



Review

Chutes and ladders in hepatitis C nucleoside drug development[☆]

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ABSTRACT

Chutes and Ladders is an exciting up-and-down-again game in which players race to be the first to the top of the board. Along the way, they will find ladders to help them advance, and chutes that will cause them to move backwards. The development of nucleoside analogs for clinical treatment of hepatitis C presents a similar scenario in which taking shortcuts may help quickly advance a program, but there is always a tremendous risk of being sent backwards as one competes for the finish line. In recent years the treatment options for chronic hepatitis C virus (HCV) infection have expanded due to the development of a replicon based *in vitro* evaluation system, allowing for the identification of multiple drugable viral targets along with a concerted and substantial drug discovery effort. Three major drug targets have reached clinical study for chronic HCV infection: the NS3/4A serine protease, the large phosphoprotein NS5A, and the NS5B RNA-dependent RNA polymerase. Recently, two oral HCV protease inhibitors were approved by the FDA and were the first direct acting anti-HCV agents to result from the substantial research in this area. There are currently many new chemical entities from several different target classes that are being evaluated worldwide in clinical trials for their effectiveness at achieving a sustained virologic response (SVR) (Pham et al., 2004; Radkowski et al., 2005). Clearly the goal is to develop therapies leading to a cure that are safe, widely accessible and available, and effective against all HCV genotypes (GT), and all stages of the disease. Nucleoside analogs that target the HCV NS5B polymerase that have reached human clinical trials is the focus of this review as they have demonstrated significant advantages in the clinic with broader activity against the various HCV GT and a higher barrier to the development of resistant viruses when compared to all other classes of HCV inhibitors.

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Contents

1. Introduction	121
2. Valopicitabine (NM283): the first clinical HCV nucleoside	123
2.1. Preclinical development	123
2.2. Synthesis of valopicitabine (NM283)	123
2.3. Clinical development	123
2.3.1. Phase I/II	123
2.3.2. Phase IIa	124
2.3.3. Phase IIb	124
2.3.4. Phase IIb	124
2.4. Conclusions	124

[☆] http://en.wikipedia.org/wiki/Snakes_%26_Ladders.

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3.	R1479 and its prodrug R1626 : balapiravir	124
3.1.	Preclinical development	124
3.2.	Synthesis of R1479 and its prodrug balapiravir (R1626)	125
3.3.	Clinical development.	125
3.3.1.	Phase I.	125
3.3.2.	Phase Ib.	125
3.3.3.	Phase IIa	125
3.3.4.	Phase IIb	126
3.4.	Conclusions	126
4.	RG7348 (MB11362)	126
4.1.	Preclinical development	126
4.2.	Clinical development.	126
4.2.1.	Phase I.	126
4.3.	Conclusions	126
5.	PSI-6130	126
5.1.	Chemical synthesis	126
5.2.	Preclinical development	127
5.3.	Clinical development.	127
5.4.	Conclusions	127
6.	RG7128 (R7128, RO5024048, mericitabine)	127
6.1.	Preclinical development and chemical synthesis	127
6.2.	Clinical development.	128
6.2.1.	Phase I.	128
6.2.2.	Phase IIb	128
6.3.	Conclusions	129
7.	IDX184	129
7.1.	Synthesis of IDX184	129
7.2.	Preclinical development	129
7.3.	Clinical development.	130
7.3.1.	Phase Ia	130
7.3.2.	Phase Ib.	130
7.3.3.	Phase IIa	130
7.3.4.	Phase I combination of IDX184 and IDX320	131
7.3.5.	Phase I.	131
7.3.6.	Phase IIb	131
7.4.	Conclusions	131
8.	PSI-6206 to PSI-7851 to GS-7977 (PSI-7977; sofosbuvir)	131
8.1.	Discovery and preclinical profiling.	131
8.2.	Synthesis of PSI-7851 and PSI-7977 (GS-7977)	132
8.3.	Synthesis of single isomer PSI-7977 (sofosbuvir, GS-7977)	132
8.4.	Clinical trials for PSI-7977 (GS-7977; sofosbuvir)	132
8.4.1.	Phase I.	132
8.4.2.	Phase IIa	133
8.4.3.	Phase IIb	133
8.4.4.	Phase III.	134
8.5.	Conclusions	135
9.	Combinations of sofosbuvir with other DAAs.	135
9.1.	Sofosbuvir (PSI-7977; GS-7977) plus daclatasvir (BMS-790052).	135
9.1.1.	Phase IIa	135
9.1.2.	Phase II	136
9.2.	Sofosbuvir plus ledipasvir (GS-5885).	136
9.2.1.	ION-1.	136
9.2.2.	ION-2.	136
9.2.3.	LONESTAR.	136
9.2.4.	ION-3.	136
9.3.	PSI-7977 (GS-7977; sofosbuvir) plus simeprevir (TMC435).	136
9.4.	Sofosbuvir (GS-7977; PSI-7977) a pyrimidine plus PSI-938 a purine nucleotide analog.	136
10.	R7432	137
11.	PSI-938 (PSI-352938)	137
11.1.	Synthesis of PSI-938	137
11.2.	Preclinical development	137
11.3.	Clinical development.	137
11.3.1.	Phase I.	137
11.3.2.	Phase IIa, NUCLEAR	137
11.3.3.	Phase IIb, QUANTUM	138
11.4.	Conclusions	138
12.	TMC649128	138
12.1.	Preclinical development	138
12.2.	TMC649128 synthesis.	138
12.3.	Clinical development.	139

12.3.1.	Phase Ia.....	139
12.3.2.	Phase Ib.....	139
12.4.	Conclusions.....	139
13.	GS-6620	139
13.1.	Preclinical development.....	139
13.2.	GS-6620 synthesis.....	139
13.3.	Clinical development.....	139
13.4.	Conclusions.....	140
14.	ALS-2200 (VX-135) and ALS-2158	140
14.1.	ALS-2258 clinical development.....	140
14.2.	ALS-2200 clinical development.....	140
14.2.1.	Phase II.....	140
15.	BMS-986094 (INX-189/INX-08189)	141
15.1.	Synthesis of BMS-986094	141
15.2.	Clinical development.....	141
15.2.1.	Phase Ia.....	141
15.2.2.	Phase II.....	141
15.3.	Conclusions.....	142
16.	Lessons learned.....	142
	Acknowledgements and disclosures.....	142
	References.....	142

1. Introduction

The hepatitis C virus (HCV) was identified in 1989 as a member of the *Flaviviridae* family of positive-stranded RNA virus (Choo et al., 1989). Infection of a human host by HCV results in a serious infection affecting about 3% of the worldwide population, according to the World Health Organization (WHO). The WHO also estimates that 4 million people contract HCV each year (WHO, 2012). Although the early stages of HCV infection are usually asymptomatic, and about 20% of infected individuals will naturally clear the virus, a majority of infections progress to chronic infection. A significant number of chronically infected individuals will eventually develop more serious liver problems such as cirrhosis, hepatocellular carcinoma (HCC), or liver failure requiring liver transplantation (Darby et al., 1997; Poynard et al., 2000; Tong et al., 1995). The global health burden of HCV-associated morbidity and mortality is expected to increase substantially during the next decades, as many chronically infected individuals progress to end stage disease with associated complications (Davis et al., 2003; Manns et al., 2007). In fact, in industrialized nations, HCV infection is already the leading cause for liver transplantations. Unfortunately, re-infection of the transplanted liver from an unknown reservoir often occurs post-transplantation (Hoofnagle, 1997). The virus is transmitted parenterally by contaminated blood most often from sharing contaminated needles, but also from improper

sterilization of medical, dental, body piercing, or tattoo equipment. Heterosexual transmission and vertical transmission (infected mother to her child during the birthing process) of HCV can also occur, but are rare (Fishman et al., 2009; Ghosn et al., 2009). Sexual practices that involve higher levels of trauma to the anogenital mucosa or that occur when there is a concurrent sexually transmitted infection, such as HIV or genital ulceration, do present a higher risk of HCV transmission (Tohme and Holmberg, 2010).

Three major drug targets have reached evaluation in humans for chronic HCV infection (Farnik and Zeuzem, 2012; Schaefer and Chung, 2012): the NS3/4A serine protease, the large phosphoprotein NS5A and NS5B RNA-dependent RNA polymerase (RdRp) (Bobeck et al., 2010; De Francesco and Migliaccia, 2005; Huang et al., 2006). The majority of drugs that target the HCV NS3/4A serine protease are peptidomimetics and represent the only two FDA approved direct acting antiviral agents for treatment of HCV infection. Although the ultimate role of NS5A in the HCV replication cycle is not fully understood, the 49 kDa NS5A protein is required for HCV replication as it is part of the membrane bound replication complex (Lemon et al., 2010). The two main categories of HCV NS5B RdRp inhibitors are non-nucleoside and nucleoside analog inhibitors (Brown, 2009; Burton and Everson, 2009; Legrand-Abravanel et al., 2010), which differ in their chemical structure, barrier to resistance, pan-genotypic activity and mode of action.

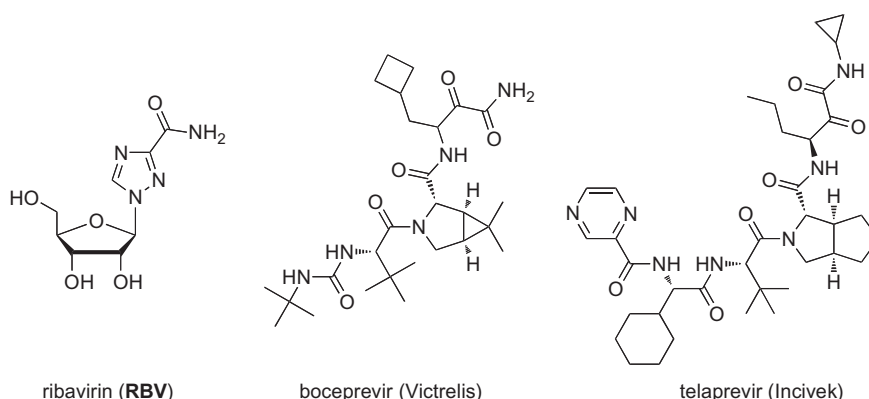


Fig. 1. Structures for ribavirin (RBV), boceprevir (Victrelis) and telaprevir (Incivek).

The current FDA-approved treatments for chronic HCV are limited to pegylated interferon- α (IFN) alone or in combination with ribavirin (RBV) with or without protease inhibitors (PI) boceprevir (Victrelis®) and telaprevir (Incivek®) (Fig. 1) (Hughes and Shafran, 2006; Pan et al., 2012; Sheridan, 2011). Non-pegylated IFNs have also been approved including the recombinant IFN alfacon1 and natural IFN, purified from the leukocyte fraction of human blood, multiferon. Unfortunately, these treatments have limited efficacy for the GT1 population, which is the most prevalent GT in the US and China and emphasize the urgent unmet medical need for new improved therapeutic agents that are pangenotypic (Manns et al., 2001; McCown et al., 2008). These treatments also have major side effects including depression, low red and/or white blood cell counts, central nervous system toxicity, fatigue, headache, flu-like symptoms, mild alopecia, thyroid dysfunction, itching, swelling of the face, eyes, lips, tongue, or throat, skin rashes, hemorrhoids, diarrhea and teratogenic effects (Fried, 2002; Gaetano and Reau, 2013; Gordon and Keller, 2005; Hezode et al., 2012; Keicher et al., 2005; Koch and Narjes, 2007; McHutchison et al., 2002; Pawlotsky, 2003; Wong and Lee, 2006).

In the clinic, the primary treatment goal for chronically infected HCV (CHC) persons is lifelong clearance of detectable HCV infection. When new anti-HCV chemical entities are evaluated in clinical trials, the prediction of lifelong clearance of HCV infection is often assessed by a surrogate endpoint termed sustained virologic response (SVR). SVR is determined at ≥ 12 weeks after discontinuation of antiviral therapy and defined as non-detectable HCV RNA in serum that is measured by sensitive assays such as a polymerase chain reaction (PCR) based determination. Two earlier predictors of SVR during clinical trials are rapid virologic response (RVR) and early virologic response (EVR) which are defined as reduction of serum HCV RNA to PCR-non-detectable levels by week 4 and achievement of $\geq 2 \log_{10}$ reduction in serum HCV RNA level by week 12, respectively. Not all persons who eventually achieve an SVR will have met the EVR or RVR milestones and conversely reaching any or all of these three categories do not guarantee lifelong clearance of detectable HCV infection. However, these guidelines are predictive of ultimate therapy success and help clinicians

determine, for example, if persons with significant side effects that fail to achieve EVR or RVR should discontinue treatment.

Nucleoside analogs that target the HCV NS5B polymerase that have reached human clinical trials will be the focus of this review as they have demonstrated significant advantages in the clinic with broader activity against the various HCV GT and a higher barrier to the development of resistant viruses when compared to all other classes of HCV inhibitors (McCown et al., 2008). The HCV NS5B polymerase catalytic site is highly conserved across the various genotypes resulting in little variation of potency of the nucleoside triphosphate inhibitors that target this protein (Asselah and Marcellin, 2012; Waheed et al., 2013). Selection of HCV mutants *in vitro* has been quite difficult with most nucleoside inhibitors and selected mutants have been found markedly unfit with little to no clinical significance (Najera, 2013; Tong et al., 2013). There have been several nucleoside analogs discovered that exhibit selective anti-HCV activity, most with modifications at the 2' and/or 4' positions of the ribose sugar. However, nucleoside analogs as a class have potential problems such as poor passive cellular permeability and the modified nucleoside may not be a suitable substrate for the three successive cellular kinases that are necessary to transform it to the biologically active nucleoside analog triphosphate (NTP) (Fig. 2). Since HCV lacks nucleoside kinase expression, conversion to the biologically active nucleoside analog triphosphate relies on the human cellular kinases (Meppen et al., 2009). In addition, antiviral activity of nucleoside analogs is heavily influenced by the levels of natural dNTP or rNTP (Fig. 2) since they compete for incorporation by viral polymerases. Nucleoside analog inhibitors of viral RNA polymerization have the additional challenge of 10–100 times higher levels of rNTPs relative to the corresponding dNTP (Kennedy et al., 2010; Medivir, 2011a,b; Traut, 1994). Additionally, another significant obstacle in the development of nucleoside analogs for HCV therapy is their potential to be substrates and/or inhibitors of host polymerases. As an example, in the recent unfortunate events related to the 2'-methyl substituted guanosine analogs **IDX184** and **INX-189**, the human mitochondrial RNA polymerase has become of significant concern (Arnold et al., 2012).

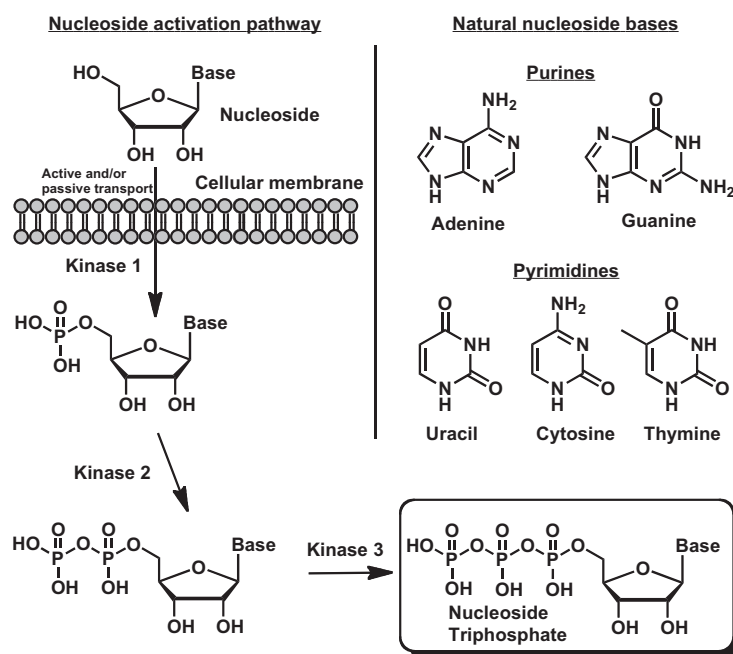


Fig. 2. Nucleosides transform via three successive cellular kinases to the biologically active nucleoside analog triphosphates. Natural ribo- and 2'-deoxyribonucleoside bases.

Prodrugs are defined as a pharmacological substance administered in an inactive (or significantly less active) form that is metabolized into an active metabolite. Prodrugs are most often employed to improve absorption, distribution, metabolism, and/or elimination (ADME) properties of the parent pharmacological substance. One of the most common applications of prodrugs is for a drug that is poorly absorbed through the gastrointestinal tract to improve oral bioavailability. A prodrug may also be used to improve the selectivity by which the drug interacts with the intended target cells or processes versus those that are not the drug's intended target. In the HCV area, nucleoside prodrugs have demonstrated both improvement of oral bioavailability and the ability to selectively deliver nucleotide analogs to liver tissue by first pass metabolism. Nucleoside analogs themselves are prodrugs of the active triphosphate form, which is recognized and incorporated by viral polymerases or reverse transcriptases. Antiviral activity of a nucleoside analog can often be improved by conversion to a prodrug as this aids in absorption and/or cellular penetration. In the case of monophosphate prodrugs, the potential rate limiting initial phosphorylation step is bypassed leading to higher intracellular nucleoside analog monophosphate levels which in many cases leads to significantly higher triphosphate levels that can translate to a significant enhancement in antiviral activity (De Clercq and Field, 2006; Hecker and Erion, 2008; Li et al., 2008; Poijärvi-Virta and Lönnberg, 2006; Sofia, 2011; Sofia et al., 2010a).

Prodrugs have played a central role in the delivery of active agents to their target tissue with improved efficiency and fewer side effects for well over 100 years. One of the earliest and enduring prodrug applications was to salicylic acid which when converted to acylsalicylic acid (Gerhardt, 1853; Von Gilm, 1859), significantly reduced irritation to the mouth and gastrointestinal tract (Fig. 3). Salicylic acid has been used for thousands of years to ease pain and reduce fever, but it was not until the period from 1826 to 1838 that salicylic acid was isolated by a series of chemists from willow tree bark (Jeffreys, 2005).

In 1856 an industrial scale process to make salicylic acid was developed from phenol and carbon dioxide (Kolbe, 1860; Schmitt, 1885), but interestingly the correct chemical structure was not assigned until 1869 that the correct chemical structure was assigned

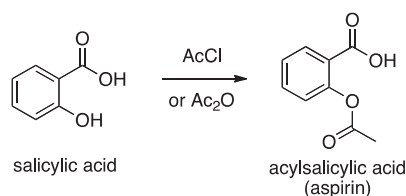


Fig. 3. Synthesis of acylsalicylic acid from salicylic acid.

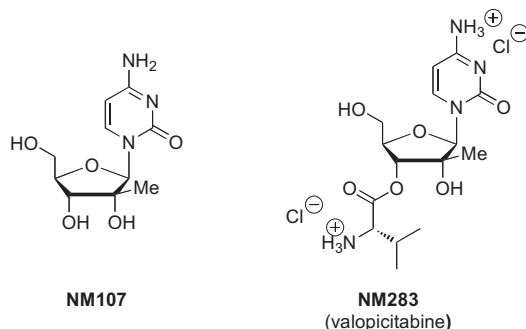


Fig. 4. Chemical structures of NM107 and NM283.

