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Evolution of HCV NS5B Nucleoside and Nucleotide Inhibitors

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Contents

Abstract Adenosine and cytidine analogs containing the 2′-*C*-methyl substituent were identified as initial hits from screening. These compounds displayed selective anti-HCV activity in a cell-based HCV replicon assay and, as their triphosphates, inhibited HCV NS5B polymerase enzyme in a cell-free assay. Since then, a number of new 2′-modified nucleoside analogs and nucleotide derivatives were synthesized and evaluated for direct inhibition of HCV replication. Potency, selectivity, and other drug-like properties were substantially optimized, and consequently more than a dozen compounds were advanced into preclinical and clinical evaluations. In the end, a prodrug of 2′-fluoro-2′-*C*-methyluridine monophosphate PSI-7977 (GS-7977, sofosbuvir) was approved for the treatment of chronic HCV infection.

Keywords HCV, Monophosphate prodrug, NS5B, Nucleoside, Nucleotide, Phosphoramidate prodrug

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1 Background

The discovery of HCV NS5B nucleoside inhibitors began primarily with screening of existing collections of nucleosides for anti-HCV activity. Historically, nucleoside analogs and derivatives containing structurally diverse nucleobase and/or ribose moieties had been synthesized and evaluated toward a wide range of biological and therapeutic utilities. Accordingly, a library of these nucleoside analogs existed when both cell-based HCV replicon and cell-free NS5B enzyme assays were available, which enabled rapid screening and identification of initial hits.

At the time that a search for HCV inhibitors was initiated, nucleos(t)ide analogs had already demonstrated their therapeutic utility as antiviral agents for herpes viruses, hepatitis B virus, and human immunodeficiency virus. Over 20 nucleos(t) ides had been identified and developed for treatment of these viral infections worldwide. For this reason, there was a high expectation that nucleos(t)ide analogs would in turn serve as an effective agent in HCV therapy. However, those viruses encode a polymerase whose primary function is DNA synthesis, and thus the majority of nucleos(t)ide antiviral drugs to treat those infections are mimic of endogenous 2′-deoxynucleosides. Since HCV NS5B is an RNA polymerase, different structural elements in the nucleoside scaffold are likely to be required for recognition by the RNA polymerase. For example, presence of the 2′-hydroxyl group as in the ribonucleoside should offer such selective recognition over DNA polymerases (Fig. 1).

To be an effective and selective inhibitor of HCV replication, a nucleos(t)ide analog must enter the infected host cell and get metabolized by host kinases to its nucleoside triphosphate (NTP), which then must selectively bind to the active site of NS5B polymerase as a substrate and become incorporated into the elongating chain of viral RNA. Unlike naturally occurring canonical nucleosides, however, the incorporation into the nascent RNA should then block further RNA polymerization, consequently resulting in inhibition of production of functionally matured HCV RNA (i.e., HCV replication) (Fig. 2). On the other hand, this nucleos(t)ide analog and its metabolites must not adversely affect normal cellular processes such as de novo nucleoside synthesis, cellular signaling, and RNA synthesis (e.g., through being incorporated into the elongating host RNA by host RNA polymerases). Since these adverse effects to host cells could indirectly inhibit HCV replication, it is important to investigate whether the observed antiviral activity is derived from direct blockage of viral replication. Mechanistically, it is the NTP species that directly interacts with HCV NS5B and blocks generation of the matured HCV RNA.

A nucleoside analog itself is essentially a biologically inactive substance that requires the metabolic transformation to the bioactive NTP species by three distinctive kinases: ribonucleoside kinase (rNK) phosphorylates the nucleoside to the nucleoside monophosphate (NMP), nucleoside monophosphate kinase (NMPK) then phosphorylates NMP to the nucleoside diphosphate (NDP), and finally nucleoside diphosphate kinase (NDPK) phosphorylates NDP to NTP (Fig. 3). Depending on the structure of nucleoside analog, however, the efficiency of any of these

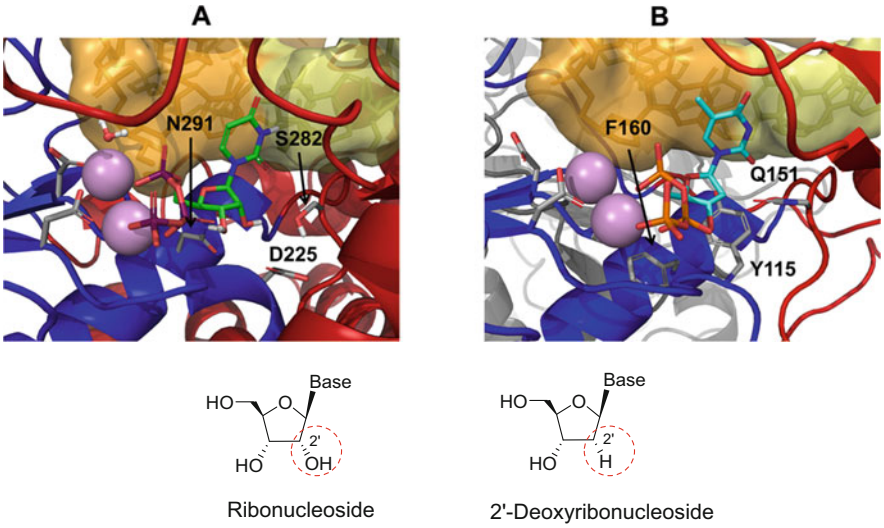

Fig. 1 RNA and DNA polymerases have different amino acid residues in the substrate-binding sites. These models show hydrophilic amino acid residues interacting with the 2′-hydroxyl group in the active site of HCV NS5B RNA polymerase (**a**) and hydrophobic amino acid residues interacting with the 2′-deoxy position in the active site of HIV RT DNA polymerase (**b**). The 2′-hydroxyl group in the ribonucleoside offers selective recognition by RNA polymerases over DNA polymerases

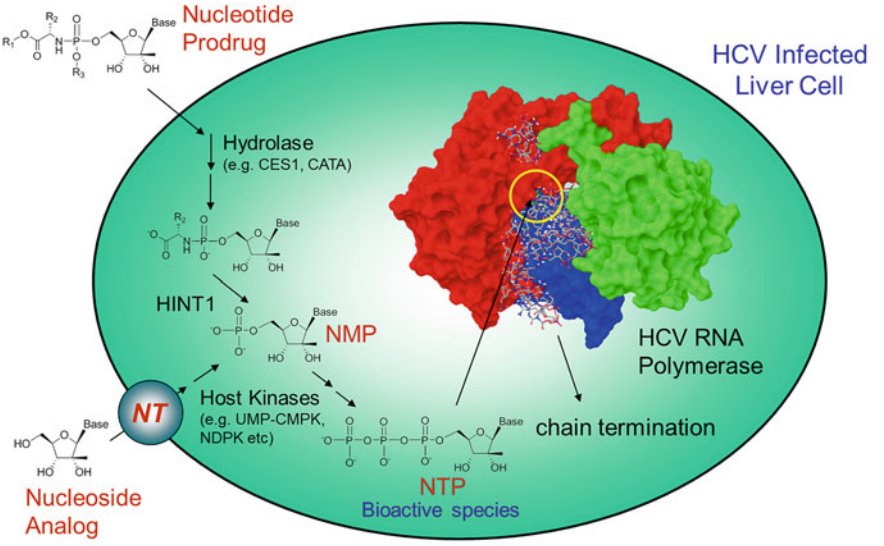

Fig. 2 Mode of action for HCV NS5B nucleos(t)ide inhibitor. Nucleoside analog or nucleotide prodrug enters the HCV-infected liver cell and is metabolized to the biologically active nucleoside triphosphate, which inhibits NS5B RNA polymerase to cause formation of degenerated HCV

