX-08189 was progressed into a Phase Ib study in treatment-naive genotype 1 HCV patients dosed once daily at either 9 mg or 25 mg. Antiviral activity was observed with a mean HCV RNA reduction of -0.71 and -1.03 log_{10} IU/ml, respectively [54,55]. These early human data demonstrated clinical proof of concept for INX-08189 and consequently, the compound is continuing to undergo clinical evaluation.

Another application of phosphoramidate prodrug technology for delivering a 2'-C-methylguanosine 5'-monophosphate is exemplified by IDX184 (17; Figure 7) [56]. In this case the phosphoramidate prodrug moiety utilized a benzyl amine in place of an amino acid for the amine substituent and an S-acetyl-2-thioethanol moiety (SATE) as the phosphate ester substituent in place of the more common aryl group. The SATE group is a known phosphate ester prodrug construct [33,57,58]. Mechanism for release of the desired 2'-C-methylguanosine 5'-monophosphate is believed to involve both CYP450-dependent and independent processes. Based on the prodrug structure one would anticipate that prodrug release would involve cleavage of the terminal thioester followed by loss of the phosphate ester via intramolecular attack of the free thiol group releasing the phosphate and an equivalent of episcufide, then enzymatic cleavage of the benzyl phosphoramidate. However, a detailed mechanism for the IDX184 (17) prodrug cleavage has not been reported to date. In the whole cell HCV replicon assay IDX184 (17) was shown to be a potent inhibitor of HCV replication (EC_{50}=0.4 µM; CC_{50}>100 mM) and was also active in the genotype 2a JFH1 replicon (EC_{50}=0.6–11 µM) [59]. At a concentration of 2.5 µM it cleared HCV replicon RNA after 14 days of treatment. Like other 2'-C-methyl nucleosides it was shown to select for the NS5B S282T mutation in the HCV replicon. In combination with the protease inhibitor IDX320, IFN-α, or RBV, IDX184 exhibited an additive or synergistic profile [60]. When administered orally to cynomolgus monkeys, IDX184 (17) produced high live triphosphate levels relative to oral administration of the parent nucleoside with what appears to be high hepatic extraction. Subsequent studies in a HCV-1-infected chimpanzee model showed that oral administration of IDX184 (17) at 10 mg/kg over 3 days produced a median viral load decline of approximately -2.3 log_{10} at day 3 and 4 [61]. Consequently, IDX184 (17) was progressed into the clinic and in a Phase Ia single ascending dose study was shown to be generally safe and well tolerated at oral doses from 5 mg to 100 mg [56]. PK assessment supported a liver target of IDX184 (17) was administered at doses from 25 to 100 mg once a day for 3 days. Day 4 viral load assessment showed that at the highest dose of 100 mg, a -0.74 log_{10} IU/ml reduction in viral load was observed [62]. Following the positive Phase I clinical results, a Phase II study was initiated in which IDX184 (17), at doses from 50–200 mg, was combined with pegylated IFN and RBV for 14 days in treatment-naive HCV genotype 1 patients [63]. At day 14 viral load reductions of -2.7 to -4.1 log_{10} IU/ml were achieved with no adverse events attributed to IDX184 (17) and with no detection of resistant virus [64]. However, in an attempt to evaluate the efficacy of a DAA combination of IDX184 (17) with the NS3 protease inhibitor IDX320, three serious adverse events were reported in a drug–drug interaction study in healthy volunteers and consequently, the programme was placed on clinical hold in September 2010, awaiting resolution of the cause of the adverse events [65,66]. In February 2011, the US Food and Drug Administration (FDA) removed full clinical hold for IDX184 (17) owing to evidence that the toxicity was likely caused by IDX320, and the programme was placed on partial clinical hold. Initiation of a Phase IIb 12-week trial of IDX184 (17) in combination with pegylated IFN and RBV is anticipated in the second half of 2011 [67].

4'-Azido ribonucleotide phosphoramidates

The 4'-C-azidocytidine nucleoside R1479 (18; Figure 8) was reported to be an inhibitor of HCV replication (EC_{50}=1.28 µM) and subsequently its 2',3',5'-tri-O-isobutyrate ester prodrug R1626 (balapiravir; 20) was
taken into clinical development as a treatment for HCV infection [32,68]. R1479 was shown to have reduced activity against the S96T HCV polymerase mutant [27]. In a Phase Ib monotherapy clinical study in HCV-infected patients dosed with 500, 1,500, 3,000 and 4,500 mg twice daily for 14 days, R1626 (20) demonstrated a mean decrease in viral load of -0.3, -1.2, -2.6 and -3.7 log10 IU/ml, respectively, thus providing proof of concept for this class of nucleosides. R1626 was subsequently taken into a Phase IIa study in genotype 1 treatment-naive patients where at a dose of 1,500 mg twice daily in combination with IFN/RBV a -5.2 log10 IU/ml reduction in viral load with an 81% rapid virological response (RVR) was observed after 28 days of therapy [30]. However, dosing was limited by neutropenia and in a Phase IIb study in combination with IFN/RBV, the development of R1626 (20) was discontinued as a result of significant haematological adverse events [69].

In an effort to increase the potency of R1479 (18), a 5′-phosphoramidate nucleotide prodrug strategy was investigated [70]. It was hoped that the increased lipophilicity introduced by the phosphoramidate group might result in enhanced passive diffusion and therefore, increased intracellular levels of the monophosphate and ultimately of the active triphosphate thus leading to a significant in vitro and in vivo advantage. In addition, if liver targeting was possible, as was described for other nucleotide prodrugs, there existed a possibility that the liabilities associated with R1479 (18) could be ameliorated by limiting systemic exposure. After an extensive structure–activity relationship (SAR) study, it was noted that preparation of the 5′-phosphoramidate derivative 21 (Figure 8) did not provide any appreciable improvement in potency over the nucleoside R1479 (18), indicating that this nucleoside is already efficiently phosphorylated and that application of the phosphoramidate technology was not able to boost its potency [70].