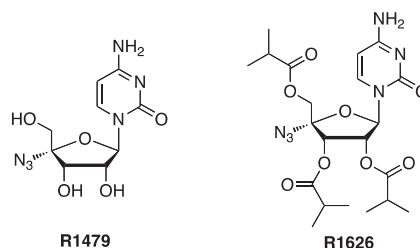


Fig. 5. 4'-Azidocytidine analogs used in the human clinical treatment of HCV.

(Schröder and Kraut, 1869). In 1897, approximately 44 years after the first synthesis, acylsalicylic acid was first prepared by Bayer and in less than two years aspirin was trademarked and was being sold worldwide. As of 2013 (160 years after the first synthesis) more than 41 kilotons (metric) of aspirin is consumed annually worldwide. Many modern prodrugs utilize the same simple ester masking of a free hydroxyl group as a prodrug strategy as is exemplified by 3'-L-valinyl ester prodrug NM283 (Fig. 4) or 2',3',5'-triisobutyrate ester prodrug R1626 (Fig. 5) and 3',5'-diisobutyryl ester prodrug RG7128 (Scheme 4) which are among the first HCV clinical candidates.

2. Valopicitabine (NM283): the first clinical HCV nucleoside

2.1. Preclinical development

2'-C-Methylcytidine (Harry-O'kuru et al., 1997) also known as NM107 (Sommadosi et al., 2008, 2004a,b,c; Sommadossi and La Colla, 2001a,b; Storer et al., 2004), was discovered by Idenix Pharmaceuticals to be a potent inhibitor of RNA viruses replication such as bovine viral diarrhea virus (BVDV), West Nile virus (WNV), dengue-2 virus, yellow fever virus (YFV) and subsequently HCV (Kowdley et al., 2013; Sommadossi and La Colla, 2001b). Despite its broad spectrum *in vitro* activity, NM107 displayed low oral bioavailability. To overcome this issue, NM283 (Sommadosi et al., 2008, 2004a,b,c; Sommadossi and La Colla, 2001a,b; Storer et al., 2004) also called valopicitabine, the 3'-O-L-valinyl ester prodrug of NM107, was developed (Pierra et al., 2005). In March 2006, Novartis announced to exercise its option from Idenix Pharmaceuticals to in-license the late stage development of valopicitabine.

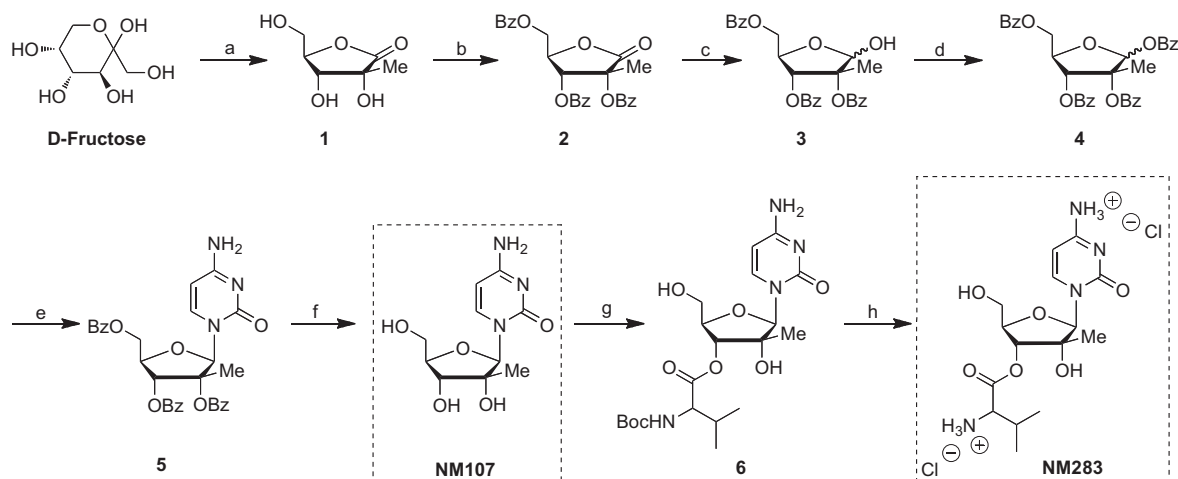
2.2. Synthesis of valopicitabine (NM283)

Large scale synthesis of NM107 and NM283 were achieved from commercially available D-fructose by first reaction with calcium oxide and then treatment with oxalic acid to give the key 2'-C-Me-lactone **1**. Subsequent benzoylation, reduction of the lactone with Red-Al and benzoylation of the lactol **3** afforded compound **4**. At this stage, Vorbrüggen type coupling with cytosine using SnCl_4 as Lewis acid followed by debenzoylation gives access to NM107. NM283 is finally obtained from NM107 after simple peptidic coupling with N-Boc-L-valine and Boc-removal under acidic condition (Scheme 1) (Pierra et al., 2005).

2.3. Clinical development

2.3.1. Phase I/II

In 2004, the first phase I/II study of valopicitabine was reported, wherein 48 subjects received valopicitabine at 50, 100, 200, 400 and 800 mg/day orally once daily for 15 days with 14 days of follow-up; a subset of subjects received 400 mg twice daily (Godofsky et al., 2004). During these escalation trials, a dose-related decrease of serum HCV RNA at Day 16 was observed ranging from 0.15 \log_{10}



Scheme 1. Synthesis of valopicitabine (**NM283**). Reagents and conditions: (a) i. CaO, water; ii. CO₂, oxalic acid, 18%; (b) BzCl, TEA, DMAP, DME, 83%; (c) Red-Al/EtOH, toluene, 99%; (d) BzCl, TEA, DMAP, DME, 52%; (e) bis(trimethylsilyl)acetamide, cytosine, SnCl₄, CAN; (f) MeONa, MeOH, r.t., 90% over two steps; (g) *N*-Boc-L-valine, CDI, DMAP, TEA, DMF/THF, 91%; and (h) HCl, EtOH, 97%.

at the lowest dose to 0.73 log₁₀ at 400 mg/twice daily. At 400 mg/day, 50% of the subjects experienced transient mild nausea and 20% had vomiting, but all completed treatment without modifications.

2.3.2. Phase IIa

Following the discovery of a synergistic antiviral effect against BVDV, *in vivo*, between **NM107**, the parent compound of valopicitabine (**NM283**) and **IFN**, a combination study with treatment-naïve subjects involving valopicitabine and **IFN** was initiated (Afdahl et al., 2005). Valopicitabine was given for 24 weeks with escalation to 800 mg/day over the first week before **IFN** was administered (1.0 µg/kg weekly) on Day 8. Along with a satisfactory tolerance, HCV RNA reductions increased to 3.2 log₁₀ IU/mL for the combination treatment group (compared to 1.0 log₁₀ IU/mL for monotherapy group). Eleven of twelve subjects treated for at least 10 weeks had substantial HCV RNA reductions superior to 2 log₁₀ and four subjects were PCR-negative (<10 IU/mL).

2.3.3. Phase IIb

In a Phase IIb clinical trial, a 48 weeks combination of valopicitabine and **IFN** was evaluated in treatment-naïve subjects with HCV GT1 (Dieterich et al., 2006; Lawitz et al., 2007). This study was divided into five treatment groups: (A) **IFN** alone (180 µg QW = once weekly) for 4 weeks, valopicitabine 800 mg/day starting at week 5; (B) valopicitabine 200 mg/day + **IFN** (180 µg QW); (C–E) valopicitabine 800 mg/day with various week 1 induction regimens + **IFN** (180 µg QW). However, due to high gastrointestinal (GI) side effects at 800 mg/day dose of valopicitabine, the protocol was amended to reduce the dose to 200 or 400 mg/day after 14–22 weeks of treatment. The rate of viral decline was shown to be valopicitabine dose-related through week 4. HCV RNA was below 600 IU/mL in most subjects by week 12 and below 20 IU/mL by week 24 and 36. Group B maintained suppression of HCV RNA through week 48 along with satisfactory tolerability.

2.3.4. Phase IIb

In another Phase IIb trial (Afdahl et al., 2005), a 72 week three arm combination study involving valopicitabine/**IFN** in non-responder subjects was designed. Subjects were randomized to: (A) valopicitabine monotherapy (800 mg/day) and 3 combinations arms: **IFN** (180 µg SQ/week) with **RBV** (1000–1200 mg/day) + (A) valopicitabine (400 mg/day); (C) dose escalation 400–800 mg/day; (D) 800 mg/day or **IFN**/**RBV** retreatment. Again, due to GI issues with the 800 mg/day dose, the maximum dose was reduced

to 400 mg/day. At week 48, HCV RNA suppression of treated persons with doses of 800 mg/day or escalated-dose 400–800 mg/day of valopicitabine, was 6-fold greater (0.8 log₁₀) than **IFN**/**RBV** treatment demonstrating the potency of valopicitabine for the treatment of non-responder persons.

2.4. Conclusions

Twelve days after Idenix announced on July 1st 2007, that a triple therapy with valopicitabine and **IFN**/**RBV** cleared HCV RNA in 72% of subjects after 12 weeks of treatment, the FDA requested that the clinical trials on valopicitabine be discontinued due “to the overall risk/benefit profile observed to date in clinical testing” (Idenix, 2007). From the beginning, this drug as plagued with GI issues and unfortunately was not potent enough to be used alone or even when combined with the standard of care (**IFN**/**RBV**).

3. R1479 and its prodrug R1626: balapiravir

3.1. Preclinical development

Discovered and developed by Hoffmann-La Roche, Inc., but aided by technology and compound libraries obtained by the 1994 purchase of Syntex Research, the 4'-azidocytidine derivative **R1479** (Ahmed et al., 2007; Connolly et al., 2005; Devos et al., 2002; Harrington et al., 2008; Martin et al., 2004) was identified as a potent and selective inhibitor of HCV RNA polymerase. **R1479** displayed an EC₅₀ of 1.3 µM in an HCV replicon system and did not exhibit cytotoxic or cytostatic behavior in the Huh-7 replicon cell line (Smith et al., 2007). The triphosphate form of **R1479** was found to be a substrate for HCV polymerase and it inhibited RNA chain extension after incorporation as a non-obligatory chain terminator with an IC₅₀ of 320 nM (Klump et al., 2006). *In vitro* and *in vivo* assessment of **R1479** revealed that like many other nucleoside analogs, **R1479** was a polar molecule with a low aqueous solubility of 10 mg/mL that led to limited absorption after oral administration (Alfredson et al., 2006; Toniutto et al., 2008). Further, **R1479** exhibited low Caco-2 permeability [(Artursson et al., 1996); Caco-2 cells are used as a model system to predict intestinal epithelial permeability] and oral bioavailability values ranging from 22% in monkey to 89% in rat. Oral absorption in human clinical studies showed 6–18% based on urine recovery.

Consequently, prodrug approaches were investigated (Alfredson et al., 2006) to improve the oral bioavailability of **R1479**. Prodrug

approaches that increase lipophilicity of a compound increase the potential for passive transport across cellular membranes and thus potentially improve absorption and transport across cellular membranes. The first approach focused on using esters and amides at the 2', 3' or 5' positions of sugar to increase the lipophilicity, the second approach focused on increased lipophilicity with liver targeted cleavage using carbamate prodrugs at N4 position of cytosine and the third approach focused on amino acid esters to potentially target the PepT1 transporter for increased active intracellular transport. A five-fold increase in plasma exposure was observed with tri-isobutyrate ester prodrug **R1626** (Ahmed et al., 2007; Connolly et al., 2005; Devos et al., 2002; Harrington et al., 2008; Martin et al., 2004) in oral bioavailability in rats and monkeys. Further, results from Caco-2 experiments (Brandl et al., 2008) indicated that the hydrolysis of the prodrug is rapid and converted to **R1479** by esterases in gastrointestinal epithelial cells. Exceptional enhancement of dose proportionality combined with a 3-fold enhancement of plasma exposure both in human studies versus oral administration of **R1479** resulted in the selection of tri-isobutyrate ester prodrug **R1626** for further clinical evaluation.

3.2. Synthesis of **R1479** and its prodrug balapiravir (**R1626**)

The synthesis of 4'-azidocytidine (**R1479**) (Connolly et al., 2005) and its triisobutyrate ester prodrug **R1626** (Sarma, 2007) is outlined in Scheme 2. Starting from uridine, 5'-iodo uridine was prepared then base eliminated to the 4'-exocyclic methylene nucleoside analog **7**. Addition of iodoazide across the double bond and benzylation gave the 5'-iodo, 4'-azido bis-benzoylated nucleoside **8**. Oxidative displacement of the 5'-iodo gave the 5'-*m*-chlorobenzyl ester **9** that was converted to the cytidine analog and debenzoylated to give **R1479**. **R1626** was then formed in good yield by reaction with isobutyric anhydride in the presence of potassium hydroxide (Scheme 2).

3.3. Clinical development

3.3.1. Phase I

Starting in 2006, balapiravir (**R1626**) was given to healthy subjects in single doses from 500 to 12,000 mg to evaluate the safety, tolerability and pharmacokinetics. High levels of **R1479** was found in plasma accompanied by low levels of both mono and diester

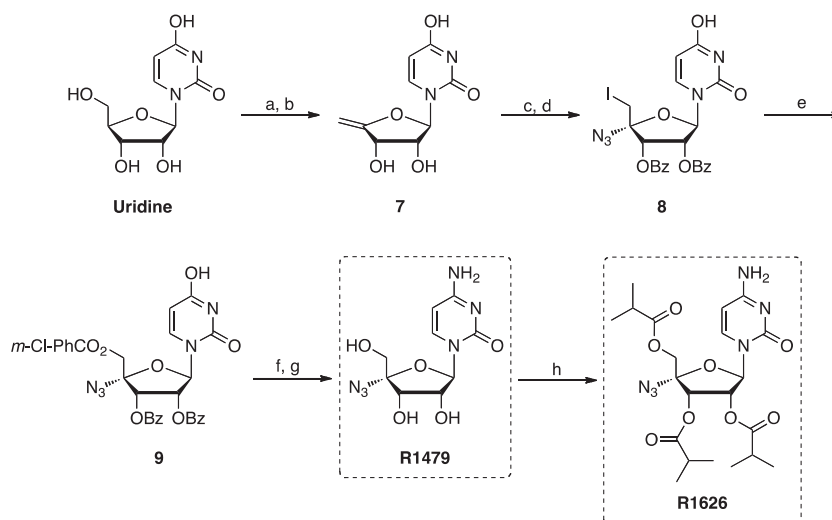
analogs. **R1479** was found in plasma in a linear amount proportional to all doses studied. Forty healthy male subjects (13–34 years old) were randomized 3:1 for treatment with **R1626** or placebo and were followed up for 7 days. The most common reported adverse effects were headache and irritation at the site of ECG electrode application. The phase I study concluded that balapiravir was efficiently absorbed, rapidly converted to **R1479** and was tolerated up to a dose of 12,000 mg when orally administered in healthy subjects (Robson et al., 2007).

3.3.2. Phase Ib

This clinical trial was designed to study the effect of **R1626** administered as monotherapy in HCV-infected persons. A total of forty-seven GT1 subjects were randomized to oral treatment of **R1626** with doses of 500, 1500, 3000, 4500 mg and placebo for 14 days (Roberts et al., 2008). As seen in the phase I study, after oral administration, **R1626** was efficiently converted to **R1479** with dose proportional pharmacokinetics observed over the entire dose range. The mean viral concentration at baseline was $6.3 \log_{10}$ IU/mL, after 2 weeks of dosing; the mean (and median) viral concentration decreased by 1.2 (0.8), 2.6 (2.7) and 3.7 (4.1) \log_{10} at 1500, 3000 and 4500 mg, respectively. **R1626** was generally well tolerated up to 3000 mg twice a day with mild side effects and no serious adverse events. However, there were increased adverse events at the highest dose of 4500 mg BID. The main adverse effect seen was reversible, mild to moderate hematological changes. This study resulted in a maximum dose of 3000 mg twice a day for **R1626** in all future clinical trials. The study concluded that treatment with **R1626** as monotherapy significantly reduced plasma HCV viral load.

3.3.3. Phase IIa

In this study drug resistance, efficacy and safety of the combination therapy of **R1626** along with IFN with and without RBV was studied over four weeks in 107 GT1 treatment-naïve patents. Subjects were randomized to 1500 or 3000 mg BID of **R1626** with IFN or 1500 mg BID with RBV. Control group received standard of care (SOC) as IFN and RBV (Pockros et al., 2008). After the four-week treatment period all four arms continued on SOC out to 48 weeks. Results were promising at week 4 with undetectable HCV RNA (<15 IU/ml) in 29%, 69% and 74% of subjects receiving 1500 mg BID dual therapy, 3000 mg BID dual therapy, and 1500 mg BID



Scheme 2. Synthesis of **R1479** and its prodrug balapiravir (**R1626**). Reagents and conditions: (a) I_2 , PPh₃, imidazole, THF, 83%; (b) NaOMe, MeOH, 75%; (c) $BnN(Et)_3Cl$, NaN_3 , 4-NMM, I_2 , CH_3CN ; (d) $BzCl$, Pyr., 74% over two-steps; (e) K_2HPO_4 , *m*-CPBA, CH_2Cl_2 , 71%; (f) 1,2,4-triazole, $POCl_3$, Et_3N , MeCN, 88%; (g) NH_4OH , MeOH, H_2SO_4 -isopropanol, 95%; and (h) isobutyric anhydride, KOH, DMAP, THF, 80%.

triple drug therapy, respectively, compared with 5% in the SOC arm. Sequence analysis of the entire NS5B coding region of the samples from these subjects revealed no known **R1479** resistance mutations (S96T or S96T/N142T) when compared with the base-line sequence. The main adverse event noted was grade 4 neutropenia that occurred in 50% of the persons in the **R1626** treatment groups and occurred with 78% of the persons in the 3000 mg BID group compared to 10% in the SOC arm. Overall, this study had safety concerns related to the drug; however, most AEs were mild (80%) or moderate (16%), but 4% were rated as severe. Strong synergistic antiviral effect was seen with **R1626**, **IFN** and **RBV**, which suggested that the dose of one or more of these drugs could be lowered to improve the tolerability without significantly compromising efficacy.

3.3.4. Phase IIb

This double-blind phase II trial assessed the optimal treatment protocol for balapiravir plus **IFN/RBV** (Nelson et al., 2012). Seven arms of treatment-naïve GT1 infected subjects ($N = 516$) were randomized and received balapiravir 500, 1000, or 1500 mg twice daily, **IFN** 180 or 90 $\mu\text{g}/\text{week}$ and **RBV** 1000/1200 mg/day or **IFN/RBV**. Safety concerns reduced the planned treatment duration with balapiravir from 24 to 12 weeks. From week 2 to week 12 undetectable HCV RNA was more common in all balapiravir groups. Ultimately, the efficacy assessment was compromised by high rates of dose modifications and discontinuations, which resulted in similar SVR rates in the balapiravir groups (32–50%) and **IFN/RBV** group (43%). Balapiravir showed dose related serious adverse events (especially hematological, infection, ocular events) and discontinued for safety reasons in 28–36% of patients (most often for lymphopenia). Balapiravir was considered possibly related to two deaths in the balapiravir/**IFN/RBV** combination groups.