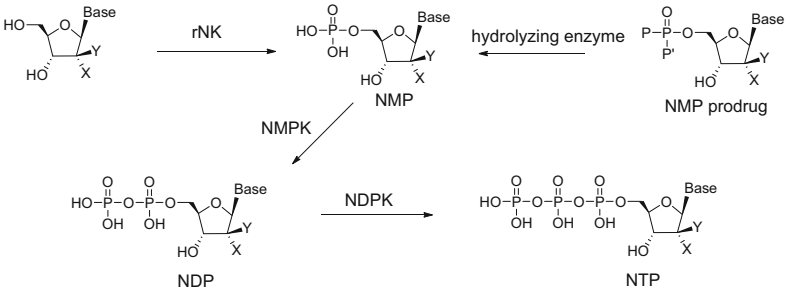

Fig. 3 A nucleoside analog is metabolized to the corresponding NTP via three distinctive kinases. NMP prodrug releases NMP, which is then transformed to NDP and NTP. *rNK* ribonucleoside kinase, *NMPK* nucleoside monophosphate kinase, *NDPK* nucleoside diphosphate kinase

enzymatic reactions may be compromised, and consequently the intracellular production of NTP may be limited. In such a case, the nucleoside analog will display a lower-than-expected antiviral activity in a cell-based assay, even if the corresponding NTP (often prepared by chemical synthesis) is found to be directly and potently active against the NS5B polymerase in a cell-free biochemical assay.

Consequently, there have been significant efforts in rescuing nucleosides that are poor substrates for the kinases yet biologically active as the triphosphates in the NS5B enzyme assay. In particular, for nucleoside analogs that are poor substrates for the first kinase in the phosphorylation cascade, monophosphate prodrug strategies have been successfully employed (Fig. 3). NMP is negatively charged at the physiological pH (i.e., pH 7.4) and thus poorly cell-permeable. However, the monophosphate prodrug that shields the charges can enter the cell and be transformed intracellularly to NMP (and subsequently to NDP and to NTP), thus bypassing the rate-limiting kinase-mediated first phosphorylation. Several prodrug tactics have been developed to efficiently deliver NMP into the target cells.

In order to achieve in vivo efficacy, sufficient levels of intracellular NTP must be maintained until viral replication is stopped and the remaining virus is cleared out from the infected cells. Unlike in vitro systems, the drug levels in the plasma of animals are dynamic and decreased over time after administration of the drug. During the transit time, a nucleos(t)ide analog present in the circulation must be taken up by the infected cells and transformed to the NTP species. The level of the intracellularly formed NTP species should then be high enough to effectively suppress the viral replication. Importantly, the intracellular half-life of the NTP should also be long enough to maintain the NTP concentration at the effective level until the nucleos(t)ide analog is re-administrated. In general, NTP is a metabolically unstable species, and its half-life varies depending on the structure of the nucleos(t)ide. Thus, designing a nucleos(t)ide analog that has a prolonged half-life as the triphosphate and meets all the requirements including potency and selectivity is challenging.

Nucleos(t)ide analogs thus meeting these requirements would have yet another hurdle to overcome – oral bioavailability. Oral administration is the most preferred

route of administration because of its simplicity and convenience. Historically, however, many nucleos(t)ide analogs suffer from poor oral bioavailability due to their high polarity and low intestinal permeability. In order to improve oral absorption of these polar drugs, a number of prodrug approaches have been explored. An ideal prodrug achieves delivery of a parent drug by attachment of a nontoxic moiety that is stable during transit but is readily cleaved to release the parent drug once reached to the target tissue. Since the hepatocytes in the liver are the major cells infected by HCV, selective loading of the parent drug into the liver over other tissues is desired. Some successful prodrug strategies employed for improved oral bioavailability and targeted liver-loading will be described in the following sections.

Finally, a major strive in direct-acting anti-HCV drug discovery and development is to achieve activity against all genotypes and high barrier of the emergence of drug-resistant variants. These are the two critically important biological properties that an antiviral agent must have to be successful in the clinic. Nucleos(t)ide analogs target the highly conserved active site of the HCV polymerase. Thus, they are expected to be equally effective across all genotypes and also have a high barrier to resistance because of poor replicative fitness of any active-site mutant variants. Accordingly, despite of a number of challenges in its drug discovery, this class has been pursued as the most attractive agent that would be a cornerstone of the treatment regimens for chronic HCV infection.

2 Early Nucleoside Inhibitors

Early on, researchers at Merck screened their own in-house collection of nucleosides for anti-HCV activity and identified two nucleoside analogs, 2′-*C*-methyladenosine (2′-*C*-MeA) and 2′-*O*-methylcytidine (2′-*O*-MeC), which inhibit HCV RNA replication (Fig. 4) [1]. In a replicon assay using a stable Huh-7 human hepatoma cell line, which supports the replication of HCV RNA and proteins, both compounds displayed antiviral activity with a 50% effective concentration (EC_{50}) of 0.25 and 21 μM, respectively. Furthermore, they were selective in that no cytotoxicity was observed up to 100 μM in HBI10A cells as measured by a MTS assay. MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] salt) is a chemical reagent to be used to quantify metabolically active cells by a colorimetric method. When incubated with actively growing replicon cells

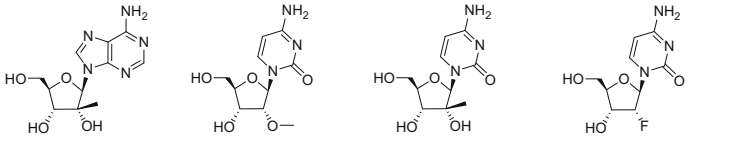

Fig. 4 Early HCV nucleoside inhibitors

in culture, these nucleoside analogs resulted in intracellular formation of the corresponding triphosphates that were potent competitive inhibitors of NS5B-catalyzed reactions in vitro, with a 50% inhibitory concentration (IC_{50}) of 1.9 and 3.8 μM (obtained with NS5BΔ21), respectively. Contrarily, the triphosphates affected the function of host cell DNA polymerases α, β, and γ only minimally. Metabolism studies with the tritium-labeled nucleosides revealed that 2′-*C*-MeA was efficiently taken up into either Huh-7 cells or HBI10A cells and converted intracellularly to the corresponding triphosphate (at 75–105 pmol/million cells upon incubation of 2 μM of the nucleoside for 23 h), while incubation with 2′-*O*-MeC yielded very little triphosphate (with intracellular concentration over 500-fold less than that produced from 2′-*C*-MeA). This explains the significantly higher inhibitory activity of 2′-*C*-MeA over 2′-*O*-MeC in the cell-based assay.

2′-*C*-Methylcytidine (2′-*C*-MeC) was also evaluated, along with 2′-*C*-MeA, which was first published by a group at Pharmasset [2]. This pyrimidine nucleoside containing the 2′-*C*-methyl substituent also actively inhibited HCV viral replication, although it was approximately tenfold less potent than 2′-*C*-MeA in a replicon assay (a 90% effective concentration EC_{90} of 10.4 vs. 1.4 μM). The structural implication of this initial result was twofold: (1) the 2′-methyl substitution on the ribose ring confers anti-HCV activity and (2) the nucleobase influences antiviral potency. This discovery prompted intense efforts toward further structural refinement of the 2′-modified nucleosides.