Further evaluation of the 4′-azido pyrimidine class of nucleosides investigated the 4′-C-azidouridine

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**Figure 8. 4′-Azido cytidine nucleoside and nucleotide phosphoramidate prodrug inhibitors of HCV replication**

<table>
<thead>
<tr>
<th>Structure</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1479 (18)</td>
<td>1.28 µM</td>
<td>&gt;100 µM</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 0.32 µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; = 0.51 µM</td>
<td>CC&lt;sub&gt;50&lt;/sub&gt; = &gt;100 µM</td>
</tr>
<tr>
<td>21</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt; = 1.28 µM</td>
<td>CC&lt;sub&gt;50&lt;/sub&gt; = &gt;100 µM</td>
</tr>
<tr>
<td>R1626 (20)</td>
<td></td>
<td></td>
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</tbody>
</table>

CC<sub>50</sub>, 50% cytotoxic concentration; EC<sub>50</sub>, 50% effective concentration; IC<sub>50</sub>, 50% inhibitory concentration.
derivative 22 (Figure 9) [71]. It was shown that the 4′-C-azidouridine (22) was inactive as an HCV inhibitor as determined in the cell-based replicon assay. However its 5′-triphosphate analogue (23) demonstrated inhibition of the HCV polymerase enzyme (IC₅₀=0.22 µM) similar to that of the triphosphate 19 (IC₅₀=0.32 µM) of the cytidine derivative 18. Speculating that poor substrate activity for cellular kinases was contributing to the lack of whole cell activity of the 4′-C-azidouridine (22), a series of 5′-phosphoramidate prodrugs was prepared. SAR around the phosphoramidate moiety showed that the nature of the substituents on the phosphoramidate had a dramatic effect on activity in the HCV whole cell assay. Since the compounds were prepared as a mixture of diastereomers at the phosphorus centre of the phosphoramidate, the diastereomers were separated chromatographically but no difference in potency was observed. Improvements in activity of >450-fold were reported, with the most active compound in the series having the 1-naphthyl l-alanine benzyl ester phosphoramidate (24) substitution pattern (EC₅₀=0.22 µM). To date, no reports have appeared that indicate further progression of a 4′-C-azidouridine 5′-phosphoramidate prodrug beyond in vitro studies.

In the 4′-azido riboside class of nucleosides, the phosphoramidate prodrug strategy was also applied to the adenosine derivative 25 (Figure 10) [72]. As in the case of the 4′-C-azidouridine, the 4′-C-azidoadoenosine (25) was shown to be inactive in the HCV replicon assay with the assumption that the lack of activity was due to its inability to be phosphorylated by cellular kinases. It was hypothesized that delivering a monophosphate prodrug derivative would both bypass the non-productive monophosphorylation step and also reduce the extent of intracellular metabolism by deamination. Preparation of a series of phosphoramidate derivatives led to the identification of several compounds, like compound 27, that showed submicromolar EC₅₀ values (0.22–0.59 µM) in the HCV replicon assay and were devoid of cellular toxicity; however, no additional data was provided to support protection from deamination nor translation of the in vitro data into an in vivo setting.

2′-α-Fluoro-2′-β-C-methyl nucleotide phosphoramidate prodrugs
In 2005, the first disclosure of a 2′-α-fluoro-2′-β-C-methylcytidine nucleoside was made with the...
**Figure 10. 4’-Azido adenosine nucleoside and nucleotide phosphoramidate prodrug inhibitors of HCV replication**

![Chemical structures of 4’-Azido nucleoside inhibitors of HCV replication.](image)

EC_{50} = >100 \mu M, CC_{50} = >100 \mu M

IC_{50} = Not determined

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CC_{50}, 50% cytotoxic concentration; EC_{50}, 50% effective concentration; IC_{50}, 50% inhibitory concentration.

**Figure 11. 2’-α-F-2’-β-C-Methylcytidine nucleoside inhibitors of HCV replication**

![Chemical structures of 2’-α-F-2’-β-C-Methylcytidine nucleoside inhibitors of HCV replication.](image)

EC_{90} = 4.5 \mu M

IC_{50} = 0.34 \mu M

---

CC_{50}, 50% cytotoxic concentration; EC_{50}, 50% effective concentration; EC_{90}, 90% effective concentration; IC_{50}, 50% inhibitory concentration.
publication of PSI-6130 (28; Figure 11) [73,74]. This class of nucleosides demonstrated unique characteristics in vitro that would eventually translate into the clinic. PSI-6130 (28) was shown to be an inhibitor of HCV replication in the cell-based replicon assay (90% effective concentration [EC$_{90}$]=4.5 µM) and its triphosphate (29) was shown to be a potent inhibitor of the HCV NS5B polymerase (IC$_{50}$=0.34 µM) with an intracellular half-life of approximately 6 h. This class of nucleosides demonstrated exquisite selectivity for HCV versus other Flaviviruses, other RNA viruses as well as for HIV and HBV. The class showed broad genotype coverage inhibiting the HCV polymerase from genotypes 1 to 6. Triphosphate 29 was also shown not to be an inhibitor of any of the human DNA or RNA polymerases. PSI-6130 (28) demonstrated a safety profile that did not exhibit any of the toxicology signals common to nucleosides including bone marrow and mitochondrial toxicity. Although the known 2′-C-methyl nucleoside S282T amino acid alteration in the HCV polymerase was shown to be resistant to PSI-6130 (28), the level of resistance was demonstrated to be relatively minimal (threefold) and difficult to raise in vitro [27,75]. However, in clinical studies PSI-6130 (28) delivered modest oral bioavailability and a significant amount of the drug was metabolized to an inactive uridine metabolite [21]. To rectify these shortcomings a prodrug was eventually developed leading to the simple 3′,5′-di-O-isobutyrate diester prodrug RG7128 (mericitabine; 30) [21].