3.4. Conclusions

In Oct-2008, following an unacceptable benefit to risk ratio revealed in later Phase IIb study and lack of superior efficacy, **R1626** was withdrawn from further consideration of future drug development in the treatment of chronic hepatitis C (Klump and Smith, 2011; Roche, 2008). Interestingly, **R1626** was evaluated in adult patients infected with dengue virus in a double-blind placebo controlled trial at 1500 and 3000 mg BID for five days. In August 2012, Roche reported **R1626** to be well tolerated but it demonstrated no viremia improvement versus the placebo arm (Nguyen et al., 2013). In our hands the parent 4'-azidocytidine nucleoside was a weak inhibitor in the HCV replicon system ($\text{EC}_{50} = 11 \mu\text{M}$ and $\text{EC}_{90} = 32 \mu\text{M}$) and it is unclear why this drug was selected to be developed in humans.

4. RG7348 (MB11362)

A collaboration and license agreement between Metabasis Therapeutics, Inc. and Roche began in 2008 for the development of new treatments for HCV infection utilizing pre-clinical candidate **MB11362** and the proprietary HepDirect® liver-targeting technology. The lead HepDirect nucleoside, renamed **RG7348**, was de-

clared a clinical candidate in the second quarter of 2009. In January 2010, Ligand acquired Metabasis and subsequently, Roche advanced **RG7348** into Phase I clinical studies (Ligand, 2010).

4.1. Preclinical development

The structure of **RG7348** has not yet been disclosed, but it is known that it is a double prodrug that acts as a potent and selective HCV NS5B polymerase inhibitor (Nieforth et al., 2012). **RG7348** was designed to provide high intra-hepatic levels of 4'-azidouridine triphosphate (Fig. 6). Very little preclinical data has been disclosed for **RG7348**. After oral dosing, the double prodrug was subsequently transformed by esterases and CYP3A4 (cytochrome P450 3A4) to 4'-azidouridine monophosphate **10**, which was converted by kinases to the active 4'-azidouridine triphosphate **11**, a potent and selective inhibitor of HCV polymerase.

4.2. Clinical development

4.2.1. Phase I

In 2012 Nieforth and co-workers reported a Phase I study on safety, pharmacokinetics (PK) and anti-HCV activity for GT1 subjects with **RG7348**. The study was a randomized, double-blind, placebo-controlled single ascending dose study in healthy subjects (part A: 60 subjects with 12/placebo and 8/cohort receiving 15, 50, 150, 300, 600 and 1200 mg), and multiple dose study in chronic HCV GT1 subjects (part B: 12 male subjects: 4/placebo and 8/**RG7348** 150 mg BID). After **RG7348** dosing, C_{max} and AUC_{inf} for (**10**) and (**11**) increased dose proportionally from 15 to 150 mg and less than dose proportionally at higher doses. **RG7348** was well tolerated in chronic HCV persons in this proof-of-concept study. Although **RG7348** was a novel liver-targeted HCV nucleotide polymerase inhibitor, which demonstrated promising safety and PK, there was limited observed antiviral effect (mean reduction in HCV RNA $<0.8 \log_{10}$ IU/mL at all time points) at the dose studied.

4.3. Conclusions

In November 2010, Roche terminated the HCV development with Ligand. There was no statement from either company that explained the basis of this decision. In January 2011, Ligand Pharmaceuticals Inc. announced a strategic relationship with Chiva Pharmaceuticals to develop multiple Ligand assets. Chiva was granted a non-exclusive HepDirect® technology license for the discovery, development and commercialization of new compounds for HCV treatment, including **RG7348**. No other advances for **RG7348** have been reported to date. Although the HepDirect technology seemed interesting, it never delivered on the promise to be liver specific and to add value to the nucleoside analogs studied.

5. PSI-6130

5.1. Chemical synthesis

β -D-2'-Deoxy-2'-fluoro-2'-C-methyl-cytidine (**PSI-6130**) was conceived from a combination of 2'-methyl of the HCV active

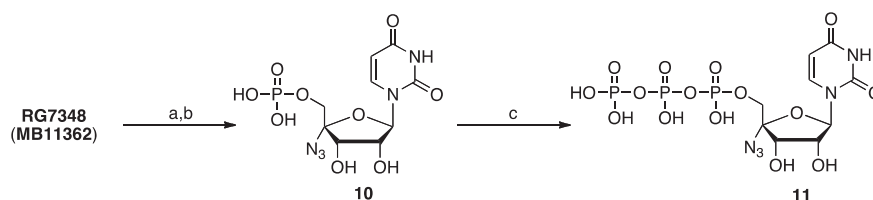


Fig. 6. Metabolism and metabolites of **RG7348**: (a) esterases; (b) CYP3A4; and (c) kinases.

NM107 with the non-selective 2'-deoxy-2'-fluorocytidine (Cedilote et al., 2008; Clark, 2005). Also, at about the same time, Pharmasset scientists discovered the modest anti-HCV activity of gemcitabine which was another 2'-disubstituted nucleoside (Stuyver, 2003). **PSI-6130** was first synthesized from commercial available cytidine in about 3% total yield (Clark et al., 2005), but a more efficient, diastereoselective and scalable process was developed using commercially available isopropylidene protected D-glyceraldehyde (Wang et al., 2009). Thus, Wittig type reaction was performed with aldehyde **12** to give the unsaturated derivative **13** (Scheme 3). After oxidative dihydroxylation, compound **15** was selectively fluorinated. Cyclisation of ester **16** under acidic conditions gave lactone **17**, which after benzylation, reduction of the lactone and further acetylation gave the key sugar **19**. Finally, introduction of the cytosine base under Vorbrüggen type coupling condition and complete debenzoylation afforded **PSI-6130** in a 6.4% overall yield.

5.2. Preclinical development

PSI-6130 is a potent and specific HCV RNA-dependent RNA polymerase (RdRp) inhibitor with EC_{50} of $5.4 \pm 2.6 \mu\text{M}$ in Huh-7 replicon cells (Clark et al., 2005; Stuyver et al., 2006). It is interesting to note that just a few years earlier the commonly used BVDV surrogate assay would not have identified the anti-HCV activity of **PSI-6130** because **PSI-6130** was found to be highly specific for HCV over other RNA or DNA viruses. The 5'-triphosphate of **PSI-6130** was found to be equally active against the wild-type GT1b replicon clone and the S282T mutant replicon (Murakami et al., 2007). **PSI-6130** showed no apparent cytotoxicity against various cell types, including human bone marrow and human peripheral blood mononuclear cells. No mitochondrial toxicity was observed with **PSI-6130** (Stuyver et al., 2006). An *in vitro* triple combination study of **PSI-6130** with IFN and RBV at 1:1:1 ratio indicated synergistic antiviral effect, with no apparent cytotoxicity effects with any of the combinations tested (Bassit et al., 2008). Cell-based metabolism studies with primary human hepatocytes indicated that **PSI-6130** is a unique compound that gives rise to two nucleoside triphosphates that are both potent inhibitors of the HCV RdRp (Fig. 7) (Ma et al., 2007; Murakami et al., 2007, 2008). **PSI-6130** can be converted to an inactive uridine analog **PSI-6206** (also known as **RO2433**) by human cytidine deaminase (CDA) and to **PSI-6130-MP** by human 2'-deoxycytidine kinase (dCK), respectively. The formed **PSI-6130-MP** produced two active nucleoside triphosphates through two separate pathways. Thus, phosphorylation of **PSI-6130-MP** to the 5'-di- and 5'-triphosphates was catalyzed by uridine-cytidine monophosphate kinase (YMPK) and nucleoside diphosphate kinase (NDPK), respectively. In addition,

metabolism studies with **PSI-6130** showed that the monophosphate of **PSI-6130** was deaminated by 2'-deoxycytidylate deaminase (DCTD) to the uridine monophosphate derivative by and subsequently anabolized to the 5'-triphosphate **RO2433-TP** by YMPK and NDPK in a second pathway (Fig. 7). The inactive uridine analog **PSI-6206** is not a substrate for dCK and cannot be converted to **PSI-6206-MP** directly. The first cellular activation step to monophosphate is the rate-limited step among the three phosphorylation steps. Interestingly, the monophosphate species of **PSI-6130** and **PSI-6206** are very rapidly transformed into their corresponding nucleoside triphosphates, since those compounds are found at the highest concentration among all phosphate species present. Finally, **PSI-6130-TP** was a more potent inhibitor of the wild-type and S282T mutant polymerase compared to **PSI-6206-TP** (Ali et al., 2008; Ma et al., 2007; Murakami et al., 2007). Notably, **PSI-6206-TP** had a significantly longer half-life than **PSI-6130-TP** (see below).

5.3. Clinical development

Early animal studies involving single-dose pharmacokinetics studies with **PSI-6130** in rhesus monkeys showed low oral bioavailability for **PSI-6130** (Asif et al., 2007). An early phase I study in humans found a significant conversion to the inactive uridine analog combined with a bioavailability of 25%.

5.4. Conclusions

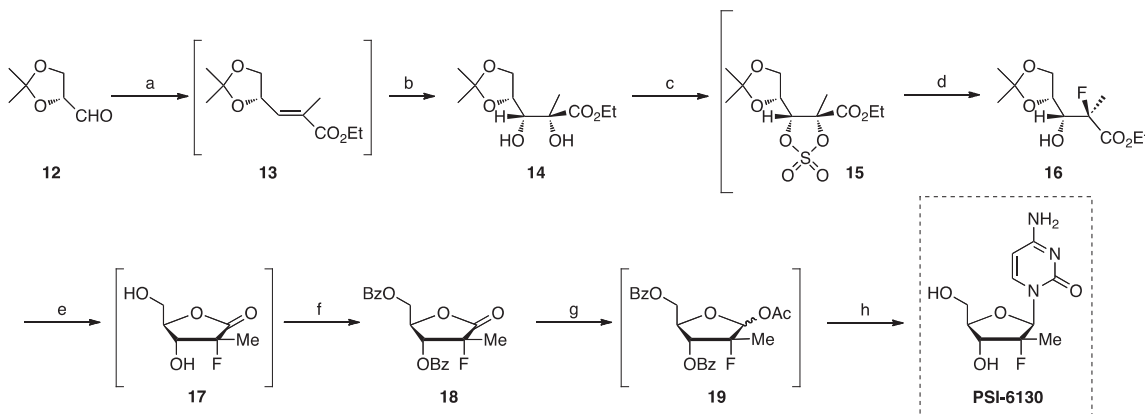
Because of these factors, a prodrug strategy was explored to potentially enhance bioavailability and provide a more selective conversion to **PSI-6130-TP**. **RG7128**, a prodrug of **PSI-6130** (refer to Section 6 of this manuscript), was ultimately selected for clinical evaluation based on its improvements in bioavailability and pharmacokinetics.

6. RG7128 (R7128, RO5024048, mericitabine)

6.1. Preclinical development and chemical synthesis

RG7128 (mericitabine) is a 3',5'-diisobutyryl ester prodrug and was prepared directly from **PSI-6130** with isobutyryl chloride in the presence of DMAP and TEA in THF/H₂O at 0 °C (Scheme 4) (Chun et al., 2007).

Mericitabine has excellent oral bioavailability but has very little plasma exposure and is quickly converted to **PSI-6130** in humans. Metabolism of **PSI-6130** proceeds by a bifurcated pathway to more potent triphosphate species **PSI-6130-TP** and less active uridine



Scheme 3. Synthesis of **PSI-6130**. Reagents and conditions: (a) $\text{Ph}_3\text{PC}(\text{Me})\text{CO}_2\text{Et}$, DCM, -40°C ; (b) KMnO_4 , acetone, 0°C , 53% over two steps; (c) i. SOCl_2 , TEA, DCM, 0°C ; ii. aq. NaOCl, TEMPO, NaHCO_3 , CH_3CN , 0°C ; (d) i. TEAF, dioxane, 100°C ; ii. $(\text{MeO})_2\text{C}(\text{Me})_2$, conc. HCl, dioxane, r.t.; (e) EtOH, conc. HCl, r.t.; (f) BzCl, pyridine, r.t., 47% from **(14)**; (g) i. $\text{Li}(\text{O}-t\text{Bu})_3\text{AlH}$, THF, -20°C ; ii. Ac_2O , DMAP, -20°C ; and (h) i. silylated N^4 -benzoylcytosine, SnCl_4 , PhCl, 65°C ; ii. NH_3 , MeOH, r.t., 29% from **(18)**.

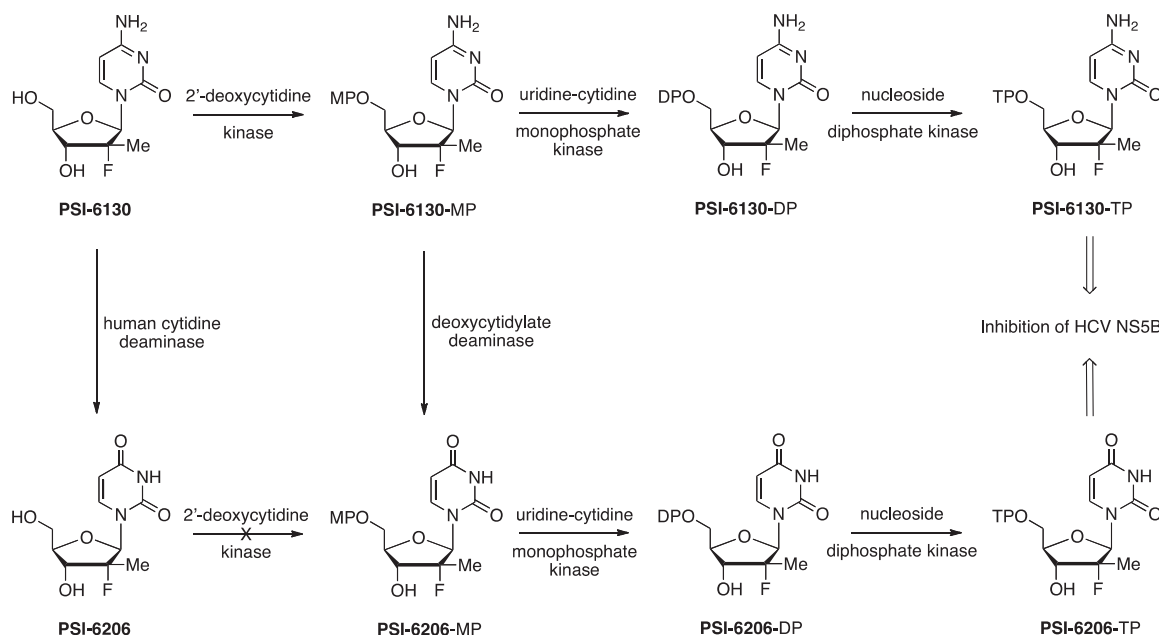


Fig. 7. Metabolism of **PSI-6130** leads to both the active triphosphate and the inactive nucleoside **PSI-6206**.

analog **PSI-6206**-TP and both serve as non-obligate chain terminators (Ali et al., 2008; Ma et al., 2007; Murakami et al., 2007, 2008). The prodrug mericitabine demonstrated potent inhibition of stable and transient replicons with EC_{50} values similar to those of **PSI-6130**. Both mericitabine and **PSI-6130** inhibited HCV GT1a (H77 strain) and GT1b (Con1 strain) subgenomic RNA replication, with similar levels of potency (mean EC_{50} values, 0.31 and 0.51 μ M, respectively) (Ali et al., 2008).

6.2. Clinical development

6.2.1. Phase I

In a two week phase I monotherapy clinical study with 32 HCV GT1 patients, mericitabine showed a mean 2.7 \log_{10} reduction in viral RNA with a dose of 1500 mg BID (Reddy et al., 2007). HCV RNA levels were unchanged at Day 15 in six subjects who were given oral placebo in this study. In a four week, randomized, placebo-controlled phase I trial, 88% and 85% of treatment-naïve subjects, who were given 1000 or 1500 mg BID of mericitabine in combination with SOC (IFN/RBV), achieved undetectable RNA levels, respectively (Rodriguez-Torres et al., 2008). By contrast, only 19% subjects in SOC control group achieved no virus detectable level.

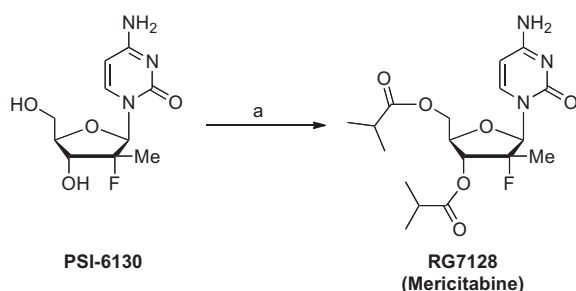
6.2.2. Phase IIb

In one of the phase IIb clinical trials (PROPEL) (Jensen et al., 2010; Wedemeyer et al., 2013), 408 GT1 and GT4 treatment-naïve subjects were randomized into one of five treatment arms: arm A:

mericitabine 500 mg BID for 12 weeks; arm B: mericitabine 1000 mg BID for 8 weeks; arm C: mericitabine 1000 mg BID for 12 weeks; arm D: mericitabine 1000 mg BID for 12 weeks; and arm E: placebo BID for 12 weeks. All subjects received IFN/RBV at standard doses for 24 or 48 weeks during and after mericitabine/placebo treatment. Subjects in arm A, B and C who achieved extended rapid virologic response (eRVR) stopped all treatment at week 24. Subjects who did not have an eRVR, and persons in arm D and E continued SOC to complete the 48 weeks treatment. Overall, the virologic response rates were higher in arms A–D than in arm E at weeks 4 and 12. However, the arms D (50.6%) and E (placebo, 51.2%) had similar overall SVR24 rates (primary outcome) with lower rates in the response-guided therapy arms A, B and C (48.8%, 42.0% and 32.9%, respectively).

These data demonstrated that treatment with mericitabine plus IFN/RBV for 8 or 12 weeks provided potent suppression of HCV RNA, was well tolerated, did not result in selection of resistant variants; however, it did not increase SVR rates compared with placebo.

An analysis from the now completed phase IIb JUMP-C trial was reported by Pockros et al. (2013). A total of 166 treatment-naïve persons infected with HCV GT1 or GT4 were randomized to one of two treatment arms. Subjects randomized to arm A were given mericitabine BID plus SOC (IFN/RBV) with response guided therapy (RGT). Persons on arm A who achieved eRVR (extended rapid virologic response; means no virus detected at week 4 and week 12) stopped all treatment at week 24. Those persons who did not achieve an eRVR also stopped the mericitabine at week 24, but continued an additional 24 weeks of SOC treatment. The subjects in control group arm B received SOC treatment for 48 weeks. The results of JUMP-C phase IIb trials showed that mericitabine was safe, and associated with higher SVR rates when compared to SOC (56.8% vs. 36.5%). The virologic response obtained at weeks 4 and 12 in the JUMP-C trial was comparable to results observed with the PROPEL trials. However, the authors note that extending treatment period from 12 to 24 weeks in the PROPEL study was not as effective in increasing SVR rates as found in the JUMP-C study. The difference in SVR rates may have contributed to differences observed in viral breakthrough rates since the PROPEL study showed an almost two-fold increase in relapse as compared to JUMP-C results (Pockros et al., 2013).