Several other 2′-modified nucleoside analogs were also explored for their potential as HCV NS5B inhibitors, which include 2′-deoxy-2′-fluorocytidine (2′-FdC), an analog of cytidine where the 2′-hydroxy group is replaced with the fluorine (Fig. 4) [3]. In a 4-day replicon assay using Huh-7 cells, 2′-FdC had a potent inhibitory activity with a 90% effective concentration (EC_{90}) of 5.0 μM and no cytotoxicity (50% reduction in cell growth $CC_{50} > 100$ μM). In addition, the 2′-FdC triphosphate (2′-FdCTP) showed IC_{50} of 14.9 μM in an NS5B polymerase enzyme assay. However, prolonged incubation of the HCV replicon cells with 2′-FdC (at 8 μM over 7 days) resulted in a significant reduction in the host ribosomal RNA levels, compared to the untreated control. A closer analysis of the cell cycle distribution of the treated replicon cells revealed that 2′-FdC caused cytostasis due to an S-phase arrest. These results suggest that apart from the desired inhibition of the NS5B enzyme, 2′-FdC itself or its metabolites inhibit one or more cellular targets. For example, 2′-FdCTP was shown to be incorporated into host cell DNA and RNA, which might be a cause for the observed toxicity. However, the exact mode of action for the cytostatic effect remains unknown. Regardless, an enticing postulate from this work is that a single point isosteric replacement of canonical nucleosides (e.g., from the 2′-hydroxy to the 2′-fluoro) may be insufficient to dissociate the undesired side effects on host cells from the desired antiviral activity and provide an acceptable selectivity for HCV NS5B.

Independently, a new structural class of anti-HCV active nucleosides was identified by researchers at Roche. This discovery was based on a rational design approach. A series of 4′-substitued 2′-deoxyribonucleosides had been previously investigated as selective inhibitors of HIV reverse transcriptase. These were specifically designed to

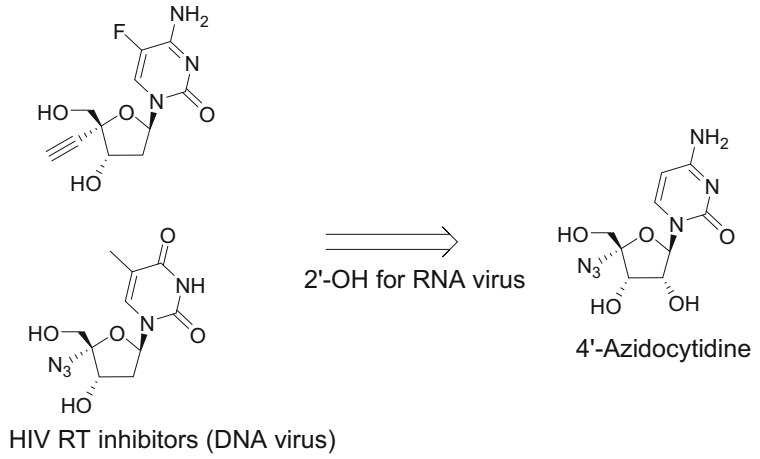

Fig. 5 Incorporation of 4′-substitutions into a ribonucleoside core for HCV activity

target DNA viruses. To build in anti-HCV activity, the 2′-hydroxy group was incorporated into these molecules. Among those synthesized and tested, 4′-azidocytidine emerged as a promising analog with an EC_{50} of 1.3 μM with no cytotoxicity up to 2,000 μM (Fig. 5) [4].

3 Advanced Nucleos(t)ide Inhibitors

3.1 Adenosine Analogs

Although 2′-*C*-MeA was shown to be a potent and selective HCV NS5B inhibitor in in vitro assays, it was far less than ideal to be developed as a therapeutic agent. Similar to naturally occurring adenosine, this compound is readily degraded by adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) present in systemic circulation, which leads to poor pharmacokinetics (PK) (Fig. 6). PK studies with rats showed rapid decrease in the plasma nucleoside levels upon intravenous administration (i.e., the plasma clearance was in excess of hepatic blood flow; >200 mL/min/kg) and no detectable drug concentration in plasma upon oral dosing (i.e., oral bioavailability of 0%). To address these issues, extensive structure-activity relationship investigation around this purine nucleoside had been conducted by a research team at Merck [5–8].

The importance of the methyl group at the 2′-*C* position for NS5B activity was reaffirmed by their work. Various ribose modifications including transfer of the methyl group to the 3′-*C* position or the 2′-O position (i.e., 2′-methoxy), incorporation of steric bulk from the methyl to the ethyl, and transposition of the stereochemistry at the 2′ position (i.e., from α-hydroxy-β-methyl to β-hydroxy-α-methyl)

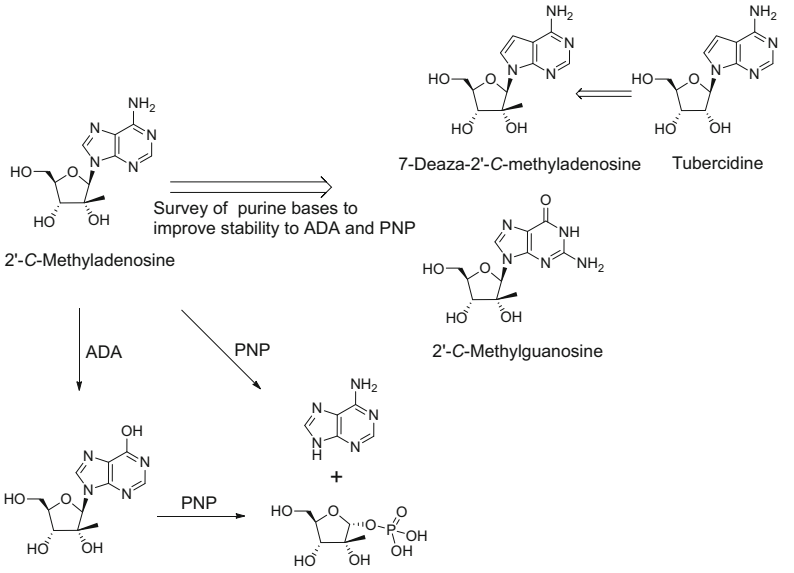

Fig. 6 7-Deaza-2′-*C*-methyladenosine and 2′-*C*-methylguanosine identified from survey of a number of purine nucleoside analogs toward achieving stability to adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP)

resulted in loss of activity. While the SAR of the ribose moiety was stringent, some flexibility was allowed for modification of the purine nucleobase moiety. Most of the nucleobases that had previously demonstrated efficient cellular uptake and conversion to the triphosphate in the context of the ribonucleoside core similarly conferred anti-HCV activity. Among those tested, two nucleosides, 7-deaza-2′-*C*-methyladenosine (7-deaza-2′-*C*-MeA) and 2′-*C*-methylguanosine (2′-*C*-MeG, discussed in detail at the following section), had emerged as new leads that exhibited antiviral activity with complete stability to ADA and PNP (Fig. 6).