RG7128 (30) demonstrated proof of concept in the clinic when administered alone or in combination with SOC [21,24,30]. In a Phase Ia multiple ascending dose study in 40 genotype 1 non-responder HCV-infected patients, RG7128 (30) was dosed at 750 mg and 1,500 mg either once daily or twice daily. The study demonstrated a dose-dependent reduction in HCV RNA levels and also showed that twice daily dosing was preferred. The 1,500 mg twice daily dose of RG7128 (30) resulted in the maximum antiviral effect producing a -2.7 log$_{10}$ IU/ml drop in viral load after 14 days of monotherapy. Subsequently, two Phase Iia studies in combination with SOC (Pegasys® and Copegus®) were initiated, one in genotype 1 treatment-naives and another in genotype 2,3 non-responder HCV-infected patients. The genotype 2/3 study was the first non-genotype 1 patient study initiated with a DAA. In the Phase Iia study treating genotype 1 patients, doses of 500, 1,000 and 1,500 mg twice daily were investigated over 28 days. Study results showed that the 1,000 and 1,500 mg doses provided the best viral load reduction and were essentially equally effective producing RVRs of 85% and 88% and viral load reductions of -5.05 and -5.09 log$_{10}$ IU/ml, respectively. In the genotype 2/3 patient study at a dose of 1,500 mg twice daily over 28 days, a -5 log$_{10}$ IU/ml reduction in viral load was observed which translated into a 90% RVR. The results of the genotype 2/3 patient study demonstrated for the first time that a DAA could be effective in the clinic in this patient population and corroborated the broad genotype coverage predicted by the in vitro data. In both genotype 1 and genotype 2/3 patient studies there were no significant adverse events reported and the safety was described as no different than placebo with SOC.

In both the genotype 1 and genotype 2/3 Phase Iia clinical studies where RG7128 (30) was dosed in combination with SOC no viral breakthroughs were observed over the course of therapy. Subsequent clonal analyses showed that there was no identified NS5B S282T variants in the treatment groups nor was there any pre-existing S282T viral variants observed at baseline in the patient population studied [75]. These data support the high barrier to resistance observed in the preclinical studies for this nucleoside class.

RG7128 (30) progressed into a Phase Iib clinical study evaluating 408 genotype 1 and 4 HCV patients when dosed with 500 or 1,000 mg twice daily daily RG7128 (30) in combination with SOC over 12 or 24 weeks assessing SVR as the primary end point. An interim 12-week analysis was reported which showed an average 83% complete early virological response (cEVR) for the 1,000 mg RG7128 12-week regimen and no evidence of drug resistance [22,76]. RG7128 (30) is expected to complete the Phase Iib study in the near term.

With the hope of demonstrating proof of concept that the combination of two DAAs could lead to an IFN-sparing clinical regimen, RG7128 (30) was combined with a protease inhibitor RG7227 (danoprevir) in a 14-day clinical study in genotype 1 patients to assess safety and viral kinetics [77]. The study demonstrated that combination of the two DAAs was safe and well tolerated and that at the highest dose levels (1,000 mg RG7128 and 900 mg danoprevir) in both treatment-naive and IFN-null responders, viral load declines of -5.1 log$_{10}$ IU/ml and -4.9 log$_{10}$ IU/ml were observed. This combination produced a viral decline that is quantitatively similar to that observed when each individual agent is combined with IFN and RBV. These results demonstrated that in the clinic the combination of a nucleoside HCV polymerase inhibitor and a protease inhibitor has the potential to provide a promising combination for future HCV therapy.

Metabolism studies on the cytidine nucleoside PSI-6130 (28; Figure 11) showed that not only was the nucleoside converted to its active triphosphate derivative 29, but that it was also converted to the uridine nucleoside PSI-6206 (31; Figure 12) in vivo [78,79]. However, this uridine nucleoside was shown not to be active as an inhibitor of HCV replication in the replicon assay. Subsequent studies indicated that the triphosphate of PSI-6206 (32; Figure 12) was a potent...
inhibitor of the HCV NS5B polymerase with an 
IC_{50}=1.19 \, \mu M \) and that this triphosphate had a long desirable intracellular half-life of 36 h [80]. Study of the phosphorylation kinetics of the uridine nucleoside 31 showed that its lack of activity was related to the lack of conversion to its 3'-monophosphate derivative and that subsequent conversion of the 5'-monophosphate to the corresponding 5'-diphosphate and triphosphate derivatives was facile. In an attempt to take advantage of the long intracellular half-life of the uridine triphosphate 32 with the hope of potentially developing a once-daily drug, a prodrug of the uridine 5'-monophosphate was pursued. Similar to the nucleosides discussed above, a phosphate prodrug approach was investigated to overcome the enzymatic blockade at the monophosphorylation step and effectively deliver the uridine nucleoside 5'-monophosphate. A phosphoramidate prodrug strategy was implemented with the expectation that first pass metabolism of the prodrug moiety in the liver would achieve efficient delivery to the liver of the uridine nucleoside 5'-monophosphate [21,81,82]. Extensive SAR around the phosphoramidate moiety showed that the preferred substitution to achieve submicromolar whole cell HCV replicon potency without cellular toxicity required a small branched alkyl ester at the terminal carboxylate ester moiety, a small alkyl group at the alpha position of the amino acid substituent and a simple phenoxy or halogenated phenoxy phosphate ester group. To achieve the required delivery of the prodrug to the liver and subsequent release of the nucleoside 5'-monophosphate in hepatocytes, stability studies in simulated gastric fluids (SGF) and simulated intestinal fluids (SIF) and stability on exposure to liver S9 fractions were carried out. Several compounds provided the desired profile which showed good stability in SGF, SIF and blood but a short half-life in liver S9 fractions, thus supporting the expectation that the phosphoramidate strategy could enable liver targeting. The 2'-\alpha-F-2'-\beta-C-methyluridine 5'-phosphoramidates were subsequently shown to generate high concentrations of the corresponding nucleoside triphosphate 32 in primary hepatocytes of rat, dog, monkey and human, and eventually showed in rat and dog PK studies that the phosphoramidates could get to the liver and generate high levels of the uridine nucleoside triphosphate 32 there. The lead compound

\[
\text{PSI-6206} \quad \text{IC}_{50}=1.19 \, \mu M
\]

\[
\text{PSI-7851} \quad \text{EC}_{90}=0.52 \, \mu M, \text{CC}_{50}=>100 \, \mu M
\]

\[
\text{PSI-7977} \quad \text{IC}_{50}=0.42 \, \mu M, \text{CC}_{50}=>100 \, \mu M
\]