Scheme 4. Synthesis of **RG7128**. Reagents and conditions: (a) DMAP, THF/H₂O, TEA, isobutryl chloride, pH 6.4, 0 °C.

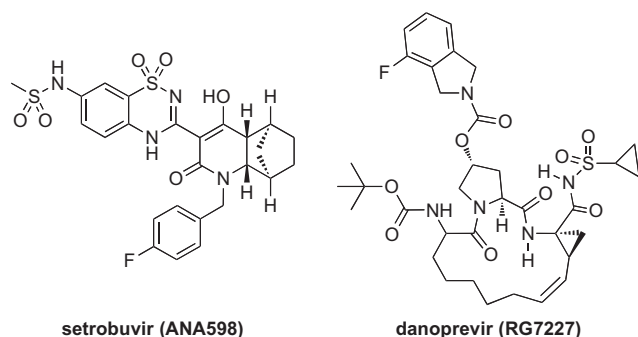


Fig. 8. Structures of setrobuvir (**ANA598**, non-nucleoside polymerase inhibitor) and danoprevir (**RG7227**, protease inhibitor).

It is worth noting that a combination of mericitabine plus SOC was also effective in treating HCV GT2/3 relapsers or non-responder subjects (Gane et al., 2010). Twenty persons with GT2/3 infections, who had not achieved SVR with previous treatments were given mericitabine 1500 mg BID plus SOC for 4 weeks, followed by SOC alone for 22–24 weeks. Ninety percent (18/20) of persons achieved RVR and 65% (13/20) achieved SVR at 12 weeks. This trial demonstrated that mericitabine plus SOC provided an effective regimen for the treatment of HCV GT2 and GT3 infected persons who have failed previous treatments.

INFORM-SVR, an ongoing phase IIb clinical trial, aims to evaluate the safety and efficacy of an all-oral IFN-free treatment with mericitabine and ritonavir-boosted danoprevir (**RG7227**, protease inhibitor, Fig. 8), with or without **RBV** in GT1a/1b treatment-naïve subjects. Among the different arms of the study, a 15% virus breakthrough rate was reported for GT1a and 1b persons (Gane et al., 2012). Viral breakthrough was associated with virus harboring protease inhibitor resistance conferring mutations. Importantly, a transient S282T mutation, an NS5B polymerase mutation associated with mericitabine resistance, was selected in one person.

Finally, ANNAPURNA, an ongoing phase IIb clinical trial is evaluating the combination of setrobuvir (**ANA598**, **RO5466731**, non-nucleoside polymerase inhibitor, Fig. 8), danoprevir (**RG7227**, **RO5190591**, protease inhibitor, Fig. 8) and ribavirin with or without mericitabine in patients with CHC who are either treatment-naïve or have previously experienced a null response to interferon-based treatment (Fig. 8). With an expected completion date of December 2013, this two-part five arm study will be looking at SVR12 and safety as end points. In part 2, based on results from part 1, further treatment-naïve subjects will receive a successful regimen from part 1, or a reduced intensity regimen, and interferon-based null-response persons will be added to the study (Roche, 2012).

6.3. Conclusions

The INFORM-SVR study is considered a milestone game changing combination since it demonstrated for the first time that an all-oral drug combination could suppress HCV replication to levels similar or exceeding SOC. It is also one of very few studies that show the selection of nucleoside analog associated resistance conferring mutations in humans, even though the apparently resistant virus was transient. The S282T mutation was not observed during other clinical triple combination trials with or without IFN, suggesting that the high barrier to resistance of mericitabine, and the low fitness of the mutant virus reduce the incidence of viral breakthrough caused by nucleoside analog resistance selection (Jensen et al., 2010; Le Pogam et al., 2010; Pockros et al., 2011, 2013). The high rate of viral suppression with mericitabine

combined with significant relapse rates without mutation selection indicates that longer treatments, higher doses, and/or other antiviral combinations may be necessary to make mericitabine a major player in HCV treatment. This may be due to the short half-life of **PSI-6130-TP**, **PSI-6130-TP** not reaching a viral reservoir(s), and/or overall lack of potency of **PSI-6130-TP**.

7. IDX184

7.1. Synthesis of **IDX184**

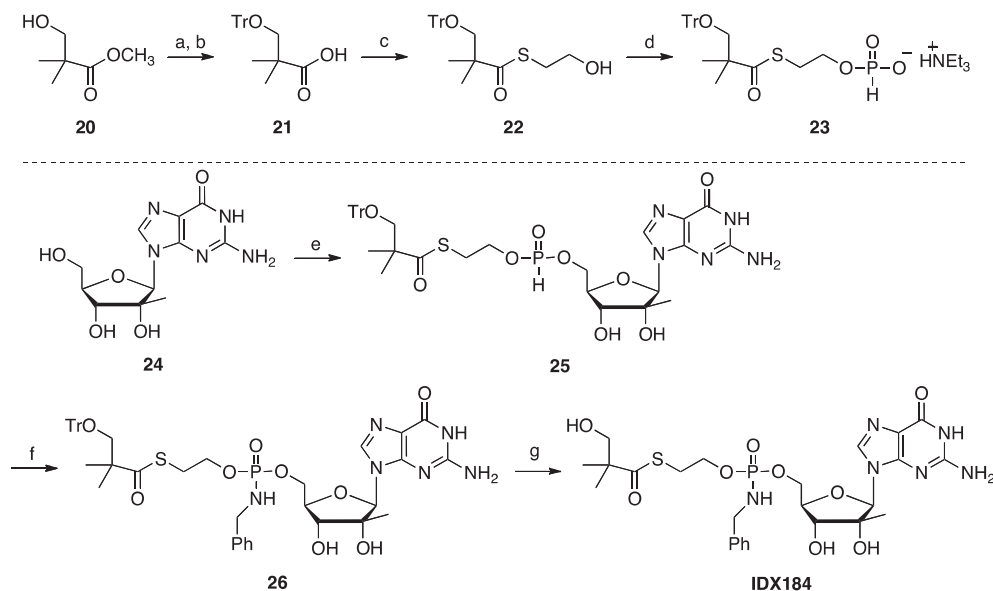
IDX184 (Cretton-Scott et al., 2010; Sommadossi et al., 2008; Surleraux and Gosselin, 2011, 2012) was designed by Idenix Pharmaceuticals as a 2'-C-methyl guanosine (2'-C-MeG) ribonucleoside that contains a S-acyl-2-thioethyl (SATE) phosphoramidate prodrug to selectively deliver the 5'-MP of 2'-C-methylguanosine ribonucleoside to human liver. The metabolism of **IDX184** to 2'-MeG-MP takes place predominantly in liver cells and presumably involves cytochrome P450 (CYP450)-dependent and -independent processes (Zhou et al., 2011). By bypassing the rate-limiting monophosphorylation process, 2'-MeG-MP is then efficiently converted to its active 5'-TP within hepatocytes by cellular kinases. The complete metabolic pathway of **IDX184** is yet to be fully elucidated. Liver targeting may result in a favorable therapeutic index by increasing the levels of the active 2'-MeG-TP in liver cells while lowering systemic exposure to the nucleoside, potentially minimizing the risk of systemic side effects (Cretton-Scott et al., 2008).

IDX184 was synthesized as shown in Scheme 5 (Sommadosi et al., 2008). Preparation of the *H*-phosphonate monoester precursor was achieved in three steps from commercially available 2,2-dimethyl-3-hydroxypropanoic acid methyl ester **20**, by protection of the alcohol followed by saponification, leading to compound **21** in 92% yield without purification. Installation of the side chain was performed by peptidic coupling between compound **21** and 2-mercaptoethanol, to generate alcohol **22**. Finally, treatment of compound **22** with phosphorus acid and pivaloyl chloride, followed by quenching the reaction with triethylammonium bicarbonate (TEAB), generated *H*-phosphonate monoester precursor **23** in 90% over two steps.

2'-C-Methylguanosine **24** and precursor **23** were then both treated with pivaloyl chloride to furnish intermediate **25**, which was further reacted with benzylamine to generate Tr-protected phosphoramidate diester **26** in quantitative yield. Classical deprotection with trifluoroacetic acid led to the isolation of **IDX184** in 39% yield (Scheme 5).

7.2. Preclinical development

In preclinical studies using an HCV replicon assay, **IDX184** exhibited 10 times greater potency than nucleosides that were in clinical development at the time. In a seven-day preclinical toxicology study of **IDX184**, no toxicities, including gastrointestinal or hematological, were observed in monkeys at doses greater than or equal to 600 mg/kg/day. In HCV GT1 infected chimpanzees, once daily oral administration of 10 mg/kg of **IDX184** produced a rapid and potent inhibition of HCV replication with mean viral load reductions of 2.5–4.0 log₁₀ after four days of dosing (Standing et al., 2008, 2009). Studies were also performed to characterize resistance mutations profiles of **IDX184**. The S282T mutation was identified in all tested cell lines and emerged around 25–77 days. Although the S282T mutant gave **IDX184** a 23-fold increase in EC₅₀, low (5%) replicon capacity of S282T replicons was also observed as previously reported by Ludmerer et al. (2005). The signature S282T mutant and other drug-related resistance mutants were only established *in vitro* for cell-based administration as ultimately



Scheme 5. Synthesis of **IDX184**. Reagents and conditions: (a) TrCl, Et₃N, DMAP, CH₂Cl₂, reflux, overnight; (b) NaOH, dioxane, reflux, 16 h, 92% over two steps; (c) i. CDI, toluene/DMF (2/1, v/v), r.t., 30 min; ii. 2-mercaptoethanol, toluene/DMF (2/1, v/v), −10 °C, 3 h; (d) i. H₃PO₃, PivCl, pyridine, 0 °C to r.t., 3 h; ii. TEAB, 90% over two steps; (e) **(23)**, PivCl, pyridine, −15 °C, 2 h, 32%; (f) Benzylamine, CCl₄, r.t., 1 h, quantitative yield; and (g) TFA, CH₂Cl₂, r.t., 30 min, 39%.

there were no mutant resistance found in clinical trials and no cross-resistance between **IDX184** and other classes of HCV antiviral agents (McCarville et al., 2011, 2010). **IDX184** demonstrated additive antiviral activity in the HCV replicon in combination with HCV protease inhibitors and **IFN** and synergistic activity in combination with **RBV**. **IDX184** also remained fully active *in vitro* against HCV containing known protease and non-nucleoside inhibitor drug resistance mutations (Zhou et al., 2011, 2009). As a result of its favorable preclinical profile, **IDX184** was selected as Idenix's lead candidate in January 2008 and Phase I clinical trials were started in July of the same year.

7.3. Clinical development

7.3.1. Phase Ia

In July 2008, a randomized, placebo-controlled, dose-escalating study was designed to evaluate the safety and tolerability of **IDX184** after oral administration in healthy subjects. Doses escalating from 5 to 100 mg were administered to forty-eight healthy subjects for three days, and PK data were collected (Idenix, 2008b). The most common adverse event was dizziness, 5.6% of occurrences for the **IDX184** treated group compared to 2.5% for the placebo cohort. Mild to moderate adverse events occurred and resolved by the end of the study. No severe adverse effects (SAE) or dose-limiting toxicity were observed. The level of **IDX184** and 2'-C-MeG in plasma remained low and dose-dependent, consistent with a liver-targeting approach. The short half-life of **IDX184** (around 1 h) in plasma suggested that **IDX184** was unlikely going to accumulate over time. In comparison, 2'-MeG in plasma has a long half-life with $t_{1/2}$ of approximate 18–42 h for doses above 25 mg, which reflected the long half-life of 2'-MeG-TP in the liver. The C_{24h} of 2'-C-MeG is 0.58–2.88 ng/mL for dose higher than 25 mg of **IDX184**. Overall, **IDX184** was safe and well tolerated at a single dose up to 600 mg/kg/day. The favorable PK supported once-daily dose administration therapy in future studies involving HCV infected individuals.

7.3.2. Phase Ib

In December 2008, a double-blind, dose-escalating study (Phase Ib) began to evaluate the safety and antiviral activity of **IDX184** in

fourty-one treatment-naïve subjects infected with GT1 chronic hepatitis C (Idenix, 2008a). These persons received 25 mg QD, 50 mg QD, 75 mg QD and 100 mg QD or placebo QD for 3 days. **IDX184** was safe and well tolerated: the occurrences of adverse events were following similar pattern as the placebo-treated group with the most frequently adverse events reported being headache and dizziness. The PK analysis data in the study was consistent with those of the previous healthy volunteers study. The mean HCV RNA reduction (log₁₀) ranged from 0.47 to 0.74 after treatment with 25–100 mg cohorts at day four. Significant HCV viral load decline occurred in persons receiving doses from 75 to 100 mg (Lalezari et al., 2012, 2009, 2010). This study helped to determine optimal doses that could be chosen for testing in later studies.

7.3.3. Phase IIa

In November 2009, a total of eighty subjects were enrolled to evaluate the short-term safety, the antiviral activity and PK profile of **IDX184** in combination with **IFN/RBV** in subjects with GT1 chronic hepatitis C infection. Over 14 days, individuals enrolled in this phase IIa, randomized, double-blind study (Idenix, 2009) were given sequential doses of 50 mg QD, 50 mg BID, 100 mg QD, 100 mg BID, 150 mg QD, 200 mg QD of **IDX184** in combination with **IFN/RBV** and **IFN/RBV** as the placebo cohort. Most adverse events were mild, and no serious adverse events were associated with **IDX184** plus **IFN/RBV** treatment. The pattern of side effects was similar between treatment of **IDX184** plus **IFN/RBV** and treatment of **IFN/RBV** alone. At day 15 of **IDX184** combination treatment, mean viral load reduction (log₁₀) was: 50 mg QD (−2.7), 50 mg BID (−4.0), 100 mg QD (−4.2), 100 mg BID (−4.1), 150 mg QD (−4.3), 200 mg QD (−3.7) of **IDX184** in combination with **IFN/RBV** and −1.5 for **IFN/RBV**. For cohorts given **IDX184** 50 mg QD, 100 mg BID and 200 mg QD, undetectable viral level (<15 IU/mL) were found in 13–29% of the persons, whereas for 50 mg BID, 100 mg QD and 150 mg QD, 40–50% of the individuals achieved undetectable viral levels. Moreover, all **IDX184** treated cohorts led to a low level of mean alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels compared to **IFN/RBV** cohort. In addition, the mean plasma concentration of 2'-MeG, which was identified as predictor of viral load started to

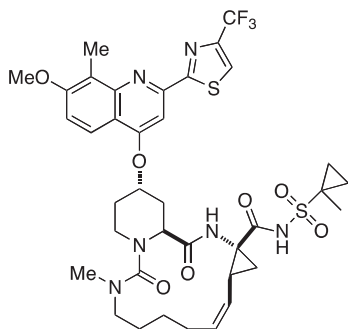


Fig. 9. Chemical structure of HCV protease inhibitor **IDX320**.

decline at Day 14 (Lalezari et al., 2013, 2010; Membreno and Lawitz, 2011).

7.3.4. Phase I combination of **IDX184** and **IDX320**

In June 2010, a clinical drug-drug interaction study was initiated to evaluate the combination of **IDX320** (a macrocyclic HCV protease inhibitor, Fig. 9) and **IDX184** in healthy subjects (Idenix, 2010). This study was designed as a double blind, multiple-dose study of twenty subjects who were administered 100 mg/day of **IDX184** in combination with 400 mg of **IDX320**. The first cohort (8:2) was either treated with **IDX320/IDX184** matching placebo for seven days followed by seven additional days with **IDX320/IDX184**, or **IDX320** matching placebo/**IDX184** matching placebo. The second cohort (8:2) was given **IDX184/IDX320** matching placebo for seven days followed by seven additional days **IDX184/IDX320** or **IDX184** matching placebo/**IDX320** matching placebo. This study was designed to evaluate safety and tolerability and to assess the potential for a pharmacokinetic drug-drug interaction between **IDX320** and **IDX184** when the two drugs were administered in combination in healthy subjects. In September 2010, the FDA placed a clinical hold on **IDX184** and **IDX320** programs based on three serious adverse events (elevated liver function tests) that occurred during this combination study in healthy volunteers. In February 2011, the FDA removed the full clinical hold on **IDX184** and placed it on partial clinical hold allowing Idenix to advance in the clinic under the partial clinical hold (Idenix, 2011a).

7.3.5. Phase I

In April 2011, an open-label study was initiated to evaluate the relative bioavailability of **IDX184** and any potential food effect in healthy male subjects (Idenix, 2011b). The purpose of this study was to compare the amount of drug in the blood after taking either a capsule or a tablet form of the drug while fasting. Additionally, the amount of drug present in the blood after eating a meal was investigated and the safety of the tablet form of **IDX184** was evaluated in healthy volunteers. Twelve subjects received 50 mg BID **IDX184** capsules without food on Day 1, 50 mg BID **IDX184** tablets without food on Day 8, and 50 mg BID **IDX184** tablets with food on Day 15. Blood levels of **IDX184** were measured in each study participant to determine pharmacokinetic parameters including bioavailability of **IDX184**, AUC and C_{max} of **IDX184** and 2'-C-MeG. Results of this study have not been disclosed at the time of this publication.

7.3.6. Phase IIb

In July 2011, Idenix initiated a double-blind study of sixty-seven randomized subjects to evaluate the safety and the antiviral activity of **IDX184** in combination with IFN/RBV at doses of 50 or 100 mg QD for 12 weeks. The common adverse events were fatigue, nausea and headache, which were generally mild to moderate.

No treatment-related cardiac or renal serious adverse events were observed during the study and the occurrences of all adverse events were similar between **IDX184**-combination therapy and IFN/RBV-therapy. Echocardiograms and N-terminal pro B-type natriuretic peptide (NT-proBNP), a biomarker that aids in the detection of congestive heart failure and cardiac dysfunction, were added in this study to address potential cardiac concerns. Heart rate, AV conduction and cardiac depolarization remained normal. Several subjects in each group developed new T-wave inversions that were not judged to be clinically significant. The increases seen in NT-proBNP levels were greater than 200% of baseline, but reversible. In the 50 and 100 mg of **IDX184** in combination with IFN/RBV cohorts, 94% and 87% of subjects, respectively, achieved undetectable virus level at a median of 8 weeks of treatment. At week 12, a 76% and 82% rate of complete EVR was observed in the 50 and 100 mg treatment arms, respectively (Lawitz et al., 2012a). In January 2012, Idenix submitted interim phase IIb data to the FDA requesting for the removal of the partial clinical hold on **IDX184**. In February 2012, the partial clinical hold on **IDX184** was removed by the FDA allowing the initiation of dosing in the phase IIb study.