In addition to providing stability to the enzymatic degradations, 7-deaza-2′-*C*-MeA exhibited potent antiviral activity comparable to 2′-*C*-MeA in the replicon assay (EC_{50} 0.3 vs. 0.25 μM). In a NS5B enzyme assay, 7-deaza-2′-*C*-methyladenosine triphosphate (7-deaza-2′-*C*-MeATP) was 17-fold more potent than 2′-*C*-methyladenosine triphosphate (2′-*C*-MeATP) (IC_{50} 0.11 vs. 1.8 μM). Furthermore, no cellular toxicity was observed with 7-deaza-2′-*C*-MeA in a MTS assay using the Huh-7 cells for up to 72 h at a 100 μM concentration. Given that the des-methyl analog tubercidin (7-deaza-adenosine, Fig. 6) is known to be highly cytotoxic, the lack of observed cytotoxicity by introduction of 2′-*C*-methyl group was remarkable. By comparison, tubercidin displayed high cytotoxicity (CC_{50} of 3 μM at 24 h, 0.15 μM at 72 h) in the same MTS assay. Additional studies were conducted to substantiate the observation. Again, no cytotoxicity was observed at up to a 1,000 μM concentration of 7-deaza-2′-*C*-MeA in both a MTS assay using

more sensitive Jurkat cells and a [^{14}C]thymidine uptake assay using Huh-7 cells, while tubercidin exhibited cytotoxicity at submicromolar concentrations.

One concern with noncanonical nucleobases such as 7-deaza-2′-*C*-MeA is the possibility for induction of mutagenic events. However, the 2′-*C*-methyl pharmacophore apparently prevented recognition by human polymerases, which may permit the use of nucleobases that otherwise would be unsuitable. Incubation of the tritium-labeled 7-deaza-2′-*C*-MeA with Huh-7 cells for 3 days showed undetectable to trace levels of incorporation of the radioactive nucleoside into cellular RNA, which provided further evidence that this nucleoside triphosphate was very specific for incorporation by the HCV NS5B polymerase.

Because of the promising antiviral and selectivity profile, 7-deaza-2′-*C*-MeA was advanced into preclinical in vivo studies. The compound exhibited a good oral bioavailability (>50%) in male beagle dogs, Sprague-Dawley rats, and rhesus macaques. The rate of compound clearance in the circulatory system was moderate in all three animal species tested (14, 1.6, 9.0 mL/min/kg, respectively). The levels of the NTP in the liver were about 100-fold higher than the plasma levels of the parent nucleoside; oral doses at 2, 20, and 200 mg/kg to male Sprague-Dawley rats resulted in the liver NTP concentrations of 12, 92, 507 μmol/kg and the plasma nucleoside exposure of 0.1, 2.0, 4.8 μM at 8 h of post-dose, respectively. The oral 50% lethal dose (LD_{50}) to female mice was greater than 2,000 mg/kg. No deaths or other treatment-related physical signs were seen during a post-dose 14-day observation period. For comparison, the mice dosed with tubercidin at 50 mg/kg died within 6 days.

To determine an antiviral efficacy in vivo, 7-deaza-2′-*C*-MeA was administered to HCV-infected chimpanzees, which resulted in dose- and time-dependent decreases in plasma viral load. In one experiment, HCV-infected chimpanzees dosed for 7 days at 0.2 and 2 mg/kg/day by intravenous administration experienced average reductions in viral load of 1.0 and >5 $\log_{10}$ IU/mL, respectively. Separately, two HCV-infected chimpanzees received daily doses of 1 mg/kg via oral administration. After 37 days of the oral dosing, one chimpanzee with a high starting viral load experienced a reduction in viral load of 4.6 $\log_{10}$, and the viral load in the other chimpanzee fell below the limit of quantification (LOQ). Importantly, viral load remained below the LOQ throughout the duration of dosing and for at least 12 days after dosing ended. The results demonstrated a robust in vivo antiviral activity of 7-deaza-2′-*C*-MeA, justifying advancement into human clinical trials (as MK-608). Despite promising results in animal studies, however, MK-608 was discontinued for undisclosed reasons after entering phase I clinical trial.

Several additional adenosine analogs were also reported to be active against HCV (Fig. 7). Further substituents on the 7 position of 7-deaza-2′-*C*-MeA (such as **7a** and **7b**), replacement of the 2′-hydroxy to the 2′-fluoro group (**7c**), and application of the *C*-nucleoside scaffold (such as **7d**-**7f**) afforded varied potency and selectivity. However, there have been no further developments on these adenosine analogs [9–14].

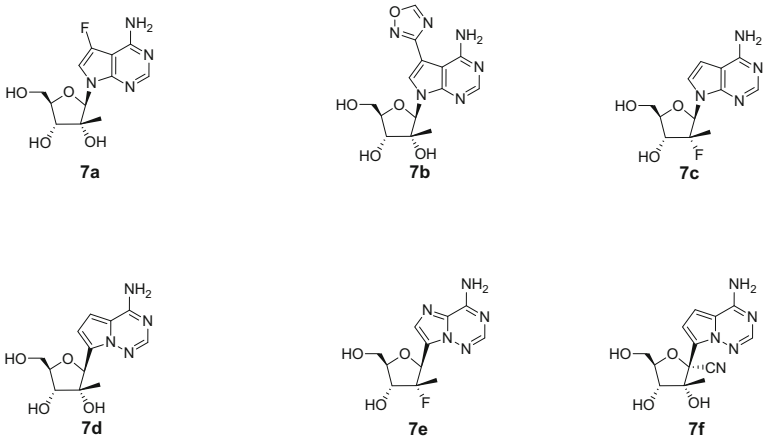

Fig. 7 Adenosine analogs with further structural variations

3.2 Guanosine Analogs

Researchers at Merck reported for the first time an analog of the guanosine series containing the signature 2′-C-methyl substituent, 2′-*C*-methylguanosine (2′-*C*-MeG) [5]. This compound exhibited a potent anti-HCV activity with EC_{50} of 3.1 μM in a cellular assay (Fig. 8). Interestingly, the corresponding NTP was exceptionally potent at inhibiting HCV NS5B enzyme activity in a cell-free assay (IC_{50} 0.13 μM). By comparison, 2′-*C*-MeA had EC_{50} of 0.26 μM and IC_{50} of 1.9 μM. The ratio of EC_{50} to IC_{50} for 2′-*C*-MeG is ~25, while that for 2′-*C*-MeA is ~0.15. This large difference was striking at a glance, but was readily explained by intracellular levels of the biologically active NTPs. The intracellular level of NTP upon incubation of 2′-*C*-MeG in the Huh-7 replicon cells were ~500-fold lower than that from 2′-*C*-MeA. This result suggested that 2′-*C*-MeG was poorly taken up by the cells and/or inefficiently metabolized to its NTP, which limits intracellular level of 2′-*C*-MeGTP, resulting in the less-than-expected EC_{50} (Fig. 8).

With this information in hand, a series of monophosphate prodrug approaches were undertaken to increase cell-based activity against HCV (Fig. 8). McGuigan and his team investigated a phosphoramidate prodrug strategy and found that L-alanine 1-naphthyl phosphoramidate prodrugs (e.g., **8-1**) of 2′-*C*-MeG afforded a significant increase in antiviral activity (EC_{50} down to 0.28 μM) with no cytotoxicity up to 50 μM [15]. This result suggested that the first phosphorylation step of 2′-*C*-MeG was rate-limiting and the subsequent phosphorylation steps leading to the NTP were well proceeded. In addition, this particular phosphoramidate prodrug enabled an efficient delivery of 2′-*C*-MeG monophosphate (2′-*C*-MeGMP) into the host cells and circumvented the limitation of the first phosphorylation step. In contrast, the adenosine prodrug counterpart (**8-2**) did not show any increase in activity (EC_{50} from 0.26 to 0.24 μM). This is because the cellular uptake and intracellular