CC_{50}, 50% cytotoxic concentration; EC_{90}, 90% effective concentration; IC_{50}, 50% inhibitory concentration.
PSI-7851 (33) was subsequently shown to be devoid of mitochondrial, bone marrow and general cellular toxicities. Combination studies with either IFN and/or RBV and other DAAs such as protease, non-nucleoside polymerase, or NS5A inhibitors showed that PSI-7851 (33) was either additive or synergistic in its effect on HCV replication in the replicon assay [83]. When evaluated in the S282T mutant replicon known to be resistant to 2′-C-methyl nucleosides, PSI-7851 (33) demonstrated a 16.4-fold resistance relative to wild type [83,84]. PSI-7851 (33), a 1:1 mixture of diastereomers at the phosphorus centre of the phosphoramidate moiety, was chosen for further development and progressed into Phase I human clinical trials.

In a single ascending dose clinical study of healthy volunteers receiving doses up to 800 mg once daily, PSI-7851 (33) was demonstrated to be generally safe and well-tolerated, with no dose-limiting toxicities. In addition, PK results exhibited a systemic exposure profile that was described as consistent with rapid uptake of the drug by the liver and low plasma exposure to the prodrug PSI-7851 (33). PSI-7851 (33) was progressed into a 3-day multiple ascending dose study in treatment-naive, HCV-infected patients with the object of evaluating safety, PK and effect on viral load. HCV RNA levels declined in a dose dependent manner with the maximal mean change from baseline of -1.95 log_{10} IU/ml at the 400 mg dose. In addition, no pre-existing or treatment-emergent S282T resistant virus was detected, nor was there evidence of viral resistance after therapy [21].

With proof of concept demonstrated in HCV patients, further development of the 2′-α-F-2′-β-C-methyluridine phosphoramidate proceeded using PSI-7977 (34; EC_{90}=0.42 µM) the more active (>10-fold) single diastereomer of PSI-7851 (33) [81]. PSI-7977 (34) was obtained by direct crystallization from the diastereomeric mixture and it was shown to have the Sp stereochemistry at the phosphorus atom thus representing the first example of the crystallization and x-ray structure determination of a nucleoside phosphoramidate. In a 28-day Phase Ia clinical study in genotype 1 treatment-naive HCV patients, in which PSI-7977 (34) was dosed at either 100, 200 or 400 mg once daily in combination with pegylated IFN/RBV, RVR rates of 88%, 94% and 93%, respectively, were observed [85,86]. In addition, no viral resistance was detected nor were there any significant adverse events reported. Recently, a 14-day monotherapy study with PSI-7977 (34) showed that HCV genotype 1 treatment-naive patients achieved an average -5.0 log_{10} decline in viral load with 88% of patients reaching undetectability (<15 IU/ml) after 14 days. This result is a significant improvement from what was observed for the diastereomeric mixture PSI-7851 (33) [87], PSI-7977 (34) has entered Phase IIb clinical trials and it is being evaluated in combination with IFN/RBV for its effects in genotype 1, 2 and 3 patients. In genotype 1 patients treated with PSI-7977 (34), 47 of 48 patients (98%) at 200 mg once daily and 46 of 47 patients (98%) at 400 mg once daily achieved an RVR at week 12 with no viral breakthrough and a promising safety profile [88]. In genotype 2 and 3 HCV patients a 400 mg once daily dose of PSI-7977 (34) in combination with IFN/RBV achieved an SVR 12 weeks after cessation of therapy in 96% of patients treated [88]. In addition to the continued Phase IIb study in combination with abbreviated durations of IFN/RBV, PSI-7977 (34) is being studied in combination with other nucleosides (PSI-352938) and with the NS5A inhibitor BMS-790052 [15,89].

Further structure–activity relationship studies around the 2′-α-F-2′-β-C-methyl class of nucleosides had shown that the guanosine derivative 35 (Figure 13) was weakly active (EC_{90}=69.2 µM) as an inhibitor of HCV in the replicon assay; however, the guanosine triphosphate 36 was shown to be a good inhibitor of the HCV RNA polymerase (IC_{50}=5.94 µM; Figure 13) [90,91]. It was also demonstrated that the guanosine triphosphate 36 was equipotent as an inhibitor of both the wild type and S282T mutant HCV polymerase: the first example of such a profile for a 2′-C-methyl nucleoside. In order to try and improve potency of this guanosine nucleoside, speculating that the first monophosphorylation was limiting, preparation of phosphoramidate prodrugs of the 2′-α-F-2′-β-C-methylguanosine 5′-monophosphate along with modifications at the C-6 position of the guanosine base were pursued [91]. This double prod drug strategy, where in addition to the metabolism of the phosphoramidate, the C-6-O-methyl group of the base is metabolized to give guanine, ultimately led to a series of HCV inhibitors having low nanomolar potency with acceptable in vitro safety and stability profiles. Selected compounds were shown to produce high levels of the guanosine triphosphate 36 in primary human hepatocytes and a radiolabelled study showed that a guanosine phosphoramidate derivative provided a desirable 3.5:4.8:1 liver to plasma ratio. The single Sp phosphoramidate diastereomer, PSI-353661 (37; EC_{90}=8 nM; Figure 13) was obtained by crystallization from the diastereomeric mixture and was shown to be the more potent of the two possible diastereomers. In vitro studies with IFN or RBV and other DAAs demonstrated that PSI-353661 (37) could be combined to produce an additive or synergistic antiviral effect [92]. PSI-353661 (37) was selected as a clinical development candidate for the treatment of HCV infections and is awaiting the start of clinical evaluation.