7.4. Conclusions

In August 2012, the FDA placed **IDX184** and **IDX19368** (another HCV nucleotide polymerase inhibitor for which Idenix had previously filed an IND, but had not initiated clinical studies) on partial clinical hold due to cardiac adverse events seen in the phase II clinical trial of **BMS-986094** (refer to Section 15 of this manuscript). All three drugs are 2'-C-methyl guanosine nucleotide prodrugs. In December 2012, Idenix completed the submission of requested cardiac safety data for **IDX184** to the FDA. Idenix had found no evidence for severe cardiac effects in all of their clinical studies to date. In the meantime, in October 2012, Idenix started a three-year observational study to evaluate the durability of sustained viral response and the kinetics of antiviral-resistant HCV in subjects who participated in studies of Idenix anti-HCV direct acting antivirals, including **IDX184**. In February 2013, the FDA communicated that **IDX184** (and **IDX19368**) programs would remain on clinical hold, and, as a result, Idenix decided to terminate the development of these programs. A reason for FDA concern over **IDX184** and related compounds is the resultant NTP derived from **IDX184** is identical to the NTP derived from **BMS-986094**. Cardiac toxicity for **BMS-986094** was demonstrated to be dose-related and the postulated reason for the cardiac toxicity was mitochondrial toxicity. Of note, 2'-C-MeG-TP has been shown to be a substrate for incorporation by human mitochondrial RNA polymerase (Arnold et al., 2012). However, cell free assays can be misleading since the nucleoside may never be phosphorylated in mitochondria. Nevertheless, Idenix decided not to pursue this drug further since by then they had identified nucleoside analogs with superior virological and toxicological profiles.

8. PSI-6206 to PSI-7851 to GS-7977 (PSI-7977; sofosbuvir)

8.1. Discovery and preclinical profiling

The presence of 2'-deoxy-2'-fluoro-2'-C-methyluridine **PSI-6206** metabolite along with its triphosphate **PSI-6206-TP** was recognized during metabolism studies on **PSI-6130** in cell culture and in monkeys (see discussion Section 5.2) (Fig. 10). These compounds were evaluated for their anti-HCV activity in replicon assay (Ma et al., 2007). While the uridine nucleoside **PSI-6206** was devoid of anti-HCV activity in culture, **PSI-6206-TP** demonstrated a potent and selective inhibition of HCV NS5B polymerase with mean IC_{50} = 1.19 μ M. Subsequent stability studies using primary human

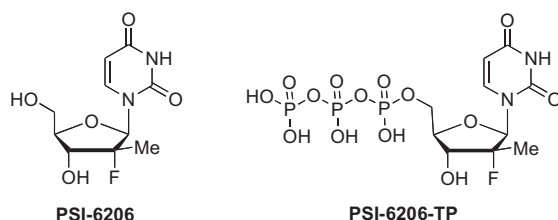


Fig. 10. Structure of 2'-F-2'-C-methyluridine, **PSI-6206**, and its triphosphate **PSI-6206-TP**.

hepatocytes showed that **PSI-6206-TP** had significantly longer half life ($t_{1/2}$ = 38 h) than its analogous cytidine triphosphate **PSI-6310-TP** ($t_{1/2}$ = 6 h) (Murakami et al., 2008).

Cellular pharmacology with **PSI-6206** demonstrated that it was very poorly phosphorylated to the monophosphate nucleotide (**PSI-6206-MP**), which could explain the lack of anti-HCV activity. The phosphoramidate prodrug approach was taken to bypass the initial unproductive phosphorylation step (Murakami et al., 2010). After *in vitro* and *in vivo* profiling of multiple potential prodrugs, phosphoramidate prodrug **PSI-7851** was selected as the lead compound. **PSI-7851**, an equal mixture of two phosphorous diastereoisomers **PSI-7976** and **PSI-7977** (Berrey et al., 2013; Chun et al., 2010; Clark, 2005; Cleary et al., 2013; Ross et al., 2010; Sofia et al., 2008) was shown to be a potent and selective inhibitor of HCV replication (EC_{50} = 0.52 μ M) with no associated bone marrow, mitochondrial or other cellular toxicity. (Fig. 11) Its potential efficiency was further demonstrated by high levels of triphosphate (**PSI-6206-TP**) formation in primary human hepatocytes and in the livers of rats, dogs and monkeys when administered *in vivo* (Sofia et al., 2010a). *In vitro* studies, showed **PSI-7851** had a broad range of antiviral activity across different HCV GTs (Lam et al., 2012). Based on impressive preclinical data, **PSI-7851** was selected for future development and progressed into phase I human clinical trials.

8.2. Synthesis of **PSI-7851** and **PSI-7977** (**GS-7977**)

Subsequently, a method was developed to separate the two **PSI-7977** and **PSI-7976** diastereoisomers. The S_p diastereoisomer **PSI-7977** (EC_{90} = 0.42 μ M) was shown to be more potent inhibitor than the R_p diastereoisomer **PSI-7976** (EC_{90} = 7.5 μ M) in replicon assays (Sofia et al., 2010a). However, when both diastereoisomers were tested in primary human hepatocytes almost equal amounts of active triphosphate (**PSI-6206-TP**) was formed, suggesting both isomers may be equally active in the liver cells (Murakami et al., 2010). Enzymatic studies showed Cathepsin A (CatA) and carboxylesterase 1 (CES) could both catalyze the first step hydrolysis (of the carboxyl ester linkage between the carboxylic acid from amino acid moiety and the isopropyl alcohol) of phosphoramidate

prodrugs **PSI-7977** and **PSI-7976**. **PSI-7977** was found to be a better substrate for CatA while **PSI-7976** was preferentially hydrolyzed by CES1 (Murakami et al., 2010). The apparent discrepancy between replicon data and primary human hepatocytes was related to the different expression of these enzymes in replicon cell lines and primary human hepatocytes (Murakami et al., 2010; Sofia et al., 2010a). Both isomers were devoid of cytotoxicity when tested against a broad range of cell lines. Mitochondrial toxicity was evaluated up to 100 μ M in both CEM and HepG2 cells and no toxicity was observed for **PSI-7977** while **PSI-7976** showed CC_{90} of 72 and 68.6 μ M for inhibition of mtDNA and rDNA, respectively in HepG2 cells (Sofia et al., 2010a). Pure S_p isomer **PSI-7977** was selected for future clinical studies due to its higher potency in replicon assay and better safety profile. To date, **PSI-7977** (now called sofosbuvir or **GS-7977**) is the only nucleotide analog HCV polymerase inhibitor that has completed phase III clinical trials and this drug is expected to receive FDA approval by the end of 2013.

8.3. Synthesis of single isomer **PSI-7977** (sofosbuvir, **GS-7977**)

The first generation synthesis of **PSI-7977** involved direct reaction of phosphoryl chloride reagent **28a** with 2'-deoxy-2'-fluoro-2'-C-methyluridine (**PSI-6206**) and separation of the two diastereoisomers by crystallization (Sofia et al., 2010a) (Scheme 6). Ultimately, with some modifications to the chemistry and workups during process development, the yield from (**28a**) to the mix of **PSI-7976** and **PSI-7977** (48:51) was improved to 45.7% from which the single isomer **PSI-7977** was isolated in 15% yield after crystallization (Ross et al., 2010).

This approach was further optimized by using *p*-nitrophenol as the leaving group of the phosphorylating agent as shown in (**28b**) (Scheme 6). In this case a 47% of a 3:1 mix of **PSI-7977** and **PSI-7976** were obtained. Separation of (**28b**) into its single diastereoisomers gave the desired chiral phosphorylating reagent **28c**, which upon reaction under basic conditions gave clean inversion at the phosphorous centers to give **PSI-7977** as the single desired S_p diastereomer. Pentafluorophenyl was used in a similar manner with the phosphorylating reagent **28d** which allowed for a multigram synthesis of single S_p isomer **PSI-7977** without the need for a chiral catalyst, chiral auxiliary or column purification (Ross et al., 2011, 2010) (Scheme 7).

8.4. Clinical trials for **PSI-7977** (**GS-7977**; sofosbuvir)

8.4.1. Phase I

In March 2009, clinical trials for **PSI-7851** (also known as **GS-9851**; mixture of diastereoisomers **PSI-7976** and sofosbuvir) were completed in 42 healthy volunteers. The administration of single dosage of 25 mg up to 800 mg QD was shown to be generally safe

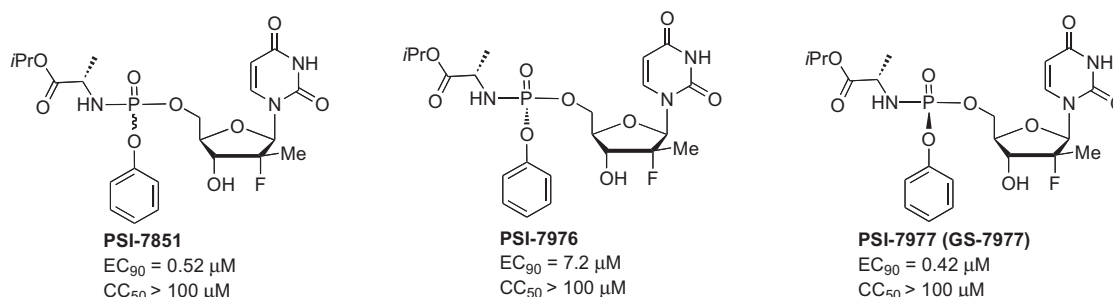
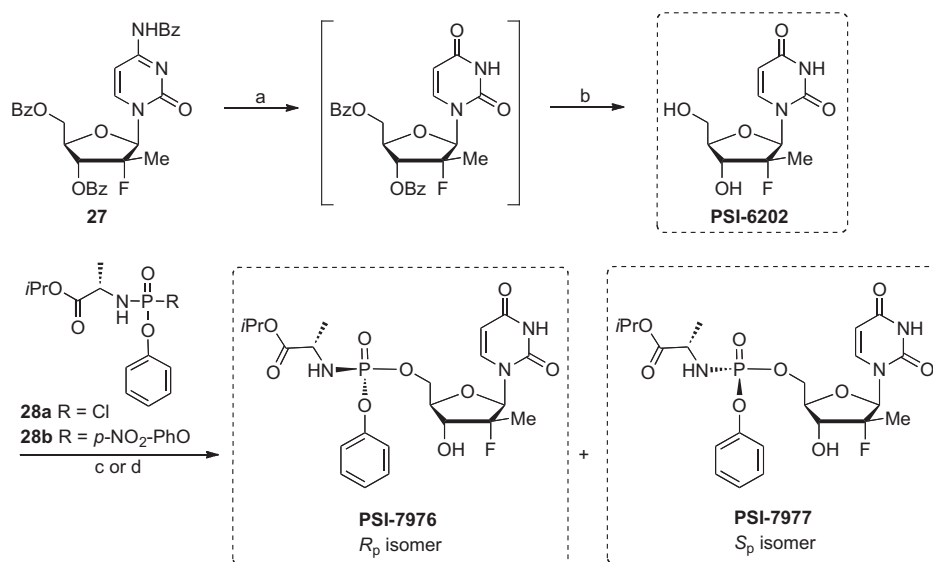
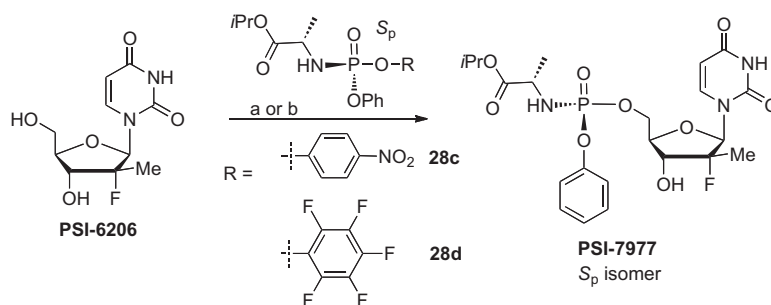


Fig. 11. Structures, anti-HCV replication activity and cytotoxicity of phosphoramidate prodrug **PSI-7851** and its diastereoisomers **PSI-7976** and **PSI-7977**.



Scheme 6. Synthesis of **PSI-7976** and **PSI-7977** (sofosbuvir, **GS-7977**) via column purification. Reaction conditions: (a) 70% aqueous acetic acid, 100 °C; (b) 25% methanolic ammonia, 0–15 °C, 78% overall yield; (c) (**28a**) (*R_p*:*S_p* = 1:1) (6.5 equiv), NMI (8 equiv), CH₂Cl₂, r.t., overnight, 6%, 1:1 mix of **PSI-7976** and **PSI-7977**; and (d) (**28b**) (*R_p*:*S_p* ≈ 1:1) (2 equiv), *t*-BuMgCl (2.1 equiv), THF, r.t., 48 h, 47%, 3:1 mix **PSI-7977**:**PSI-7976**.



Scheme 7. Synthesis of single isomer **PSI-7977** (sofosbuvir, **GS-7977**). Reaction conditions: (a) THF, *t*-BuMgCl (2.1 equiv) added over 30 min at r.t., stirred 30 min then (**28c**), r.t., 60 h, 40%; and (b) THF, *t*-BuMgCl (2.1 equiv) added over 30 min at –5 °C, stirred 30 min, warmed up to 20 °C and stirred for 30 min and then cooled to 5 °C then (**28d**) (1.2 equiv) was added over 30 min, stirred for 18 h at 5 °C, 68%.

and well tolerated with no adverse events when compared with the placebo arm and with a pharmacokinetic profile consistent with once-daily dosing (Denning et al., 2012). Phase Ia trials with **PSI-7851** also evaluated safety and tolerability for three day monotherapy at different doses (50, 100, 200, 400 mg) in treatment-naïve subjects. Reductions in viral load were observed without any serious adverse events when compared to the placebo arm (Lawitz et al., 2013c). In fact, the 400 mg QD dose led to a decrease of 1.76 log₁₀ IU/mL after three days of treatment compared to the related mericitabine, which after 14 days at 1500 mg with QD dosing, gave a 1.48 log₁₀ IU/mL drop.

8.4.2. Phase IIa

The safety, antiviral activity and tolerability of three different doses of sofosbuvir in combination with **IFN/RBV** were assessed in treatment-naïve persons with GT1 HCV infection. Sixty-four subjects were randomized (1:1:1:1) to receive one of three once-daily doses of sofosbuvir (100, 200 and 400 mg) or placebo plus **IFN/RBV** for 28 days, which was followed by 44 weeks treatment with **IFN/RBV** alone therapy. All who received sofosbuvir experienced rapid virologic response (RVR) of 88%, 94% and 93% for 100, 200, and 400 mg dose, respectively. In comparison, only 21% of the subjects achieved RVR in the control arm. No viral breakthrough was observed during 28 days treatment; however, eight

individuals had relapsed during **IFN/RBV** treatment (four on sofosbuvir 100 mg; two on sofosbuvir 400 mg and two on placebo arm). SVR24 rates were higher for subjects in all arms with sofosbuvir treatment (56% for 100 mg dose, 83% for 200 mg dose, and 84% for 400 mg dose) than those in the **IFN/RBV** arm (43% achieved SVR after 24 weeks). The most frequent adverse effects during 28 days treatment were fatigue, nausea, chills, headache and joint pain. No serious adverse events or discontinuation was reported in first 28 days. Sofosbuvir 200 and 400 mg doses were selected for future studies (Rodríguez-Torres et al., 2013).

8.4.3. Phase IIb

Several phase IIb clinical trials have addressed the safety and efficacy of sofosbuvir treatment in the treatment of HCV GTs. Results from four studies (PROTON, ELECTRON, QUANTUM and ATOMIC) are summarized below.

8.4.3.1. Proton. In this double-blind, randomized study the efficacy of sofosbuvir in combination with **IFN** and **RBV** in treatment-naïve persons with HCV GT1, 2 and 3 was evaluated. A total of 121 treatment-naïve subjects with GT1 infection were randomly assigned to three different groups (2:2:1 ratio) to receive **IFN/RBV** with 200 mg sofosbuvir, 400 mg sofosbuvir, or placebo for 12 weeks followed by additional **IFN/RBV** treatment for 12 or 36 weeks

depending on the subject's response. Among GT1 persons, 98% of subjects on triple therapy achieved RVR compared to 19% in the placebo group. 88% of those on 200 mg dose of sofosbuvir and 91% of those on 400 mg sofosbuvir achieved SVR12 while less than 50% achieved SVR in placebo cohort. All IL28-BT/T GT persons included in the study (13/13) achieved SVR12 following triple therapy. Finally, combination therapy with sofosbuvir was even more effective for treatment-naïve persons with GT2/3 HCV infections. In this cohort, all 24 subjects who stayed on therapy achieved SVR12 (Gane et al., 2013b; Lawitz et al., 2013b; Pham et al., 2004; Radkowski et al., 2005).

8.4.3.2. Electron. This study was designed to evaluate the efficacy of sofosbuvir with **RBV**, with or without **IFN** in treatment-naïve or treatment-experienced persons with GT1, 2, or 3 HCV infections. Sofosbuvir monotherapy, reductions in **RBV** dose, and changes in duration of therapy were also evaluated. One hundred and fifty-five subjects with all three HCV GTs were divided into eleven treatment groups (Radkowski et al., 2005). In a randomized cohort, forty treatment-naïve subjects with HCV GT2 and GT3 infections were divided into four arms. All groups received sofosbuvir (400 mg QD) + **RBV** BID for 12 weeks. Three of the arms also received **IFN** for 4, 8 and 12 weeks, respectively. SVR24 was achieved for all subjects enrolled in the four arms (cohorts 1–4) (Gane et al., 2013b, 2012). In an additional arm (cohort 5), wherein ten treatment-naïve persons with HCV GT2 or GT3 received sofosbuvir monotherapy for 12 weeks, SVR24 rates were 60%. A shortened 8-week triple therapy arm (cohort 6) was also evaluated in ten GT2 or GT3 subjects and SVR24 was achieved for all participating subjects. In cohort 7, twenty-five treatment-naïve subjects with GT2 or GT3 were treated with sofosbuvir plus **RBV** for 8 weeks. Full (100%) SVR was achieved in this cohort. Cohort 8 evaluated treatment of ten GT2/3 subjects with sofosbuvir+ reduced dose **RBV** (800 mg) and resulted in a reduced SVR rate of ~60%. Twenty-five treatment-experienced subjects with GT2 and GT3 HCV infections were treated with sofosbuvir and **RBV** (cohort 9). All subjects in this arm achieved RVR while 68% attained SVR at 12 weeks. Finally, a 12-week **IFN**-free sofosbuvir plus **RBV** treatment was evaluated in ten GT1 null-responders (cohort 10) and in twenty-five GT1 treatment-naïve subjects (cohort 11). Treatment-naïve subjects with GT1 achieved SVR12 of 84% while only one out of ten null-responder subjects achieved SVR12 (Table 1).

In summary, 12-week treatment with sofosbuvir and **RBV** resulted in 100% SVR24 in treatment-naïve, GT2/3 subjects. This regimen was less optimal for treatment-experienced subjects with GT2/3 or null-responders with GT1 infections. It was concluded that duration of less than 12 weeks or reduced **RBV** dose might adversely impact treatment efficacy. Reduced dosage of **RBV** in treatment-

naïve difficult GT1 subjects in 24 weeks treatment was less efficient (SVR4 56%) than full dose **RBV** (77%). Addition of sofosbuvir to **IFN** + **RBV** regimen considerably improved the response rate among all GTs and shortened the duration of therapy. **IFN**-free combination showed high percent of cure rate in untreated individuals, but was less efficient in treatment-experienced patients or null-responders.