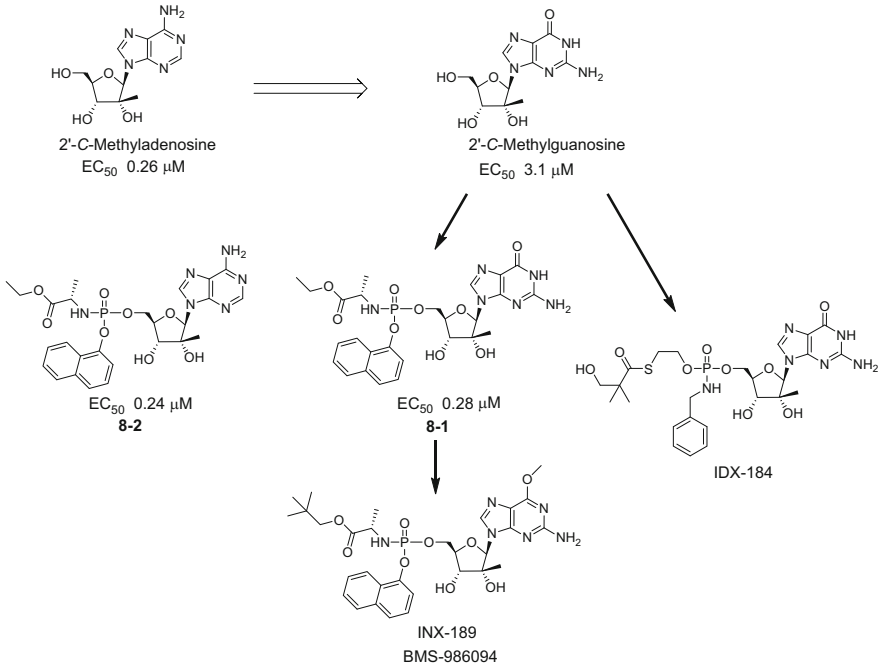

Fig. 8 Discovery of 2′-*C*-methyl guanosine and its monophosphate prodrugs

phosphorylation of the parent nucleoside 2′-*C*-MeA is already efficient so that the prodrug approach is not beneficial for NTP loading into the cells.

Continued efforts in this approach ultimately led to identification of INX-189 (Fig. 8), which is a double prodrug where a methyl group is added on the 6-*O* position of the nucleobase that is hydrolyzed upon entry to the host cells [16]. This modification was necessary to further increase the lipophilicity of the molecule for enhanced cellular permeability. A number of 6-*O*-methyl 2′-*C*-MeG prodrugs with various amino acid ester promoieties were prepared and evaluated, and a prodrug with L-alanine *neo*-pentyl ester was found to be optimal. INX-189 was substantially more potent than the earlier prodrug **8.1** (EC_{50} 0.013 μM vs. 0.28 μM) with an in vitro therapeutic index of >500 (CC_{50} 7.0 μM). Intracellular 2′-*C*-MeGTP concentration when incubated with this prodrug at EC_{90} of 40 nM in the replicon cells was 2.4 pmol/million cells (i.e., ~240 pmol/g of liver tissue), and intracellular half-life of the triphosphate was ~24 h. Subsequently, in vivo pharmacokinetic studies were conducted to determine if liver 2′-*C*-MeGTP levels required for the 90% viral inhibition could be achieved at reasonable doses. At oral doses of ≥5 mg/kg in rats, the concentrations of 2′-*C*-MeGTP in the liver exceeded the EC_{90} soon after dosing and remained at or above the level for 72 h. Similarly, the EC_{90} level in the liver was achieved at 25 mg/kg oral dose in cynomolgus monkeys.

Mitochondrial toxicity has been observed with certain classes of nucleoside analog drugs, which was ascribed to inhibition of the mitochondrial DNA polymerase. This unwanted side effect has been well documented in the case of reverse transcriptase inhibitors used to treat HIV infection. The potential liability of INX-189 with regard to mitochondrial toxicity therefore was assessed in tissue culture studies with both a liver-derived cell line (HepG2) and a lymphocyte cell line (CEM). Treatment of the compound for 3 or 14 days produced no change in the ratio of mitochondrial genome copy number to cellular DNA, which was indicative of a lack of mitochondrion-specific toxicity [17].

INX-189 (later known as BMS-986094) was subsequently advanced to clinical development. The compound was shown to be effective and well tolerated in the early phase of trials. In a phase 1b trial, significant antiviral effects were observed in the 9, 25, 50, and 100 mg cohorts with median HCV RNA reduction at day 7 by 0.64, 1.0, 1.47, and 2.53 $\log_{10}$ IU/mL, respectively, compared with -0.20 $\log_{10}$ IU/mL in placebo patients. In an expanded phase Ib trial with higher dosages (once-a-day 200 mg) as monotherapy or in combination (once-a-day 100 mg) with ribavirin for 7 days, BMS-986094 demonstrated potent and dose-dependent antiviral activity with a median HCV RNA reduction from baseline of 4.25 $\log_{10}$ IU/mL.

During a phase II trial with a longer duration of the drug treatment, however, a serious safety issue was identified. A 20-year-old male treated with 200 mg dose experienced rapidly progressive heart failure and died. Additionally, 14 of 34 patients treated with BMS-986094 longer than a week had some evidence of cardiac dysfunction. BMS-986094 also had an adverse impact on the kidney, with most of the patients having increases in serum creatinine levels regardless of the degree of systolic dysfunction. The patient who died developed acute renal failure, while another required temporary hemodialysis. However, the serum creatinine levels of all other patients were improved once the drug was stopped.

To understand these finding better, a series of in vitro and in vivo investigative studies were conducted [18]. Independent researchers reported that 2′-*C*-MeGTP was rather efficiently incorporated by mitochondrial RNA polymerase and BMS-986094 induced functional reduction of mitochondrial respiration in a cultured cell line and in freshly isolated rat cardiomyocytes at clinically relevant concentrations, which contradicts the earlier assessment. Intriguingly, tissue analysis from cynomolgus monkey PK studies revealed relatively high concentrations and slow clearance of the active metabolite 2′-*C*-MeGTP in the heart and kidney, which may be associated with the observed cardiac and renal toxicity in the clinical trials. However, the link between the tissue accumulation of the metabolite and the clinically observed toxicity remains to be further investigated.

Another 2′-*C*-MeG monophosphate prodrug, IDX-184 (Fig. 8), also had an improved antiviral activity in a replicon assay (EC_{50} 0.3–0.45 μM) and in a JFH-1 infectious system (EC_{50} 0.06–0.11 μM), as compared to the parent 2′-*C*-MeG [19, 20]. In vitro metabolism experiments indicated that IDX-184 was efficient at delivering the monophosphate in the replicon cells with a boosted level of intracellular 2′-*C*-MeGTP (~100-fold), corroborated with the increased antiviral activity in the cell-based assay. In particular, IDX-184 was predominantly metabolized in the

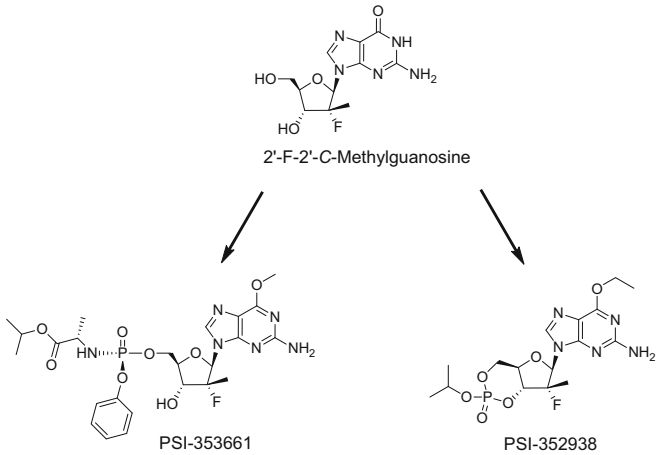

Fig. 9 Discovery of 2′-fluoro-2′-*C*-methylguanosine and its monophosphate prodrugs

liver via both cytochrome P (CYP)-dependent and cytochrome P (CYP)-independent processes, opening up the possibility of a liver-targeted delivery that could enhance the therapeutic margin by limiting potential off-target tissue toxicity. Consistent with the in vitro results, oral pharmacokinetic studies with IDX-184 in rats and monkeys showed very high extraction (approximately 95%) by the liver, with low plasma levels of the parent 2′-*C*-MeG, suggesting preferential loading of 2′-*C*-MeGTP into the liver over other tissues. IDX-184 was progressed to clinical trial, demonstrating efficacy in early trials. However, the clinical development was discontinued due to a concern of toxicity.