Another 2′-α-F-2′-β-C-methyl nucleoside having a 7-ethyl-7-deaza adenosine base unit (38; Figure 14) was reported to be an efficient inhibitor of the
Figure 13. 2′-α-F-2′-β-C-Methylguanosine nucleoside and nucleotide phosphoramidate prodrug inhibitors of HCV replication

EC₉₀=69.2 µM
EC₉₀=5.94 µM

Ψ-SI-353661
EC₉₀=0.008 µM

CC₅₀, 50% cytotoxic concentration; EC₉₀, 90% effective concentration; IC₅₀, 50% inhibitory concentration.

Figure 14. 7-Deaza-7-ethynyl-2′-α-F-2′-β-C-methyladenosine nucleoside and nucleotide phosphoramidate prodrug inhibitors of HCV replication

EC₅₀=24 µM
EC₅₀=0.4 µM

Ψ-SI-353661

Ψ-SI-353661

R=Me, Ph
Ar=Ph, p-Me-Ph, p-F-Ph, p-MeO-Ph, 1-naphthyl, 2-naphthyl
No observed activity

EC₅₀, 50% effective concentration; IC₅₀, 50% inhibitory concentration.
HCV NS5B polymerase (IC₅₀=0.4 μM) yet its activity in a whole cell Huh7 replicon assay was reported to be low (EC₅₀=24 μM) [93]. Consequently, a small series of phosphoramidate prodrugs (40) of 2'-α-F-2'-β-C-methyl-7-ethynyl-7-deaza adenosine (38) was reported, however, no antiviral activity was observed for these prodrugs and the series was not progressed further [94].

3',5'-Cyclic phosphate nucleotide prodrugs
The use of a 3',5'-cyclic phosphate prodrug construct for delivering a nucleoside 5'-monophosphate into cells is a relatively uncommon prodrug motif [95–97]. Its application has only recently been selectively applied in the development of nucleotide inhibitors for HCV polymerase. Little is known about the mechanism by which these cyclic phosphates are metabolized to the

Figure 15. C-6-Hydrazido sulfonamide 2'-C-methyladenosine and 2'-C-methyl-2-amino-adenosine nucleoside and nucleotide cyclic phosphate prodrug inhibitors of HCV replication

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>EC₅₀</th>
<th>CC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>41</td>
<td>H</td>
<td>300 μM</td>
<td>&gt;300 μM</td>
</tr>
<tr>
<td>42</td>
<td>NH₂</td>
<td>82 μM</td>
<td>&gt;300 μM</td>
</tr>
<tr>
<td>43</td>
<td>H</td>
<td>100 μM</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>NH₂</td>
<td>23.8 μM</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>H</td>
<td>68.5 μM</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>NH₂</td>
<td>1.7 μM</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>H</td>
<td>0.039 μM</td>
<td>&gt;50 μM</td>
</tr>
<tr>
<td>48</td>
<td>NH₂</td>
<td>0.008 μM</td>
<td>&gt;50 μM</td>
</tr>
</tbody>
</table>

CC₅₀, 50% cytotoxic concentration; EC₅₀, 50% effective concentration.
The first reported example of a 3',5'-cyclic phosphate prodrug inhibitor of HCV replication was methylcytidine-3',5'-cyclic phosphoramidate (Figure 15) [99]. The parent nucleosides 41, 42 were essentially inactive (EC₅₀≥92 µM) as inhibitors of HCV replication in the replicon assay and subsequently were shown not to be substrates for adenosine kinase, suggesting an inability to be phosphorylated to the respective monophosphates 43 and 44. Preparation of the 3',5'-cyclic phosphate derivatives 45, 46 resulted in significant improvement in replicon potency (EC₅₀=68.5 and 1.7 µM) relative to both the 5'-monophosphate derivatives 43 and 44 (EC₅₀=100 and 23.8 µM) and the parent nucleosides 41 and 42. This suggests that even though 44 and 45 retain a phosphate monoacid, the cyclic phosphate construct sufficiently reduces the polarity relative to the 5'-monophosphates 43 and 44 to improve cell penetration. To further enhance cell permeability characteristics, the acid of the cyclic phosphates was converted to the SATE 47 and 48 resulting in substantial improvement in HCV replicon potency [99]. The SATE cyclic phosphate prodrugs 47 and 48 demonstrated a 7,000- to 11,000-fold improvement in potency (EC₅₀=0.463 to 0.008 µM) relative the parent nucleosides with no observed cytotoxicity. Further development of these novel inhibitors has not been reported.

Another application of the 3',5'-cyclic phosphate prodrug approach focused on the study of 3',5'-cyclic phosphoramidates in an attempted to improve the activity of 2'-C-methylcytidine (Figure 16) [100]. It was postulated that relative to the 5'-phosphoramidate prodrug, the 3',5'-cyclic version would reduce the molecules rotational degrees of freedom and potentially improve entry into cells. In addition, use of a cyclic phosphate would remove the need for a phenolic substituent on the phosphoramidate moiety. Preparation of 2'-C-methylcytidine 3',5'-cyclic phosphoramidates like 49 (Figure 16) lead to prodrugs which were a mixture of diastereomers that were ultimately separated chromatographically. In some cases there was a substantial difference in activity between the prodrug diastereomers, however, in no case did any of the prodrugs demonstrate significant improvement in anti-HCV activity over 2'-C-methylcytidine itself. Contrary to the results from the HCV replicon assay, several cyclic phosphoramidate analogues did produce enhanced levels of nucleoside triphosphate when incubated with primary human hepatocytes (two- to sevenfold). Subsequent in vivo studies in the hamster showed a lack of oral bioavailability as measured by the levels of nucleoside triphosphate in the liver, possibly due to poor absorption or gastrointestinal instability. However, subcutaneous administration did show a 10- to 50-fold improvement in the levels of nucleoside triphosphate relative to that produced with 2'-C-methylcytidine. No further developments on this class of nucleotide prodrugs have been reported.