8.4.3.3. Atomic. This phase IIb clinical trial evaluated the combination of sofosbuvir with **IFN**/**RBV** for 12- or 24-weeks in treatment-naïve GT1, GT4, and GT6 persons (a total of 332 subjects were enrolled). Additionally, treatment-naïve persons with GT1 were treated with sofosbuvir in combination with **IFN**/**RBV** followed by 12 week treatment with sofosbuvir alone or in combination with **RBV**. The results from this study suggest that 12 week triple combination therapy was as effective as 24 week treatment for this group of subjects. Furthermore, it was found that sofosbuvir, with or without **RBV** after initial treatment, can be effective in achieving high SVR rate (Kowdley et al., 2013).

8.4.3.4. Phase II sponsored by the National Institute of Allergy and Infectious Diseases (NIAID). Initiated in 2011, NIH investigators recruited 60 GT1 HCV treatment-naïve subjects, into a two-part open label study (Gilead, 2011). As a proof of concept, in the first part, ten patients with mild to moderate liver fibrosis were treated with 400 mg/day of sofosbuvir and weight-based **RBV** for 24 weeks. All nine subjects who completed the 24-week course had an undetectable level of the virus 24-weeks post treatment and were deemed cured. Subsequently, in the second group of fifty subjects where thirteen suffered from serious liver damage, all took 400 mg/day sofosbuvir with the subjects being split between weight-based and low-dose (600 mg/day) **RBV**. SVR-24 rates of 68% were observed in the weight-based group and 48% in the low-dose group (Osinusi et al., 2013). Seven grade 3 adverse events were reported (anemia, neutropenia, nausea, hypophosphatemia, and cholelithiasis or pancreatitis), but none resulted in discontinuation of treatment. This NIH-sponsored study of Gilead's sofosbuvir combined with **RBV** demonstrated a safe and effective interferon-free approach for even some of the toughest HCV cases.

8.4.4. Phase III

Multiple phase III clinical trials have been initiated to evaluate the safety and efficacy of HCV treatments that contain sofosbuvir. Results from four trials (FUSION, FISSION, POSITRON and NEUTRINO) are summarized below.

8.4.4.1. Fusion. In June 2012, the randomized, double-blind trial was initiated in 200 treatment-experienced individuals with GT2 and GT3 who were assigned to two treatment groups to receive

Table 1
Participants with HCV infection and results of the ELECTRON study.

Cohort #	# of patients	Genotype	Sofosbuvir (weeks)	RBV (weeks)	IFN (weeks)	SVR (%)
1	10 ^a	GT2 and 3	400 mg QD (12)	1000 mg BID (12)	(4)	SVR24 (100)
2	10 ^a	GT2 and 3	400 mg (12)	1000 mg BID (12)	(8)	SVR24 (100)
3	10 ^a	GT2 and 3	400 mg (12)	1000 mg BID (12)	(12)	SVR24 (100)
4	10 ^a	GT2 and 3	400 mg (12)	1000 mg BID (12)	None	SVR24 (100)
5	10 ^a	GT2 and 3	400 mg (12)	None	None	SVR24 (60)
6	10 ^a	GT2 and 3	400 mg (8)	1000 mg BID (8)	(8)	SVR24 (100)
7	25 ^a	GT2 and 3	400 mg (8)	1000 mg BID (8)	None	SVR24 (100)
8	10 ^a	GT2 and 3	400 mg (8)	800 mg BID (8)	None	SVR24 (60)
9	25 ^b	GT2 and 3	400 mg (8)	1000 mg BID (8)	None	RVR (100) SVR12 (68)
10	10 ^c	GT1	400 mg (12)	1000 mg BID (12)	None	SVR12 (10)
11	25 ^a	GT1	400 mg (12)	1000 mg BID (12)	None	SVR12 (84)

^a Treatment-naïve patients.

^b Treatment-experimented patients.

^c Null-responders.

sofosbuvir (400 mg daily) with **RBV** (1000 or 1200 mg) for duration of 12 or 16 weeks. The study also included persons with compensated cirrhosis at baseline. In the 12-week treatment group the SVR12 rate was 86% for GT2 and 30% for GT3 infected subjects. In the 16-week treatment group SVR12 rate was 94% for GT2 and 62% for GT3 subjects. No adverse side effects were observed that were attributed to sofosbuvir treatment (Gilead, 2013d).

8.4.4.2. Fission. This randomized, active-controlled study, initiated in December 2012, aimed to compare 12-week course with sofosbuvir 400 mg/**RBV** with 24-week standard of care treatment **IFN/RBV** treatment in genotype 2 and 3 treatment-naïve persons. The study met its primary efficacy end point with 67% of subjects achieving SVR12 in both groups. All common adverse effects ($\geq 10\%$) occurred more frequently in **IFN/RBV** arm. Most frequently observed adverse events ($\geq 10\%$) in the sofosbuvir/**RBV** group were fatigue, headache, nausea, insomnia and dizziness (Gilead, 2013a).

8.4.4.3. Positron. This randomized, double-blind, placebo controlled study was initiated in March 2012 and was designed to evaluate sofosbuvir plus **RBV** for persons who were **IFN**-intolerant or ineligible/unwilling to take **IFN**. Two hundred and seven subjects with GT2 and GT3 HCV infections were enrolled. Of those, 15% had compensated cirrhosis and 53% were GT2. Among individuals with GT2, 93% achieved SVR12, among those with GT3 and the small percentage that had cirrhosis, 61% also achieved SVR-12 (Gilead, 2012); however the overall SVR12 rate was 78% in GT3 and GT4 combined.

8.4.4.4. Neutrino. This study was initiated in June 2012 in 327 treatment-naïve subjects with GT1, GT4, GT5 and GT6 to evaluate treatment outcomes of a 12-week course of sofosbuvir with **IFN** and **RBV** treatment. Overall, triple combination was found to be superior with 90% of subjects achieving SVR12 compared to a predefined historic control SVR rate of 60 percent. The most common adverse events (reported in $>20\%$) were fatigue, headache, nausea, insomnia and anemia (Gilead, 2013a).

8.5. Conclusions

Sofosbuvir, **PSI-7977** (now **GS-7977**) is the most promising pan-genotypic antiviral drugs against HCV infection identified to date. In combination with SOC or other DAAs such as protease and NS5A inhibitors (see below), it demonstrated high percentage of cure rate among all GTs. Like earlier, anti-HCV nucleoside analogs sofosbuvir showed a clear synergistic effect when dosed in combination with **RBV**. Two small and separate early clinical studies showed that **RBV** could lower HCV viral load, but did not cure any subjects tested (Di Bisceglie et al., 1992; Reichard et al., 1991). In our hands, **RBV** has no anti-HCV activity in the Huh7 replicon system suggesting that the antiviral effect may be due to a host effect (Bassit et al., 2008).

SVR rates were consistently lower among GT3 patients in phase III studies reporting preliminary data. Early *in vitro* genotype studies with sofosbuvir found similar EC₉₀ values for inhibition of GT1b (Con1)-, GT1a (H77)-, and GT2a (JFH-1)-derived replicons and for GT1b chimeric replicons containing the NS5B region from the J6 GT2a isolate and from GT2b and GT3a isolates (Lam et al., 2012). Thus, there was no published preclinical indication of lower efficacy versus GT3 and it remains to be seen if this pattern holds true as more data are disclosed.

On April 8, 2013, based on these clinical studies, Gilead submitted a new drug application to U.S. FDA for the use of sofosbuvir and **RBV** as an all-oral regimen in GT2 and three individuals and in combination with **IFN/RBV** for the treatment-naïve persons with GT1, 4, 5 and 6 HCV infection (Gilead, 2013c). On October 25, 2013, an FDA advisory committee voted unanimously (15–0) in favor of approval of the use of sofosbuvir in persons infected with GT2 and GT3 in combination with **RBV**. The committee also recommended (15–0) sofosbuvir in combination with **IFN** and **RBV** for treatment of chronic HCV in treatment-naïve adults with GT1 and GT4 infections.

On December 6, 2013 Gilead Sciences, Inc. announced that the FDA had approved sofosbuvir (Sovaldi™) 400 mg tablets, a once-daily oral nucleotide analog polymerase inhibitor for the treatment of chronic HCV infection as a component of a combination antiviral treatment regimen. Sofosbuvir was approved for HCV genotypes 1, 2, 3 or 4 infection, including those with hepatocellular carcinoma meeting Milan criteria (awaiting liver transplantation) and those with HCV/HIV-1 co-infection.

9. Combinations of sofosbuvir with other DAAs

9.1. Sofosbuvir (**PSI-7977**; **GS-7977**) plus daclatasvir (**BMS-790052**)

The all-oral, **IFN/RBV**-free, combination of direct acting antivirals (DAA) involving sofosbuvir an NS5B polymerase inhibitor, and daclatasvir (**BMS-790052**) (Fig. 12), an NS5A inhibitor, has been explored as an attractive approach toward HCV treatment (Sulkowski et al., 2012a,b).

9.1.1. Phase IIa

The first combination study of sofosbuvir and daclatasvir with or without **RBV** was reported in a phase IIa trial (AI444-040 study) in treatment-naïve subjects with GT1a/1b, GT2, or GT3 HCV infection (Sulkowski et al., 2012a). Eighty-eight subjects were enrolled to receive sofosbuvir (400 mg QD) and daclatasvir (60 mg QD) in combinations with or without **RBV** for 24 weeks. HCV RNA levels were monitored up to 48 weeks after discontinuation of antiviral therapy. Based on the interim results, overall, more than 95% of all treatment-naïve subjects with any GT tested achieved SVR4, but in GT1a/1b subjects the SVR4 was 100% (HCV RNA <10 IU/mL). The combination of sofosbuvir and daclatasvir was generally

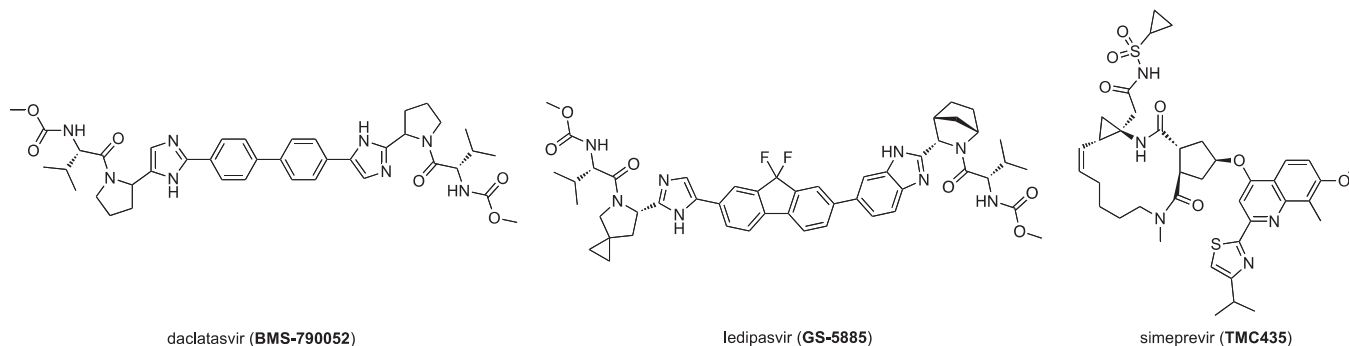


Fig. 12. Structures of daclatasvir, ledipasvir and simeprevir.

well tolerated, and, of special significance, **RBV** only contributed to adverse effects and had no effect on virologic response.

An additional eighty-two GT1a/1b subjects were randomized to the A1444-040 study to receive sofosbuvir (400 mg QD) and daclatasvir (60 mg QD) in combination with or without **RBV** for 12 weeks. In a recent update of the study (Sulkowski et al., 2012b), with a total of 170 treatment-naïve individuals with GT1a/1b, GT2, or GT3 HCV infection enrolled to receive sofosbuvir (400 mg QD) and daclatasvir (60 mg QD) in combinations with or without **RBV** for 24 weeks, in GT1a/1b, GT2, or GT3 and 12 weeks in GT1a/1b more than 93% of all treatment-naïve subjects achieved SVR. Importantly, 43 out of 44 (98%) GT1 subjects achieved SVR24 and 41 out of 44 (93%) of GT2 and GT3 subjects achieved SVR24. *IL28B* GT, viral subtype, and the administration of **RBV** had no effect on virologic response. The combination of sofosbuvir and daclatasvir were generally safe and well tolerated. The development of anemia in ≤21% subjects was only observed with **RBV** treatment.

9.1.2. Phase II

The safety and efficacy of sofosbuvir (400 mg QD) and daclatasvir (60 mg QD) in combination with or without **RBV** for 24 weeks was evaluated in forty-one GT1 subjects who experienced virologic failure with previous telaprevir- or boceprevir-based triple therapy. Sofosbuvir plus daclatasvir arm and sofosbuvir plus daclatasvir plus **RBV** arm demonstrated high SVR12 rate of 100% and 95%, respectively (Sulkowski et al., 2013).

9.2. Sofosbuvir plus ledipasvir (GS-5885)

The multipart phase II ELECTRON study aimed to evaluate the safety and efficacy of sofosbuvir-containing regimens with or without **IFN** and/or **RBV** in GT1, GT2 and GT3 individuals (Gane et al., 2013b) (See Section 8.3.3.2 for additional details). One arm of this study focused on an all-oral regimen in GT1 treatment-naïve and null-responders in which subjects were treated with sofosbuvir and **RBV** over 12 weeks (Gane et al., 2013a). Other arms of the study were similar in design, but also included ledipasvir (**GS-5885**, Fig. 12), a direct acting antiviral NS5A inhibitor. Among treatment-naïve subjects ($N = 25$) and null-responders ($N = 10$) who received sofosbuvir plus **RBV** without ledipasvir, SVR12 rates were 84% and 10%, respectively. However, all individuals [treatment-naïve ($N = 25$) and null-responders ($N = 10$)] receiving ledipasvir in combination with sofosbuvir and **RBV** showed 100% SVR12 regardless of treatment history (Gane et al., 2013a). Common adverse events included anemia, depression and headache.

There are currently several ongoing phase II and phase III clinical studies for evaluating the safety, tolerability and antiviral efficacy of a once daily fixed-dose combination tablet containing ledipasvir (90 mg), and sofosbuvir (400 mg) with or without **RBV** (1000 or 1200 mg/day divided BID) in GT1 HCV infected subjects. The latest updates from these trials are summarized in the sections below (Gilead, 2013b).

9.2.1. ION-1

In October 2012, Gilead launched a phase III trial for evaluating safety and efficacy of sofosbuvir and ledipasvir with and without **RBV** for 12 or 24 weeks among treatment-naïve GT1 subjects. Each arm enrolled fifty individuals (total 200 subjects). Based on the initial results of the 12-week arms, the trial will expand to include 800 subjects (Gilead, 2013b).

9.2.2. ION-2

A second phase III study for fixed-dose sofosbuvir/ledipasvir began in January 2013. The study plans to enroll 400 treatment-experienced GT1 subjects who have failed previous **IFN**-containing regimens (with or without protease inhibitors). This study will

evaluate treatment response in a 12-week regimen with **RBV** and a 24-week regimen with or without **RBV** (Gilead, 2013b).

9.2.3. LONESTAR

A new Phase II clinical trial is now underway to study sofosbuvir/ledipasvir for 12 weeks and of sofosbuvir/ledipasvir with and without **RBV** for 8 weeks. This is the first trial to enroll hundred GT1 treatment-naïve persons for the shorter 8-week treatment duration. Treatment with sofosbuvir plus ledipasvir (with or without **RBV**) for 12 weeks will also be evaluated for treatment-experienced GT1 persons who have failed the protease inhibitor-containing regimen (Gilead, 2013b).

9.2.4. ION-3

In May 2013, Gilead started a Phase III, multicenter, randomized, open-label study to evaluate the safety, tolerability and antiviral efficacy of sofosbuvir/ledipasvir (400/90 mg) with or without **RBV** (1000 or 1200 mg/day divided BID) administered once daily for eight weeks versus sofosbuvir/ledipasvir (SOF/LDV) administered once daily for 12 weeks in treatment-naïve non-cirrhotic subjects with chronic GT1 HCV infection (Gilead, 2013e). The primary outcomes of this study are to determine the sustained virologic response twelve weeks after discontinuation of therapy (SVR12), as well as monitoring the number/frequency of adverse events and potential laboratory abnormalities.

9.3. PSI-7977 (GS-7977; sofosbuvir) plus simeprevir (TMC435)

Simeprevir (**TMC435**) (Fig. 12) is an HCV protease inhibitor currently in phase III clinical trials for HCV infection. In November 2011, a phase II clinical trial (the COSMOS study) evaluating the combination of sofosbuvir and simeprevir with or without **RBV** in individuals who are HCV GT1 null-responders to prior **IFN**/**RBV** with METAVIR stage either F0–F2 (cohort 1) or F3–F4 (cohort 2) was initiated. The study aims to randomize ninety subjects per cohort in a 2:1:2:1 ratio to the following arms: simeprevir 150 mg once daily + sofosbuvir 400 mg once daily for 24 weeks with or without **RBV**, or simeprevir 150 mg once daily + sofosbuvir 400 mg once daily for 12 weeks with or without **RBV**. For cohort 1, a safety and efficacy interim analysis was conducted when approximately 20% of the planned randomized patients reached the end of treatment or permanently discontinued treatment early. Interim data demonstrated RVR in 13/15, 8/11, 20/23, 5/10 in simeprevir with sofosbuvir and **RBV** (24 weeks), simeprevir and sofosbuvir (24 weeks), simeprevir with sofosbuvir and **RBV** (12 weeks), and simeprevir plus sofosbuvir (12 weeks), respectively (Lawitz et al., 2013a). Similar RVR rates were observed with simeprevir plus sofosbuvir + **RBV** treated for 12 or 24 weeks. However, lower RVR rates were observed without **RBV** for 12 (50%) and 24 week (72%) treatments. Interim SVR4 results from the 12 week arms of cohort 2 in the ongoing COSMOS study, including treatment-naïve or previous null-responder HCV patients all with METAVIR score F3–F4 were recently reported. Treatment for 12 weeks with simeprevir and sofosbuvir, with or without ribavirin, led to SVR4 rates of 96% and 100%, respectively (Medivir, 2013). All arms of this study at 12 weeks were generally considered safe and well tolerated. However, anemia and increased bilirubin was observed in 13.0% and 9.3% of the patients in the **RBV** containing arms, respectively. These events were not observed in the non-**RBV** containing arms.

9.4. Sofosbuvir (GS-7977; PSI-7977) a pyrimidine plus PSI-938 a purine nucleotide analog

For information on the phase II NUCLEAR and QUANTUM trials refer to Sections 11.3.2 and 11.3.3 of this manuscript.

10. R7432

In 2010, Roche initiated a phase I clinical trial of **R7432** in a dose-escalation study with sixty persons enrolled (Roche, 2010). This study was discontinued by February of 2012 (Roche, 2011a,b). Unfortunately, the chemical structure of **R7432**, the clinical trial results and an explanation for the discontinuation has not been disclosed.

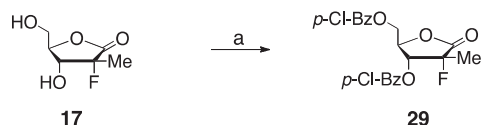
11. PSI-938 (PSI-352938)

Purine nucleoside derivatives of mericitabine were designed to be second-generation agents with improved potency, pharmacokinetics, and resistance profiles. The guanosine derivative **PSI-938** (also known as **PSI-352938**) was a result of these studies (Chun et al., 2010; Clark, 2005; Du et al., 2009; Reddy et al., 2010; Sofia et al., 2010b). This derivative is a cyclic phosphate prodrug of a 2'- α -F-2'- β -C-methyl class of nucleosides. The choice for this particular prodrug was based on observations from previous studies which showed that nucleotide cyclic phosphate prodrugs were precursors of the nucleotide monophosphate with enhanced cellular permeability *in vitro* (Gunic et al., 2007).