A guanosine analog with 2′-fluoro and 2′-C-methyl groups was only weakly active in a HCV replicon assay (EC_{90} 69 μM), but its triphosphate was potent at inhibiting HCV NS5B polymerase enzyme (IC_{50} 5.9 μM). Monophosphate prodrug approaches were applied, and more than 1,000-fold increased potency was achieved. PSI-353661 and PSI-352938 were chosen for clinical development, but their clinical evaluations were put on hold, presumably based on toxicity findings from nonclinical safety studies (Fig. 9) [21, 22].

3.3 Cytidine Analogs

The first pyrimidine analog discovered to be a selective inhibitor of HCV replication was 2′-*C*-methylcytidine (2′-*C*-MeC). Similar to a majority of other nucleosides, the compound was not sufficiently orally bioavailable, which hampered further development. To overcome the limitation, 3′-*O*-valinyl ester prodrug of 2′-*C*-MeC (NM283, valopicitabine) was devised (Fig. 10) [23, 24]. This prodrug utilized an active intestinal absorption via a peptide transport mechanism, which had been a

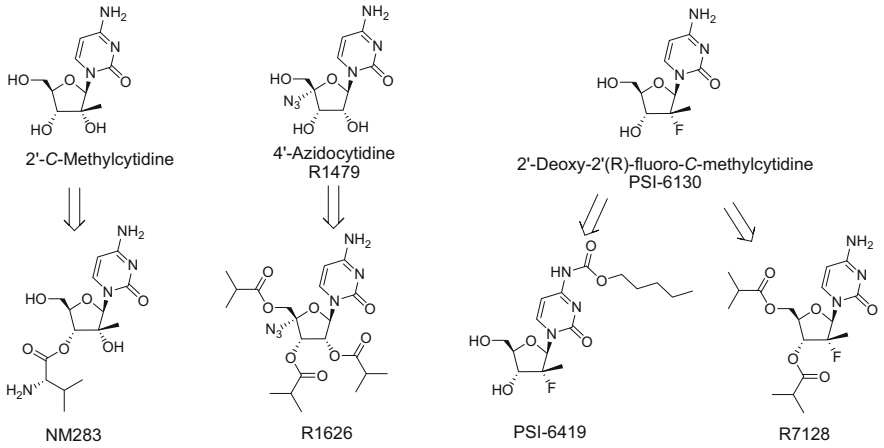

Fig. 10 Ester prodrugs of cytidine analogs for increased oral bioavailability

successful strategy employed in several nucleoside analogs. The valinyl ester prodrug was highly soluble (423 g/L in water as the dihydrochloride salt), stable in acidic media (e.g., half-life of 6 days at pH 4.5), and rapidly converted into the parent 2′-*C*-MeC in both human plasma and whole blood, which are all desirable attributes for an oral prodrug. As expected, the apparent bioavailability of 2′-*C*-MeC following oral administration of NM283 was greatly improved (34%F in rats). The prodrug also exhibited a good PK profile in monkeys and demonstrated an antiviral efficacy in the HCV-infected chimpanzee. NM283 was eventually progressed to clinical development. In a monotherapy study with HCV-infected patients, HCV viral load reductions of 0.15–1.21 $\log_{10}$ IU/mL were observed at 2 weeks with 50–800 mg/day of NM283 in a dose-dependent manner. High doses (e.g., 800 mg/day) were necessary to achieve a clinically meaningful efficacy. However, NM283 was associated with dose-limiting gastrointestinal disturbances, nausea, and vomiting, with higher prevalence in cohorts receiving doses of 400 mg/day or above. Eventually, the development of NM283 was discontinued based on a conclusion that the benefits of NM283 did not outweigh the gastrointestinal toxicities [25].

Another pyrimidine nucleoside 4′-azidocytidine (R1479) was also advanced to clinical evaluation (Fig. 10) [26]. R1479 had a potent anti-HCV activity (EC_{50} of 1.3 μM and IC_{50} of 0.67 μM) with no cytotoxic or cytostatic effects. Dose-dependent viral load reduction was evident, but observed plasma exposure of R1479 was low due to poor oral absorption. Accordingly, a prodrug campaign was undertaken. Several prodrug strategies including appendages of liver-targeted promoieties (e.g., N^6-alkyloxycarbonyl as in capecitabine), peptide transporter-targeted promoieties, and lipophilic carboxylic acid ester promoieties were investigated. Over 100 structurally diverse compounds covering a range of clogP were synthesized and evaluated for their stability, permeability, and oral bioavailability in cynomolgus monkeys. Among them, R1626 (balapiravir) (Fig. 10), a tri-isobutyl ester prodrug of R1479, was found

to have optimal properties with increased oral bioavailability and improved in vitro antiviral activity. This compound was then progressed to clinical trials. In a phase 1b study, treatment-naïve patients infected with HCV genotype 1 were treated with R1626 orally at doses of 500 mg, 1,500 mg, 3,000 mg, or 4,500 mg or placebo twice daily (BID) for 14 days with 14 days of follow-up. Doses up to and including 3,000 mg BID were well tolerated in the study. However, there was an increase in frequency of adverse events (e.g., neutropenia) at the highest dose (4,500 mg). R1626 was efficiently converted to R1479, with dose-proportional pharmacokinetics observed over the entire dose range. Dose-dependent and time-dependent reductions in HCV RNA were observed. Mean decreases in viral load after 14 days of treatment with doses of 500, 1,500, 3,000, and 4,500 mg were 0.32, 1.2, 2.6, and 3.7 $\log_{10}$ IU/mL, respectively. However, the compound was not progressed further likely due to the insufficient efficacy.

The earlier hit 2′-deoxy-2′-fluorocytidine (2′-FdC) was followed up by a research group at Pharmasset. Further structural modification of the sugar ring, namely, addition of the key 2′-*C*-Me moiety, led to the identification of 2′-deoxy-2′(R)-fluoro-2′-*C*-methylcytidine (PSI-6130) [27–29]. This change maintained the potent anti-HCV activity and yet afforded much improved selectivity. PSI-6130 showed EC_{90} of 4.6 μM in a HCV replicon assay, but little or no activity against bovine diarrhea virus and other flaviviruses, indicating that it was a highly selective HCV inhibitor. PSI-6130 is efficiently phosphorylated to the corresponding NTP in the cells. The steady-state inhibition constant (K_i) for PSI-6130 triphosphate with the wild-type NS5B enzyme was 4.3 μM, compared well with those of 2′-*C*-MeCTP (K_i 1.6 μM) and 2′-*C*-MeATP (K_i 1.5 μM). Furthermore, PSI-6130 showed little or no cytotoxicity/cytostasis against a variety of cell types and no mitochondrial toxicity. Incubation of the compound in monkey and human whole blood revealed deamination leading to formation of a metabolite 2′-deoxy-2′(R)-fluoro-2′-methyluracil (PSI-6206) which had no anti-HCV activity on its own. This biologically inactive metabolite was also detected in vivo. PK studies in rhesus monkeys revealed significant metabolism upon oral administration of PSI-6130. The oral bioavailability of PSI-6130 was 24%, but the total bioavailability including the parent drug PSI-6130 and the deaminated metabolite PSI-6206 was 64%. In addition, the plasma exposure of PSI-6206 following administration by the oral route was three- to fourfold higher than for the intravenous route, suggesting that deamination likely took place predominantly during first-pass metabolism in the liver or the gastrointestinal lining. PSI-6130 was advanced into clinical evaluation. In a phase I study with healthy male volunteers, single oral doses of PSI-6130 were generally well tolerated, and no serious adverse events were seen with doses up to 3,000 mg [30].