Figure 16. 2'-C-Methylcytidine-3',5'-cyclic phosphoramidate prodrug inhibitor of HCV replication

![Chemical Structure](image)

| Slow eluting diastereomer | EC₉₀=4 µM, CC₉₀=100 µM |

CC₉₀ 50% cytotoxic concentration; EC₉₀ 50% effective concentration.

Figure 17. O-6-Ethyl-2'-α-F-2'-β-C-Methylguanosine-3',5'-cyclic phosphate prodrug inhibitor (PSI-352938) of HCV replication

![Chemical Structure](image)

| PSI-352938 | EC₉₀=1.37 µM, CC₉₀=100 µM |

CC₉₀ 50% cytotoxic concentration; EC₉₀ 90% effective concentration.

desired 5'-monophosphates; however, based on the reported HCV data and recent clinical data [98], it is clear that this approach is an effective prodrug strategy for delivering in vivo a nucleoside 5'-monophosphate into cells.

2'-C-Methyl nucleotide 3',5'-cyclic phosphate prodrugs

The first reported example of a 3',5'-cyclic phosphate prodrug construct for inhibiting HCV was reported in an attempt to develop a set of 2'-C-methyl ribonucleosides with modified purine bases where the purine base has a hydrazido sulfonamide at the C-6 position (Figure 15) [99].
2′-α-F-2′-β-C-Methyl nucleotide 3′,5′-cyclic phosphate prodrugs
The 3′,5′-cyclic phosphate prodrug strategy was also applied to the 2′-α-F-2′-β-C-methyl class of nucleosides and ultimately resulted in the clinical development candidate PSI-352938 (50; Figure 17) [101]. PSI-352938 (50) demonstrated for the first time human clinical proof of concept for use of the cyclic phosphate prodrug approach to deliver a 5′-monophosphate [98]. In the case of the 2′-α-F-2′-β-C-methyl class of nucleosides the 3′,5′-cyclic phosphate prodrug approach was explored in an attempt to deliver 6-substituted guanosine derivatives [101]. This approach was taken because the parent 2′-α-F-2′-β-C-methylguanosine nucleoside 35 was a weak inhibitor in the HCV replicon assay even though its corresponding 2′-α-F-2′-β-C-methylguanosine triphosphate (36) was a good inhibitor of the HCV polymerase (IC\textsubscript{50}=5.94 µM) and equipotent against both the wild type and S282T mutant polymerases. In the case of the 2′-α-F-2′-β-C-methylguanosine series, unlike in the case of other 2′-C-methyl nucleosides, simple alkyl and aryl esters of the 3′,5′-cyclic phosphate were explored and SAR optimization focused around both the 6-position of the guanosine base and the phosphate ester moiety. These 2′-α-F-2′-β-C-methyl 6-substituted guanosine 3′,5′-cyclic phosphate esters were shown to be metabolized to the 2′-α-F-2′-β-C-methylguanosine triphosphate (36); however, the mechanistic details of the metabolism have yet to be reported. It was ultimately determined based on potency, cytotoxicity profile, gastrointestinal stability characteristics, and triphosphate production \textit{in vitro} and \textit{in vivo} that the Rp diastereomer PSI-352938 was the optimal candidate for clinical development. PSI-352938 was shown to have an EC\textsubscript{50}=1.37 µM with no cytotoxicity in multiple cell lines nor any observed mitochondrial toxicity or bone marrow toxicity when tested up to 100 µM \textit{in vitro}. When tested against the HCV NS5B polymerase isolated from genotypes 1 to 4, the triphosphate 36 of PSI-352938 (50) was shown to be equipotent against all genotypes and PSI-352938 demonstrated in the replicon assay to be additive or synergistic in combination with IFN and RBV and with other DAAs [83,102]. In addition, the combination of PSI-352938 and the uridine nucleotide phosphoramidate PSI-7977 (34; Figure 12) was shown to effectively clear both the wild type and S282T mutant replicons [102,103]. \textit{In vitro} and \textit{in vivo} studies showed that high triphosphate levels were produced in primary human hepatocytes and in rat liver (area-under-the-curve [AUC\textsubscript{0-24}]=41,893 ng h/g) when PSI-352938 (50) was administered orally at a 50 mg/kg dose. PSI-352938 (50)

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|}
\hline
\textbf{Drug (compound number)} & \textbf{Class} & \textbf{Inhibition HCV replicon, µM} & \textbf{EC\textsubscript{50}, µM} & \textbf{Maximum effect (dose, mg)} & \textbf{Log\textsubscript{10} IU/ml (days\textsuperscript{a})} & \textbf{Maximum effect (dose, mg)} & \textbf{Log\textsubscript{10} IU/ml (days\textsuperscript{a})} & \textbf{Status of program} \\
\hline
\textbf{Nucleoside prodrugs} & & & & & & & & \\
\hline
RG7128 & 2′-F,2′-methyl cytidine & EC\textsubscript{50}=2.5 & >100 & 1,500 & -2.7 (14) & 1,000 & -5.05 (28) & Completed Phase II \textit{in vitro} studies showed that high \textit{in vivo} triphosphate levels were produced in primary human hepatocytes and in rat liver (area-under-the-curve [AUC\textsubscript{0-24}]=41,893 ng h/g) when PSI-352938 (50) was administered orally at a 50 mg/kg dose. PSI-352938 (50)