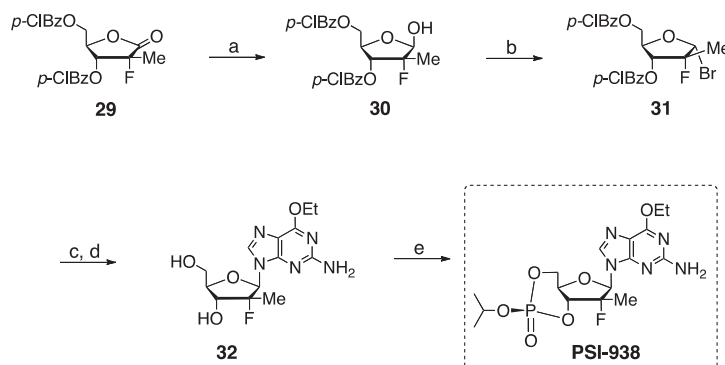
11.1. Synthesis of PSI-938

Pharmasset reported a multi-kilogram process synthesis of **PSI-938** requiring no chromatography (Reddy et al., 2011; Wang et al., 2009). 2'- α -F-2'- β -C-methyl-2'-deoxy lactone **29** was prepared as described in Scheme 8. Benzoylation was originally used to complete the synthesis of a key intermediate similar in structure to compound **29** (Scheme 8). However, a switch to 4-chlorobenzyl protection afforded more crystalline intermediates that allowed for a multi-step synthesis that did not require any column chromatography.

The conclusion of the **PSI-938** synthesis is shown in Scheme 9. The *p*-chlorobenzoyl protected lactone **29** was reduced to lactol **30**, and it is interesting to note that upon standing the β/α anomeric mixture isomerized to the thermodynamically more stable β -lactol. An Appel reaction at low temperature then furnished bromo derivative **31**. Glycosylation of compound **31** with 2-amino-6-



Scheme 8. Synthesis of **PSI-938** intermediate lactone **29**. Reagents and conditions: (a) *p*-Cl-BzCl, pyr., r.t., 70%.



Scheme 9. Synthesis of **PSI-938**. Reagents and conditions: (a) LiAl(*t*-BuO)₃H, THF, -20 °C, 61%; (b) CBr₄, PPh₃, CH₂Cl₂, -20 °C, 1 h, 79%; (c) 2-amino-6-chloropurine, *t*-BuOK, *t*-BuOH, CH₃CN, 50 °C, 16 h, 65%; (d) NaOEt, EtOH, reflux, 1 h, 92%; and (e) isopropylidichlorophosphate, NMI, Et₃N, CH₂Cl₂, 52%.

chloro purine followed by treatment with sodium ethoxide in ethanol provided the unprotected 6-ethoxy purine nucleoside **32**. After extensive investigation, conditions were found for the formation of the 3',5'-cyclic phosphate **PSI-938** (*cis*-R_p configuration) in high diastereoselectivity (>95%); it was finally isolated in 52% yield and greater than 99.5% diastereoselectivity after direct crystallization.

11.2. Preclinical development

In an HCV replicon cell assay, **PSI-938** had an EC₉₀ of 1.37 μ M with no cytotoxicity up to 100 μ M (Reddy et al., 2010). It demonstrated prolonged stability in simulated gastrointestinal fluid, simulated intestinal fluid, human plasma, and human liver S9 (*in vitro* model for liver stability). Expanded cytotoxicity data showed no cytotoxicity in a variety of cell lines and no mitochondrial toxicity. Rats treated with the compound had high levels of the triphosphate in the liver. Interestingly, **PSI-938** was equally active against the NS5B S282T mutant replicon. It also showed a high barrier of resistance and a different resistance profile than that of **RG-7128** (Lam et al., 2011). **PSI-938** was found to be metabolized by a phosphorylation pathway distinct from **RG-7128**. These promising results lead Pharmasset to nominate **PSI-938** as a preclinical candidate in June 2009 (Pharmasset, 2009).

11.3. Clinical development

11.3.1. Phase I

A phase I single ascending dose trial began in early 2010 (Pharmasset, 2010). Healthy male and female volunteers received a single dose of **PSI-938** ranging from 100 to 1600 mg. No dose-limiting toxicity was identified. In August 2010, a multidose ascending dose trial was conducted in forty subjects chronically infected with HCV GT1. Subjects received 100 mg QD, 200 mg QD, 300 mg QD, and 100 mg BID for seven days. **PSI-938** demonstrated potent antiviral activity with an average drop of 4 log₁₀ IU/mL in mean HCV RNA from baseline. A consistent decline of HCV RNA levels was observed with no viral breakthrough. **PSI-938** was shown to be safe and well tolerated. No adverse events were reported as well as any subject discontinuations (Pharmasset, 2011c).

11.3.2. Phase IIa, NUCLEAR

The combination of sofosbuvir and **PSI-938** represented the first once daily dual-nucleotide IFN/RBV-free study for HCV treatment (Lawitz et al., 2011). Sofosbuvir, a pyrimidine, and **PSI-938**, a purine, are both NS5B polymerase inhibitors. In March 2011, Pharmasset published the results of the Phase IIa NUCLEAR trial in HCV GT1 treatment-naïve subjects. The study evaluated the safety, PK and

antiviral activity of **PSI-938** and sofosbuvir administered as monotherapy or in combination for seven to fourteen days. Forty subjects were enrolled in cohorts 1–4 receiving **PSI-938** (300 mg QD) as monotherapy and/or sofosbuvir (400 mg QD) in combinations, and the treatment regimens resulted in median drops of HCV RNA that ranged from 5.0 to 5.2 log₁₀ IU/mL. **PSI-938** was generally safe and well tolerated over 7–14 days, with no adverse events or subject discontinuations. HCV RNA rapidly declined in the first three days followed by continued decrease until end of treatment or assay limit of detection (LOD) was reached. Of the 24 subjects who received the combination treatment, 22 (92%) achieved HCV RNA levels of <15 IU/mL after fourteen days of treatment. Of note, sofosbuvir and **PSI-938** had similar HCV RNA reductions over seven days. The NUCLEAR study suggested that the combination of sofosbuvir and **PSI-938** was generally safe and well tolerated with no viral breakthrough and no significant pharmacokinetic interaction during the seven to fourteen day therapy. These promising results were followed by FDA Fast Track designation in August 2011 (Pharmasset, 2011d).

11.3.3. Phase IIb, QUANTUM

In September 2011, Pharmasset initiated the Phase IIb QUANTUM trial in approximately 450 adults of all viral genotypes to assess safety and SVR rates at 12 or 24 weeks for **PSI-938**, **PSI-7977** (sofosbuvir), and various combinations of the drugs with and without **RBV** (Pharmasset, 2011c). The trial evaluated interferon-free regimens of sofosbuvir 400 mg QD and **PSI-938** 300 mg QD with and without **RBV** over 12 or 24 weeks in treatment-naïve patients. The trial also evaluated **PSI-938** monotherapy. SVR24 was the primary endpoint of the trial with patients equally randomized across the following arms: (1) **PSI-938** only; (2) **PSI-938** and sofosbuvir; (3) sofosbuvir and **RBV**; (4) **PSI-938**, sofosbuvir, and **RBV** and (5) a placebo control of 24 weeks.

Unfortunately, routine safety monitoring detected “laboratory abnormalities associated with liver function” among the 235 individuals in the study who were receiving treatment with **PSI-938** alone or in combination with sofosbuvir or sofosbuvir and **RBV** (Pharmasset, 2011a). The remaining 12 and 24-week sofosbuvir and **RBV** combination arm continued unchanged to generate data in support of the NEUTRINO trial (see Section 8.4.4.4).

11.4. Conclusions

In December 2011, **PSI-938** was dropped from the follow-up phase IIb QUANTUM trial and further clinical evaluation due to abnormalities in liver function (Pharmasset, 2011b). No future plans for **PSI-938** have been reported. It is unfortunate that a relatively high dose of this drug was used for the main studies, since it is likely that a lower dose would have also been effective with no apparent untoward effects especially when used with sofosbuvir.

12. TMC649128

12.1. Preclinical development

TMC649128 (Johansson et al., 2008; Jonckers et al., 2011) is a nucleoside NS5B polymerase inhibitor and has demonstrated an attractive pre-clinical profile: it displayed *in vitro* activity across multiple HCV GTs with a high genetic barrier to resistance. It was disclosed in May 2008, when Medivir entered into an R&D agreement with Ortho Biotech Products LP, an affiliate of Tibotec, to develop medications to treat HCV infection targeting NS5B virus polymerase. Under the agreement, Medivir and Tibotec jointly performed preclinical studies to identify potential compounds. As an effort to explore 4'-azido-2'-modified cytidines as potential HCV polymerase inhibitors known for their antiviral HCV activities, Medivir's scientists designed and synthesized 4'-azido-2'-deoxy-2'-methyl-cytidine **33** (Nilsson et al., 2012) (Fig. 13), which exhibited a moderate anti-HCV activity (Johansson et al., 2008). Although all the ester prodrug derivatives only exhibited similar or slightly lower activity compared with (**33**), the isobutyryl ester (**TMC649128**) demonstrated greatly improved mean maximum plasma concentration (C_{\max} = 4.65 μ M) and hence a larger area under the curve (AUC_{0-t} = 12.7 3 μ M \times h) and oral bioavailability (F = 65%).

12.2. TMC649128 synthesis

TMC649128 was synthesized as shown in Scheme 10: Glycosylation of **4** with silylated uracil in the presence of tin chloride allowed for the formation of compound **33**. Treatment of compound **33** with ammonia in methanol led to the clean deprotection of the benzoyl group, leading to compound **34** in quantitative yield. Protection of the 3'- and 5'-hydroxyl groups with a TIPDS protective group, followed by deoxygenation via free-radical chemistry gave compound **36** in 97% yield with selectivity of 93:7 (Li and Piccirilli, 2003). Deprotection of the TIPDS group with TBAF generated compound **37** in high yield. Nucleoside **37** was then converted to the olefin compound **38** (Verheyden and Moffatt, 1975). Treating exocyclic olefin with iodine and benzyltriethylammonium azide, followed by benzoylation, provided 4'-azido nucleoside **40** in 81% yield over two steps. Oxidative nucleophilic substitution of compound **40** with *m*-chloroperbenzoic acid/*m*-chlorobenzoic acid in DCM afforded protected azido derivative **41**, which was readily converted to 4'-azido-2'-deoxy-2'-methyl cytidine (**42**) by treatment with 1-H-tetrazole and 4-chlorophenyldichlorophosphate followed by ammonia deprotection. Final acylation of both 3'- and 5'-hydroxy groups led to the formation of **TMC649128**.

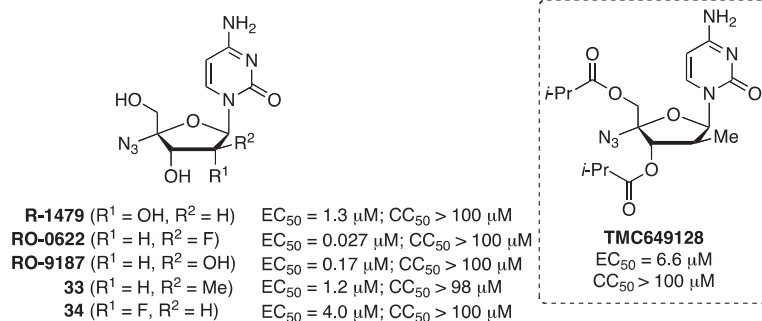
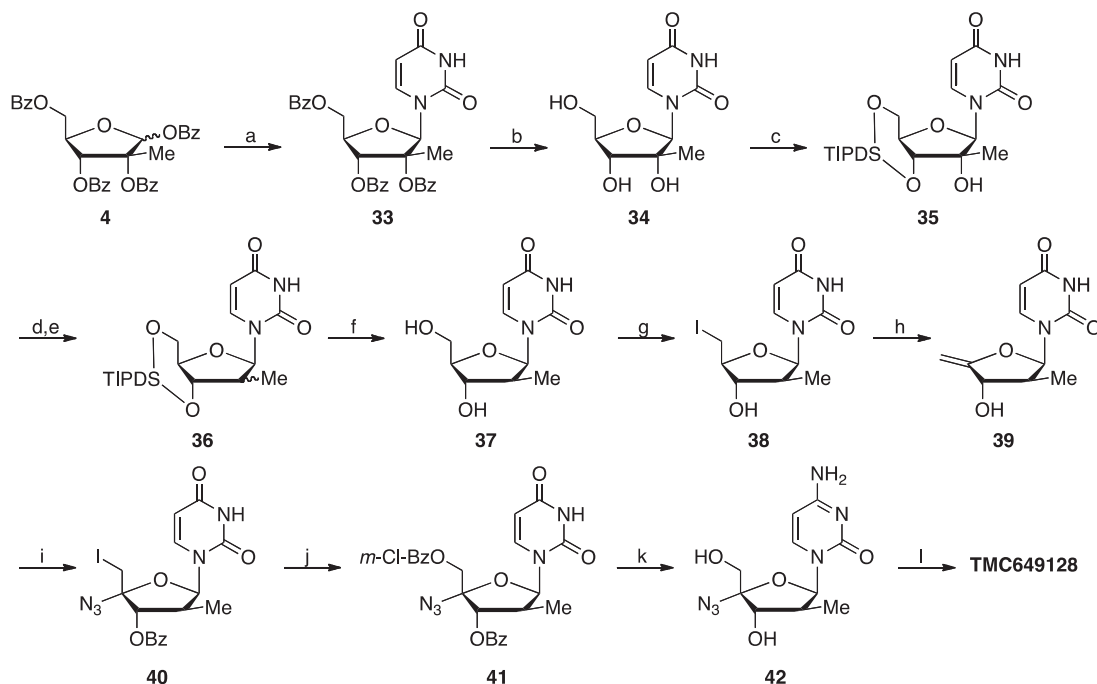


Fig. 13. Structure of prodrug **TMC649128** and related nucleosides.



Scheme 10. Synthesis of 4'-azido-2'-deoxy-2'-methyl cytidine (**TMC649128**). Reagents and conditions: (a) bis(trimethylsilyl)uracil, SnCl_4 , CH_3CN , r.t., 20 h, 96%; (b) NH_3 , CH_3OH , 0–4 °C, 2 days, >99%; (c) TIPDSCl₂, imidazole, DMF, r.t., 2 h, 65%; (d) ClCOCO_2Me , DMAP, CH_3CN , r.t., 1 h; (e) $n\text{-Bu}_3\text{SnH}$, AIBN, toluene, reflux, 2 h, 97% (β/α 93:7, de 86%) over two steps; (f) TBAF/THF, r.t., 1 h, >99% (β/α 93:7); (g) I_2 , PPh₃, imidazole, THF, 76%; (h) NaOMe, MeOH, 75%; (i) i. I_2 , NMO, $\text{Bn}(\text{Et})_3\text{NN}_3$; ii. BzCl , NMP, DMAP, THF, 81% over 2 steps; (j) $m\text{-CPBA}$, $(\text{NH}_4)_2\text{SO}_4$, DCM, 68%; (k) i. pyridine, 2-chlorophenyldichlorophosphate, 1-*H*-tetrazole; ii. dioxane, NH_3 ; iii. NH_3 , MeOH, 61% over 3 steps; and (l) $i\text{-PrCOCl}$, TEA, DCM, r.t., >99%.

12.3. Clinical development

12.3.1. Phase Ia

In January 2011, Medivir announced the start of phase Ia trial of the hepatitis C polymerase inhibitor **TMC649128**. This first in human, double-blind, randomized, placebo-controlled trial, was successfully completed in June 2011 to assess the safety, tolerability, and pharmacokinetics of increasing single oral doses of **TMC649128** in nine healthy volunteers. After each administration of the drug and up to four weeks after the last administration, safety assessments (blood and urine tests, blood pressure, pulse, electrocardiogram, and physical examination) were performed (Medivir, 2011b; Tibotec, 2011b).

12.3.2. Phase Ib

In June 2011, Tibotec initiated a phase Ib, double-blind, randomized and placebo-controlled study in GT1 HCV-infected individuals to evaluate the safety, tolerability, pharmacokinetics and antiviral activity of multiple ascending doses of **TMC649128** given as monotherapy and in combination with **IFN** and **RBV**. The pharmacokinetic/pharmacodynamic relationship for antiviral activity, active metabolite and safety of **TMC649128** and its metabolite were assessed. The short-term safety and tolerability of the co-administration of **TMC649128** and **IFN** plus **RBV** during multiple dosing for 14 days in treatment-naïve GT1 HCV-infected participants was explored. The short-term antiviral effect of the combination of **TMC649128** with **IFN** and **RBV** was also preliminary assessed (Medivir, 2011c; Tibotec, 2011a).

12.4. Conclusions

In November 2011, this study was terminated, since the antiviral activity was considered insufficient to warrant further clinical development of **TMC649128** (Medivir, 2011a).

13. GS-6620

13.1. Preclinical development

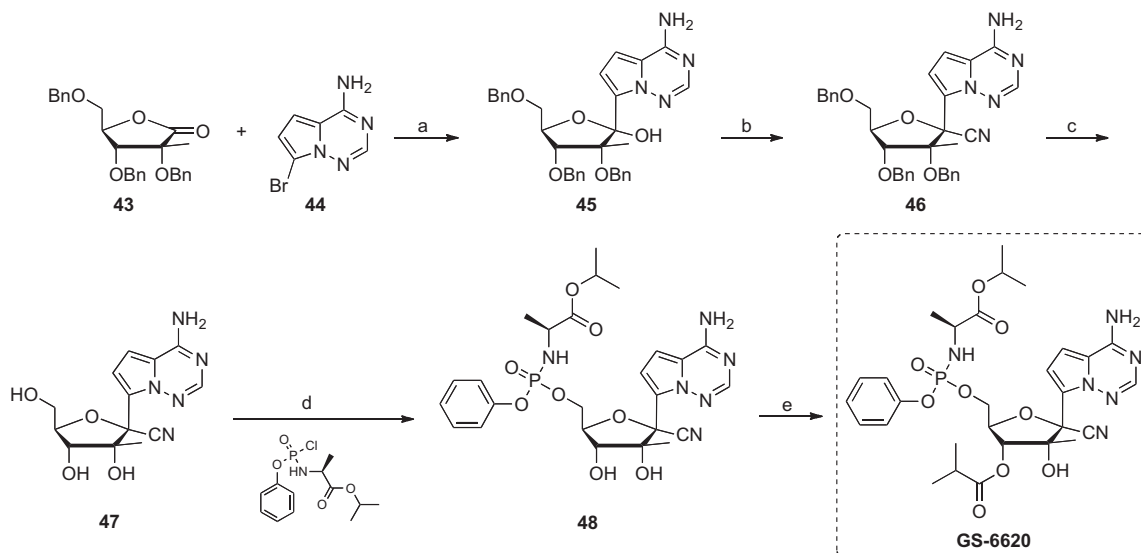
GS-6620 (Butler et al., 2011, 2009; Cho et al., 2011a,b; Cho and Wolckenhauser, 2011) is a pangenotypic, liver-targeted nucleotide prodrug developed by Gilead Sciences for the treatment of HCV. **GS-6620** was shown to have a favorable inhibitory profile *in vitro* whereby the incorporation of corresponding triphosphate resulted in chain-termination. Cell culture resistance selection assays reported the selection of the S282T mutation in the NS5B viral polymerase. This mutation conferred >200-fold resistance in *in vitro* experiments with purified recombination S282T mutant enzyme (Fenaux et al., 2011). Oral administration of **GS-6620** to animals resulted in efficient delivery of the nucleoside triphosphate metabolite into the liver (Lawitz et al., 2012b).