To improve oral absorption and drug exposure while suppressing formation of the uridine metabolite, a prodrug campaign was undertaken with PSI-6130. PSI-6419 (N^4-pentyloxycarbonyl PSI-6130) was designed inspired by capecitabine, an orally bioavailable nucleoside analog prodrug. However, this prodrug did not yield high levels of PSI-6130 in serum, when administered orally to cynomolgus monkeys. Additional prodrug approaches were then investigated, which resulted in discovery of

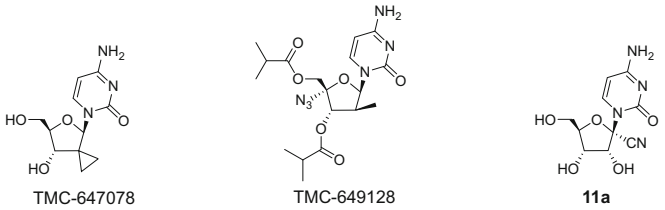

Fig. 11 Additional cytidine analogs for anti-HCV activity

R7128 (3′,5′-diisobutyrate prodrug of PSI-6130) with an improved oral bioavailability. R-7128 (mericitabine) (Fig. 10) was progressed to clinical development [30]. In a phase I dose range finding PK study in healthy subjects, single oral doses of R-7128 (500–9,000 mg) revealed efficient delivery of PSI-6130. While plasma exposure to the prodrug R-7128 was negligible, plasma levels of the parent PSI-6130 and the uridine metabolite PSI-6206 were increased dose proportionally. At dose of 1,500 mg, for example, the C_{max} for PSI-6130 was 7.5 μg/mL, median t_{max} 2.0 h, $t_{1/2}$ 5.6 h, and $AUC_{(0\text{-inf})}$ 59 μg h/mL. In subjects administered a dose of 1,500 mg with food, the respective C_{max}, median t_{max}, $t_{1/2}$, and $AUC_{(0\text{-inf})}$ were 8.9 μg/mL, 3.0 h, 5.2 h, and 71 μg h/mL. In 14-day monotherapy studies in patients with genotype 1 HCV infection, a 1,500 mg BID dose afforded the mean decline in HCV RNA of 2.7 $\log_{10}$ IU/mL with the range of the decline being 1.2–4.2 $\log_{10}$ IU/mL after completion of the treatment. Once-a-day (QD) administrations of 750 mg and 1,500 mg were less effective, resulting in viral load reduction by 2.1 and 1.5 $\log_{10}$ IU/mL, respectively. R7128 was moved to phase II studies with longer treatment duration as well as a combination regimen with PEG-IFN-α and ribavirin. However, those studies showed no clear clinical benefits, and no further clinical development had been reported since then.

Some additional cytidine analogs with modifications of the ribose ring such as TMC-647078, TMC-649128, and **11a** were identified as anti-HCV inhibitors (Fig. 11). These new cytidine analogs exhibited no advantages over the previous analogs with respect to potency and selectivity [31–33].

3.4 Uridine Analogs

Metabolism studies with PSI-6130 in primary human hepatocytes revealed multiple phosphorylated metabolites (Fig. 12). In addition to PSI-6130 and its 5′-phosphorylated derivatives as predicted, the deaminated derivative of PSI-6130 (PSI-6206) and its corresponding phosphorylated metabolites were present in the PSI-6130-treated hepatocytes [34]. Both triphosphate species are enzymatically active (IC_{50} of 0.13 μM for the CTP and 0.52 μM for the UTP). Interestingly, both species were formed equally well, but an intracellular half-life of the UTP was much longer than that of the CTP (38 h vs. 4.7 h). 2′-F-2′-*C*-MeU (PSI-6206) was

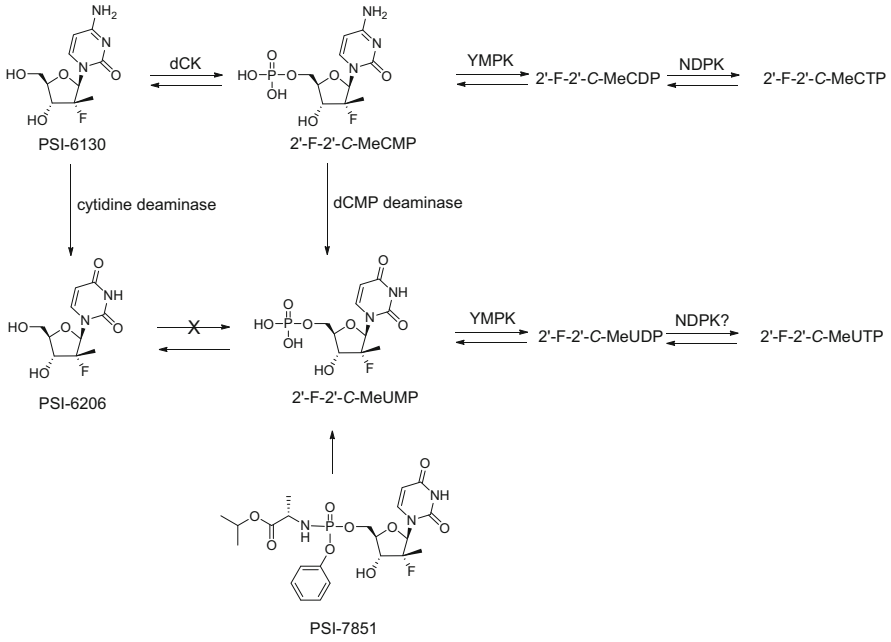

Fig. 12 Metabolic pathway of PSI-1630 and discovery of PSI-7851

intrinsically inactive in a cell-based HCV replicon assay and found to be not metabolized to its phosphorylated species. Therefore, 2′-F-2′-*C*-MeUMP present in the human hepatocytes was most likely formed through the deamination of 2′-F-2′-*C*-MeCMP by the cellular dCMP deaminase and subsequently further phosphorylated to the corresponding UDP and UTP. Accordingly, the monophosphate strategy was applied, which ultimately led to the discovery of PSI-7851 [35, 36].