\textbf{Nucleotide prodrugs} & & & & & & & & \\
\hline
PSI-7977 & 2′-F,2′-methyl uridine PA & EC\textsubscript{50}=0.42 & >100 & 400\textsuperscript{a} & -1.95 (3) & 400 & -5.3 (28) & Ongoing Phase Iib study \\
PSI-352938 & 2′-F,2′-methyl guanosine 3′,5′-cyclic phosphate & EC\textsubscript{50}=1.37 & >100 & 200 & -4.64 (7) & ND & ND & Ongoing Phase Iib study Full clinical hold changed to partial clinical hold \\
IDX184 & 2′-OH,2′-methyl guanosine & EC\textsubscript{50}=0.4 & >100 & 100 & -0.74 (3) & 150 & -4.1 (28) & Ongoing Phase Iib study \\
INX-08189 & 2′-OH,2′-methyl guanosine PA & ED\textsubscript{50}=0.01 & 7 & 25 & -1.03 (7) & ND & ND & Ongoing Phase Iib study \\
\hline
\end{tabular}
\caption{Nucleoside/nucleotide prodrugs: comparison of Phase I and Phase Ila human clinical trial results\textsuperscript{a}}
\end{table}

\textsuperscript{a}Comparison across clinical studies. Numbers in brackets represent number of days on the drug. Combination with interferon only no ribavirin present. No direct 50% effective concentration [EC\textsubscript{50}] available, represents EC\textsubscript{50} of the parent nucleoside R1479. Result from PSI-7851 the diastereomeric mixture. EC\textsubscript{50} 50% cytotoxic concentration; EC\textsubscript{50} 50% effective concentration; GI, gastrointestinal; GT, genotype; ND, no data available; PA, 5′-Phosphoramidate; SOC, standard of care.
was taken into a Phase I clinical study and was shown to be generally safe and well-tolerated when dosed up to 1,600 mg in healthy volunteers [104]. In a multiple ascending dose study in HCV genotype-1-infected patients, PSI-352938 was administered at doses of 100, 200 and 300 mg once daily and 100 mg twice daily over 7 days to assess safety, tolerability and antiviral response [98]. In the 40 patients studied HCV RNA levels declined consistently through the 7-day treatment period with a mean HCV viral load change from baseline of -4.31 log_{10} IU/ml, -4.65 log_{10} IU/ml, -3.94 log_{10} IU/ml and -4.59 log_{10} IU/ml for patients receiving 100 mg once daily, 200 mg once daily, 300 mg once daily and 100 mg twice daily. For those patients receiving the 200 and 300 mg doses 11 of 16 patients achieved HCV viral load below the limit of quantification. In addition, there were no reported adverse events in this study [98]. The early potency achieved by PSI-352938 (50) is comparable to that observed with NS3 protease inhibitors and PK parameters supported once-daily dosing. These results represent the first demonstration of clinical efficacy for a cyclic phosphate prodrug and represent the most potent nucleoside evaluated to date in a clinical setting (Table 1).

PSI-352938 (50) was further evaluated in a dual nucleotide DAA combination study with the pyrimidine nucleotide prodrug PSI-7977 (34) to evaluate the need for IFN in the treatment of HCV infection [87]. In this proof of concept study, no significant PK interactions between PSI-352938 (50) and PSI-7977 (34) were observed and no viral breakthrough was detected during therapy. This dual nucleotide combination demonstrated at day 14 a \(-4.6–5.5\) log_{10} decline in viral load with an average of 94% of patients having viral loads below the limit of detection (<15 IU/ml). This study is the first proof of concept that two nucleosides/nucleotides can be combined as a possible DAA treatment regimen. A Phase II study is being planned for this dual nucleotide DAA combination [87].

Cyclic 1-aryl-1,3-propanyl nucleotide phosphate esters: HepDirect prodrugs

Preparation of a nucleoside 5’-monophosphate cyclic diester using a 1-aryl-1,3-propanoyl prodrug moiety to accomplish liver targeting has been called HepDirect prodrug technology [33,105]. HepDirect technology was developed specifically to deliver drugs to the liver by taking advantage of liver-specific enzymes which would cleave the prodrug moiety thus revealing the active drug in hepatocytes. HepDirect prodrugs have been reported to be very stable in aqueous solutions, blood and non-hepatic tissues other than that of the gastrointestinal tract. For HepDirect technology, activation is achieved via a cytochrome P450 mediated oxidation (Figure 18). The described mechanism for nucleoside prodrug cleavage starts with hydroxylation of the C-4 tertiary carbon of the pro-moiety by CYP3A4 followed by rapid ring opening and then β-elimination to reveal the nucleoside monophosphate and an aryl vinyl ketone byproduct. Although

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**Figure 18.** HepDirect prodrug technology and the prodrug decomposition pathway.
release of aryl vinyl ketone as a byproduct of HepDirect cleavage raises toxicological concerns, it is hypothesized that this byproduct would be rapidly scavenged by high levels of glutathione in the liver, thus minimizing the associated risks. The HepDirect liver targeting prodrug approach was demonstrated in the clinic with pradefovir, a HepDirect prodrug version of adefovir [33].

2′-C-Methyladenosine HepDirect prodrugs

2′-C-Methyladenosine (9; Figure 6; EC_{50}=0.3 \mu M) was shown to be active as an inhibitor of HCV replication in the subgenomic replicon assay, however it was also determined to be an efficient substrate for adenosine deaminase, thus limiting its potential in vivo [106,107]. Since it was hypothesized that preparation of a 5′-monophosphate prodrug would block deamination and increase therapeutically levels of 2′-C-methyladenosine triphosphate (10) in the liver, a HepDirect prodrug approach was investigated. An SAR study evaluated activation in rat hepatocytes by modifications to the aryl substituent of the HepDirect prodrug moiety [108]. This assessment showed that the prodrugs were generally efficiently cleaved and converted to the nucleoside triphosphate 10 (Figure 6) particularly those with halogen substituted phenyl 51 or pyridyl groups (Figure 19). In vivo intravenous administration was able to achieve substantial levels of nucleoside triphosphate in rat liver, but oral administration resulted in a > fivefold lower level of liver triphosphate and only a 5% oral bioavailability. To circumvent this bioavailability issue the HepDirect prodrug approach was combined with other prodrug groups that would protect the 2′- and/or the 3′-hydroxyl groups or the N-6 amino group of the base (Figure 19). The combination of HepDirect with a 2′,3′-cyclic carbonate 53 appeared to be the most fruitful in that it provided a 2- to 10-fold improvement in the amount of nucleoside triphosphate observed in the liver compared to compounds incorporating only the HepDirect moiety. Unfortunately, no inhibition of HCV replicon activity was provided for any of the HepDirect derivatives to compare their potency to other prodrug approaches and no additional data have been forthcoming on the status of these agents.