13.2. GS-6620 synthesis

The synthesis of **GS-6620** is outlined in Scheme 11 (Cho et al., 2013). The tribenzylated 2'-C-Me sugar **43** was efficiently connected to the heterocyclic base **44** under lithium-halogen exchange conditions. The 1'-cyano group was introduced under Lewis acid conditions to give **46** which was followed by a boron trichloride promoted debenzilation to give C-nucleoside analog **47**. Formation of the 5'-phosphoramidate and 3'-isobutyl ester are performed utilizing standard nucleoside protocols to give **GS-6620**.

13.3. Clinical development

The favorable preclinical characteristics of this compound made it a candidate for phase I clinical trials wherein safety, tolerability, pharmacokinetics and antiviral activity were evaluated in a multicenter, randomized, placebo controlled, multiple-dose escalation



Scheme 11. Synthesis of **GS-6620**. Reagents and conditions: (a) (**44**), 1,1,4,4-tetramethyl-1,4-dichlorodisilylene (1.0 equiv) and *n*-BuLi (3.3 equiv), THF, -78°C followed by addition of (**43**), 1 h, 65–80%; (b) TMSCN (6 equiv), TMSOTf (4 equiv), CH_2Cl_2 , -15 to 0°C , 2 h, 85–95%; (c) BCl_3 (3 equiv), CH_2Cl_2 , 0°C , 1 h, 90%; (d) *N*-methylimidazole, trimethylphosphate and THF, 0°C to r.t., 2 h, overall yield 59%; and (e) i. *N,N*-dimethylformamide dimethyl acetal, CH_3CN , r.t., 1 h; ii. isobutyric acid, DCC, CH_3CN , r.t., 48 h; iii. water-TFA, 0°C to r.t., 64 h, overall yield 73%.

first-in-man study in 2010. Out of the ninety treatment-naïve subjects enrolled in the study, twelve showed signs of mild adverse events related to treatment. Two subjects who received the highest dose of treatment (900 mg BID) had virus levels below the limit of detection at the end of the five-day dosing period.

13.4. Conclusions

Although **GS-6620** was safe and well tolerated over five day of administration period, it was found that exposure to the drug was markedly lower in humans as compared to pre-clinical animal studies with substantial intra- and inter-subject variability seen in all cohorts. Gilead Sciences indicated that without further optimization of drug delivery to improve PK and antiviral activity, future clinical development would not occur for **GS-6620** (Lawitz et al., 2012b).

14. ALS-2200 (VX-135) and ALS-2158

In June 2011, Vertex licensed both a purine and pyrimidine nucleoside prodrug inhibitors of HCV NS5B polymerase from Alios Bio-pharma (Vertex, 2011). Both uridine nucleoside analog **ALS-2200** (**VX-135**) and adenosine nucleotide analog **ALS-2158** (Beigelman et al., 2010, 2012a,b; Smith et al., 2012) entered phase I clinical trials in December 2011. The exact structure of these nucleoside analogs has not yet been disclosed.

14.1. ALS-2258 clinical development

In an early clinical study, the purine **ALS-2158** did not show sufficient antiviral activity and the study was discontinued in September 2012 (Vertex, 2012b).

14.2. ALS-2200 clinical development

On the other hand, preliminary seven-day viral kinetic data with the pyrimidine prodrug **ALS-2200** (**VX-135**) administered at 200 mg QD (Marcellin et al., 2012) demonstrated a median drop of 4.18 \log_{10} (range -3.6 , -5.2) in HCV RNA in combination with **RBV** in treatment-naïve chronic HCV subjects with GT1. Five of

eight subjects achieved HCV RNA levels below the limit of quantification, and two of these five achieved HCV RNA levels below the limit of detection after seven days of dosing. Similar results were obtained with monotherapy of **ALS-2200** (200 mg, once-daily, seven days). Adverse events during the study were mild to moderate with no subject discontinuations.

14.2.1. Phase II

As of November 2012 (Vertex, 2012a,d) future multiple phase II developments of **ALS-2200** (**VX-135**) were envisaged: the first one was initiated with GlaxoSmithKline's NS5A inhibitor **GSK2336805** (Vertex, 2012d) (Fig. 14) but was terminated in June 2013 when Vertex and GSK mutually decided to cease the collaboration for a phase II study and prioritized other projects. A second study is planned for the second half of 2013 with **VX-135** in combination with Janssen's protease inhibitor simeprevir (**TMC435**, Fig. 12) in patients with GT1 HCV (Vertex, 2012a). Vertex also planned to begin a study of **VX-135** in combination with protease inhibitor Incivek® (telaprevir), the company's existing drug for persons with chronic GT1 HCV. Two additional studies include evaluating the safety and efficacy of **VX-135** plus **RBV** in treatment-naïve subjects with chronic Hepatitis C and planned study to evaluate the safety, efficacy, pharmacokinetics and pharmacodynamics of **VX-135** and daclatasvir in GT1 chronic hepatitis C subjects. All of these Phase II studies will evaluate safety, tolerability and SVR12 using 12 week all oral regimens in subjects with GT1 HCV. So far, Vertex completed dosing of 100 and 200 mg of **VX-135** in combination with **RBV** in Europe and both doses were well tolerated with no discontinuations. No serious adverse events have been reported and no

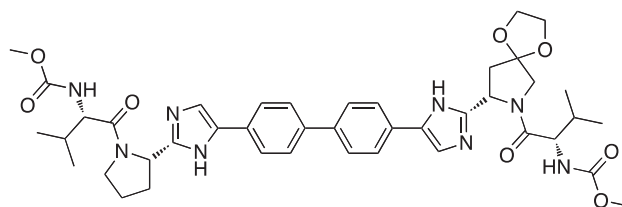


Fig. 14. Structure of **GSK2336805**.

liver or cardiac safety issues have been identified. Most recently, in April 2013, Vertex started a study of 100 and 200 mg doses of **VX-135** in combination with daclatasvir (**BMS-790052**), an NS5A inhibitor being developed by BMS (Vertex, 2012c), with results expected in early 2014. However, in July 2013, the FDA notified Vertex that a partial clinical hold had been placed on Vertex's ongoing phase II US study of **VX-135** (Vertex, 2013). This partial hold prevented further evaluation of the 200 mg dose of **VX-135** due to reversible elevated liver enzymes in patients receiving 400 mg of **VX-135** in combination with **RBV** in a European phase II study. Evaluation of the 100 mg dose of **VX-135** in combination with **RBV** in the U.S. continued. In addition, the recently initiated dosing of 100 and 200 mg of **VX-135** in combination with daclatasvir as part of a phase II study in New Zealand also continued.

15. BMS-986094 (INX-189/INX-08189)

15.1. Synthesis of **BMS-986094**

BMS-986094 (Chamberlain et al., 2010; McGuigan and Perrone, 2008) is a double prodrug of 2'-C-methylguanosine triphosphate developed by Inhibitex and later acquired by Bristol-Myers Squibb (Fig. 15). The synthetic route started with trimethylsilyl (TMS) triflate mediated glycosylation of tetrabenzoyl 2'-C-methyl sugar **4** and 6-chloroguanine. The resulting benzoylated 6-chloro nucleoside **49** was then converted to unprotected 6-O-methyl analog **50** by substitution of the chlorine with sodium methoxide in methanol at room temperature. For the prodrug portion, the phosphorochloridate **53** was prepared in two steps by treatment of 1-naphthol with phosphorus oxychloride in presence of triethylamine followed by reaction with *t*-BuCH₂-L-valine ester. Finally, reaction of (**53**) with (**50**) was conducted in THF in presence of

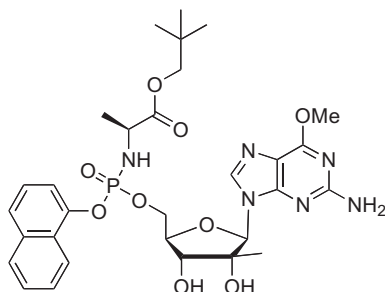
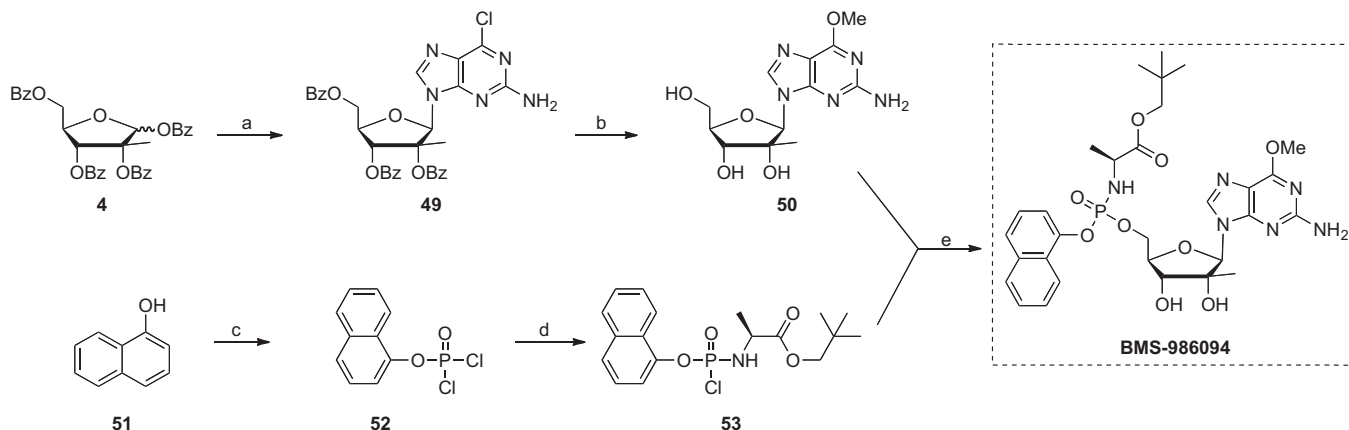


Fig. 15. Structure of **BMS-986094**.



Scheme 12. Synthesis of **BMS-986094**. Reagents and conditions: (a) 2-amino-6-chloropurine, DBU, MeCN, TMSOTf, 65 °C, 4 h, 79%; (b) NaOMe, MeOH, r.t., 24 h, 76%; (c) POCl₃, Et₃N, Et₂O, −78 °C, 1 h then r.t., overnight, 95%; (d) *t*BuCH₂-L-valine ester, Et₃N, DCM, −78 °C, 1 h then r.t., 2 h; and (e) NMI, THF, r.t., overnight, 18%.

N-methylimidazole (NMI) to generate the target compound, **BMS-986094**, in 18% yield as a mixture of diastereoisomers (Scheme 12) (McGuigan et al., 2010a,b). As the anti-HCV activity and estimated half-life values were very similar between the two phosphorous diastereomers, **BMS-986094** was tested as a mixture in the latter studies.

This 1-naphthyl neopentyl alanine phosphoramidate derivative of 6-O-methoxy-2'-C-methylguanosine showed potent inhibition in an HCV replicon 1b assay (EC₅₀ = 0.01 μM, EC₉₀ = 0.04 μM) and demonstrated a 500 fold greater potency than the parent 6-O-methoxy nucleoside (Kolykhalov et al., 2009; McGuigan et al., 2010a,b). However, in comparison with other clinical anti-HCV agents, **BMS-986094** was relatively toxic in multiple cell lines, with CC₅₀ values of 0.32, 2.23, 2.81, and 15.0 μM in Huh7 (human hepatoma), HepG2 (human hepatoma), BxPC3 (human pancreatic), and CEM (human T lymphocyte) cells, respectively (Furman et al., 2011). Other studies showed that **BMS-986094** caused bone marrow toxicity in the low micromolar range along with toxicity in kidney, blood, skeletal muscle, CNS and intestinal cells. Additional studies suggested that **BMS-986094** could cause unique tissue specific toxicity based on *in vitro* assay results, with the lowest CC₅₀ values in bone marrow cells (Hutchins et al., 2009; Standing, 2012). Furthermore, in a 14-day study, no appreciable nucleoside analog-associated mitochondrial toxicity was observed in CEM and HepG2 cells treated with **BMS-986094** up to 1 μM (Vernachio et al., 2011). It is not clear why higher concentrations were not reported.

15.2. Clinical development

15.2.1. Phase Ia

In a first-in-human, single-ascending oral dose (3–100 mg or a matching placebo) phase Ia study, **BMS-986094** was well tolerated with pharmacokinetics that supported once daily dosing (Patti et al., 2011). In a separate double-blind, placebo-controlled, dose-escalation study, 70 treatment-naïve HCV GT1 subjects dosed with **BMS-986094** at 9, 25, 50, 100, 200 mg once-daily for seven days resulted in a mean decrease in viral load of −0.62, −1.02, −1.53, −2.54 and −4.25 log₁₀ IU/mL, respectively. Doses of 9, 25 and 100 mg once-daily were also investigated in combination with **RBV** over seven days, producing −0.75, −1.56 and −3.79 log₁₀ IU/mL reduction in viral load, respectively. The drug was well tolerated at all doses (Rodriguez-Torres et al., 2011).

15.2.2. Phase II

After **BMS-986094** was granted Fast-Track Status by the FDA in March 2011, various Phase II trials were scheduled to commence in

2011 to continue investigating drug combinations for longer treatment periods. After Bristol-Myers Squibb bought Inhibitex in January 2012, **INX-189** was renamed **BMS-986094**. A 12 week Phase II clinical study in GT2/3 treatment-naïve individuals, in which **BMS-986094** was dosed at either 0, 25, 50 or 100 mg in combination with **RBV** and **IFN** resulted in RVR rates of 58%, 82%, 78% and 75%, cEVR rates of 92%, 93%, 100% and 88%, and eRVR rates of 58%, 75%, 78% and 71%, respectively. No virologic breakthroughs were observed over the course of therapy (Lawitz et al., 2012c).

15.3. Conclusions

The development of **BMS-986094** was discontinued as a result of a series of significant adverse events: in August 2012, a subject receiving 200 mg once daily dosing died of heart failure and another eight subjects were diagnosed with severe kidney and heart damage (Sheridan, 2012). The study was suspended and further development of **BMS-986094** was discontinued; however, a follow-up safety evaluation in subjects who participated in prior clinical studies is ongoing (BMS, 2012). The cause(s) of these serious side effects are still under investigation at this time. It is not clear if the observed toxicity was due to the nucleoside triphosphate itself or due to potential toxic metabolites, such as neopentyl alcohol, 1-naphthanol and methanol, released during the enzymatic cleavage of the prodrug moiety or because of the extremely high levels of nucleoside triphosphates produced. Cardiac toxicity for **BMS-986094** was demonstrated to be dose-related and the postulated reason for the cardiac toxicity was mitochondrial toxicity. As mentioned above, 2'-C-MeG-TP has been shown to be a substrate for incorporation by human mitochondrial RNA polymerase (Arnold et al., 2012). Toxicities observed during preclinical *in vitro* studies should have resulted in closer monitoring of clinical toxicities that would have led to an earlier discontinuation of the clinical study of **BMS-986094**.

16. Lessons learned

To date, nucleoside inhibitors appear to be the most promising of the three classes of direct-acting antiviral agents currently in phase III clinical studies. Recent progress in the development of nucleoside prodrugs, particularly sofosbuvir, solidifies our belief that this agent could become the best-in-class and backbone of future HCV combination chemotherapy. As such, sofosbuvir is an important milestone in the fight against HCV. The unforeseen surprises noted with nucleoside inhibitors in development are drug specific and not a class effect. The de-risking events based on carefully conducted preclinical developments and clinical trials suggest that we are on the verge of a huge game changer in the field of HCV with proof-of-concept already established for an all-oral cure for HCV. It is clear that early preclinical toxicology and pharmacology can make or break a newly drug preferably before clinical trials are begun. The tragic events related to **BMS-986094** only re-emphasizes the need for researchers to fully preclinically evaluate compounds using robust predictive toxicological tests and thorough toxicity screening in various cell-based systems and particularly mitochondrial toxicity which is paramount for the success of a new chemical entity, especially for nucleoside analogs. To assess mitochondrial toxicity, the use of the newly developed mitovir (*mitochondrial* dysfunction caused by *antiviral* ribonucleoside) score was recently proposed (Arnold et al., 2012). The mitovir score can be readily obtained during preclinical studies and may be useful to quantify the mitochondrial toxicity potential of compounds. The possibility exists that some infected persons might be more susceptible to mitochondrial toxicity when treated with nucleoside analogs due to their inherent mitochondrial or nuclear transcription

dysfunction. The bar has been raised with the FDA when it comes to toxicity associated with nucleoside analogs as is reflected by their actions with **IDX184** and **VX-135**, although exact details and guidance has not yet been forthcoming.

It is also clear that the prodrug selected may enhance oral bioavailability, but may also concurrently increase toxicity by delivering the active metabolite in high levels to undesirable compartments causing significant off-target side effects and even death. Thus, for treatment of HCV infection it is important that the drug is primarily liver targeted due to first pass effect with extensive hepatic extraction and that nucleoside triphosphate production and metabolism remain largely localized in the liver, the site of HCV replication with low systemic exposure. In the context of a viral liver infection, it is ideal for the nucleoside prodrug to be quickly metabolized and preferentially activated in the liver such that it does not go into systemic circulation and result in off-target exposure of the nucleoside or its triphosphate. For example, the side effects that were noted with both **IDX184** and **INX-189** would indicate that the drug was escaping first pass metabolism and reaching some degree of systemic exposure. Additionally, the intracellular half-life of the nucleoside triphosphate might be more important than the amount of NTP formed. After all, the active metabolite has to persist in the liver long enough to suppress and eliminate viral load. Furthermore, an oral prodrug that is unstable in gastric fluid/GI tract or the plasma may never reach its target to deliver the nucleoside monophosphate to the liver. It is also clearer now that the most desirable nucleoside analogs are those that are pangenotypic and will be effective in all HCV infected persons, especially the difficult-to-treat persons with advanced liver disease including those with decompensated cirrhosis. It is also becoming apparent that one pill may not fit all especially for underdeveloped countries where access and cost of the best combinations may become a severe obstacle to cures (Hagan and Schinazi, 2013).

The HCV drug development tsunami will continue and be broadly felt across many areas of healthcare in the next decade. It is anticipated that the prospects of a cure for millions of infected persons will in time become possible despite the cost of treatment since we have a moral obligation to provide life saving medicines that cure globally. However, it will take courageous leadership at various levels of society and government to succeed in eradicating HCV globally (Hagan and Schinazi, 2013).

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