PSI-7851 was highly potent in a HCV replicon assay (EC_{90} 0.52 μM) and, when incubated in primary human hepatocytes, produced intracellularly a significant level of the biologically active 2′-F-2′-*C*-MeUTP (~500 μM after 48 h of incubation with 100 μM of PSI-7851). The compound showed essentially no toxicity when tested in expanded cell lines including Huh7, HepG2, BxBC3, and CEM. In addition, no inhibition of mitochondrial DNA synthesis up to the highest concentration tested (50 μM) was observed. Oral administration of PSI-7851 in dogs and cynomolgus monkeys showed an adequate level of the plasma exposure of the prodrug PSI-7851 and the liver exposure of 2′-F-2′-*C*-MeUTP. Nonclinical single-dose acute toxicity studies determined a no-observed-adverse-effect-level (NOAEL) of >1,800 mg/kg. PSI-7851 was then advanced to clinical development and demonstrated a promising efficacy in chronic HCV-infected patients. Once-a-day oral administration of 400 mg of PSI-7851 afforded the mean decline in HCV RNA of 2.0 $\log_{10}$ IU/mL at day 3. By comparison, twice daily administration of 1,500 mg of R7128 resulted in the decline in HCV RNA of only 1.0 $\log_{10}$ IU/mL. Since PSI-7851 was a mixture

of diastereomers at the phosphorous center of the phosphoramidate moiety, it was not ideal for drug development. Thus, a single stereoisomer from the mixture, PSI-7977 (GS-7977, sofosbuvir), was eventually selected for further development. A full account of discovery and development of sofosbuvir will be discussed in the ensuing chapter.

As a result of the discovery of PSI-7851 and PSI-7977 exhibiting impressive efficacy and tolerability in the clinical setting, prodrugs of other uridine nucleosides were investigated. Unlike 2′-*C*-MeC, the 2′-*C*-methyl-substituted uridine (2′-*C*-MeU) was only weakly active in a HCV replicon assay, while its triphosphate 2′-*C*-MeUTP was equally potent as 2′-*C*-MeCTP in a NS5B enzyme assay. Therefore, monophosphate prodrug approaches were again investigated to improve potency, which resulted in discovery of a series of compounds with submicromolar EC_{50} in replicon assays (Fig. 13). Researchers at Alios BioPharma has explored thiomonophosphate prodrugs of various 2′-*C*-methyl-substituted nucleosides for anti-HCV activity and identified a uridine thiophosphate prodrug ALS-2200 [37]. The compound had a potent activity in a replicon assay ($EC_{50} < 1$ μM) and its thiotriphosphate (thio-NTP) had enzymatic activity comparable to the oxo-NTP counterpart. Incubation of the prodrug in plated human hepatocytes produced the thio-NTP intracellularly, suggesting that the observed replicon activity comes from the thio-NTP. Because the sulfur atom is introduced, two diastereoisomeric thio-NTPs can be possibly formed. Interestingly, however, only one isomer was found from the metabolism study. ALS-2200 is a mixture of the two diastereoisomers at the phosphorus, and one single diastereoisomer, VX-135 (absolute stereochemistry information not available), was advanced to clinical development. Although VX-135 demonstrated efficacy at reducing viral loads in HCV-infected patients, it

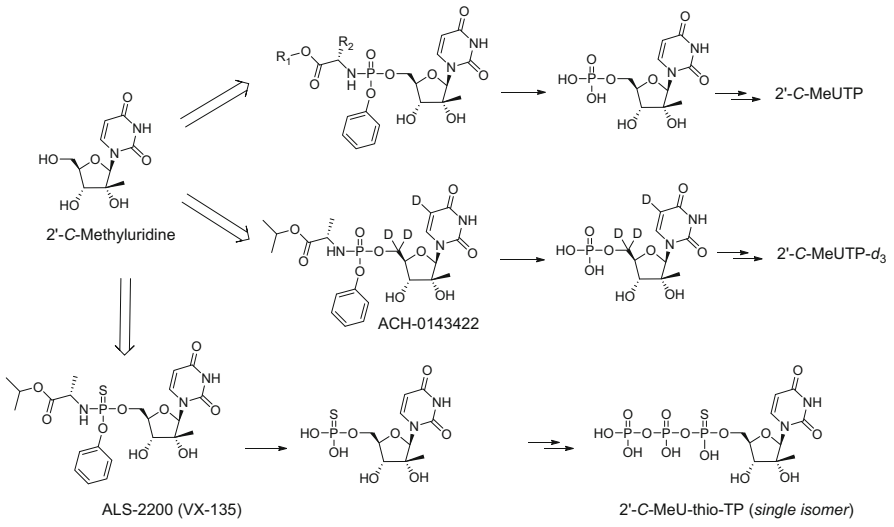

Fig. 13 Uridine monophosphate prodrugs

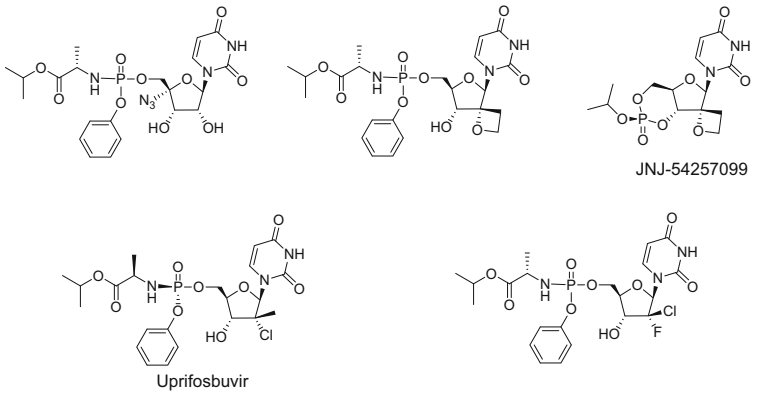

Fig. 14 Ribose-modified uridine monophosphate prodrugs for anti-HCV activity

showed liver toxicity in some patients who received a 400 mg dose. Lowering dose could avoid toxicity at the expense of reduced or compromised efficacy. For this reason, further clinical development was stopped. A research team at Achillion Pharmaceuticals investigated a prodrug of deuterated 2′-*C*-MeU, ACH-0143422, where the 5′-deuterium had a significant positive effect on in vitro metabolism [38]. The 5′-deuterium increased the stability toward dephosphorylation of the 5′-monophosphate, thereby increasing the bioactive triphosphate pool, which might result in increased efficacy in the clinic. ACH-0143422 entered clinical studies. However, it did not progress beyond phase II clinical studies.

Independently, a number of structurally related uridine derivatives were evaluated for anti-HCV activity (Fig. 14) [39–44]. Various modifications on the ribose ring were tolerated, and all these uridine analogs required monophosphate prodrug approaches for cellular activity.

4 Conclusion

Prior to initiation of discovery campaigns for anti-HCV agents, a track record of success in identifying and developing nucleoside and nucleotide drugs to treat other viral infections had been achieved, and a significant knowledge on chemistry, biology, pharmacology, and toxicology around this class of compounds had been accumulated. Accordingly, the search for anti-HCV agents appeared straightforward at first. The previous antiviral agents were all structural mimic of endogenous 2′-deoxynucleosides targeting DNA polymerases. Since HCV NS5B is an RNA polymerase, however, new structural elements had to be incorporated for selective recognition by the RNA polymerase. In 2003, it was reported for the first time that 2′-modified nucleoside analogs had selective anti-HCV activity. Since then, a number of new structural modifications were made in order to find a compound

with optimal properties such as potency, selectivity, and oral bioavailability. More than a dozen compounds were advanced into clinical trials. All these compounds but one were unsuccessful in advancing further due to insufficient antiviral efficacy or unacceptable drug-associated toxicity. A prodrug of 2′-fluoro-2′-*C*-methyluridine monophosphate PSI-7977 (GS-7977, sofosbuvir) was approved in 2013 by the US Food and Drug Administration and became a cornerstone of current treatment regimens for chronic HCV infection.

Compliance with Ethical Standards

Conflict of Interest The author is an employee of Gilead Sciences, Inc.

Ethical Approval This is a review article that does not contain any studies with human participants or animals performed by the author.

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