Bis(S-acyl-2-thioethyl) nucleotide phosphate prodrugs: SATE prodrugs

The bis(S-acyl-2-thioethyl) phosphate strategy (SATE) has been applied widely in the field of nucleoside 5′-phosphate prodrugs [33,34]. As described above in the case of IDX184 which contains a SATE monoester, an acid and an equivalent of episulfide is liberated as a result of each thioester cleavage (Figure 20). Episulfide
is highly reactive and is a known mutagen, consequently it has been assumed that release of such an agent from a pronucleotide compound would be of some concern [109].

The only reported application of the bis-SATE prodrug method to nucleotide HCV inhibitors was toward 2′-α-F-2′-β-C-methyl 7-ethynyl-7-deaza adenosine 38 [94]. In this case the activity of the SATE prodrug 54 (EC\textsubscript{50}=8 µM) was threefold better than the parent nucleoside (Figure 21). However, no additional data or progress has been reported for this compound or on the use of bis-SATE prodrugs for delivering nucleotides for inhibiting HCV.

**Discussion**

Never before has the use of phosphate prodrugs played such a significant role in the discovery and development of nucleoside-based drugs as has been the case in the area of inhibitors of HCV. The translation of multiple examples of phosphate prodrugs of nucleotides into the clinic and the demonstration of clinical proof of concept and beyond are equally unprecedented. HCV has offered a unique opportunity to apply phosphate prodrug technology. Since HCV is a disease of the liver, drug hunters are able to leverage the robust characteristics...
of liver metabolism and first pass metabolism as the means for achieving target organ drug levels and obviating the need for maintaining high circulating levels of a potentially unstable prodrug. However, because of liver targeting, decomposition of the phosphate prodrug to the desired nucleoside 3’-monophosphate does release prodrug byproducts into the liver at potentially high concentrations. This release of sometimes potentially reactive or toxic metabolites does pose a concern in developing prodrugs of nucleoside monophosphates. This concern is particularly relevant to HCV patients with advanced liver disease where liver function is compromised and therefore, may have a lower capacity to clear or process metabolites. Some of the prodrug strategies implemented for delivering nucleoside monophosphates such as the SATE or HepDirect strategies are known to release reactive metabolites that should be of concern in assessing their long term safety potential. Other prodrug strategies such as the phosphoramidate release different metabolites such as phenol or naphthol. Although phenol is readily cleared by the liver, the toxicity of substituted phenols may be of concern in deciding to progress a nucleotide prodrug forward into development [110,111]. Each of these liability concerns must be put into context of dose and PK. It is possible to circumvent any toxicity concerns if the dose of drug administered is sufficiently small to not warrant concern or if the PK profile of the metabolites limits exposure. Clearly the increased potency of these nucleotide prodrugs relative to the first generation nucleoside inhibitors increases the therapeutic index and reduces the chances of observing undesired side effects. However, long term animal toxicity studies and clinical safety assessment over extended dosing periods will ultimately address the concerns over any potential human toxicity.

The nucleotide phosphate prodrugs which have entered clinical study have demonstrated superiority over the first generation nucleoside inhibitors developed for treating HCV (Table 1). The use of the nucleoside 5’-monophosphate prodrugs has demonstrated dramatic potency enhancements relative to first generation nucleoside inhibitors in the cellular subgenomic replicon assay, higher levels of triphosphate in whole cells and in livers of animals when dosed orally. To a large extent this improved in vitro and whole animal profile has translated into the clinical setting. In the clinic, dosing frequency has been reduced from twice daily to once daily, drug load has been greatly reduced and impressive viral load declines and RVR rates have been achieved. However, in vitro HCV replicon potency alone can not itself justify the robust initial clinical results observed for these phosphate prodrugs. The least potent inhibitor in the subgenomic replicon assay, PSI-352938 (50; Figure 17), produced the greatest viral load decline in human monotherapy studies, therefore issues such as PK must be playing a critical role in the overall human clinical response. How the human body handles these prodrugs is yet to be determined. A question that remains unanswered is whether there are specific transporters that play a clinically meaningful role in prodrug uptake into the liver or transport through the gut.

It has been suggested that nucleosides/nucleotides will eventually become the backbone of anti-HCV therapy, as they have for HIV HAART. Their broad genotype coverage and resistance profile put them in a unique position and make them the drug class of choice to combine with DAAs of different mechanisms of action. With the identification of both purine and pyrimidine nucleotide prodrugs with different resistance profiles and metabolic pathways, the possibility also exists for combining two nucleosides/nucleotides into one regimen as has been done in the HIV field. Studies to explore different combinations of DAAs including dual nucleotide combinations have begun. Results in a 14-day dual nucleotide proof of concept study with PSI-7977 (34) and PSI-352938 (50) appear promising. It is the hope that these studies will pave the way forward for potentially new therapeutic paradigms in the treatment of HCV which include the elimination of IFN. However, issues such as safety, tolerability and the extent of a SVR after 12 or 24 weeks of therapy will help determine which of the current and future nucleotide produgs will successfully reach the market. At this stage long term safety is still unknown and only PSI-7977 (34) has demonstrated an SVR in the clinic, but only on a small patient population. Longer term studies are underway to better define the risk:benefit profile of these agents. However, these agents provide great hope for patients suffering from HCV.

Disclosure statement

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