Review

HCV’s days are numbered: next-generation direct-acting antivirals and host-targeting agents

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Introduction

Hepatitis C represents a viral pandemic, affecting approximately 170 million people worldwide [1]. Of all HCV infections, 50–80% lead to chronic hepatitis [2]. Approximately 20% of patients with chronic hepatitis C progress to liver cirrhosis, which is associated with the development of hepatocellular carcinoma in almost 20% of the cases [3]. For these reasons, hepatitis C is one of the leading indications for liver transplantation and liver-related morbidity and mortality [4]; therefore, successful treatment of HCV infection is a global health priority.

Since 2001, the standard of care (SOC) for chronic hepatitis C viral infection has been a combination of pegylated interferon (PEG-IFN)-α and ribavirin (P/R) [5,6]. Despite long treatment durations and abundant side effects that may cause treatment discontinuation in a high proportion of patients, only 40–50% of the treated HCV genotype (G)1 patients achieve sustained virological response (SVR). SVR, defined as undetectable HCV RNA at 6 months after the end of therapy, is the equivalent of being cured [5,6].

In 2011, the SOC for chronic hepatitis C G1 changed with the advent of two first-generation HCV NS3 protease inhibitors (PIs), boceprevir and telaprevir, given in combination with P/R. These drugs increase the chances of clearing HCV viral infection by up to 70% in previously untreated patients [7,8]. Moreover, 50% of the treated patients achieve undetectable HCV RNA in serum after the first 4 weeks of HCV PI exposure (rapid virological response [RVR]), which makes them candidates for shorter treatment duration (24–28 weeks) [7,9]. However, this new SOC is far from being ideal. The emergence of new resistance-associated variants (RAVs), complicated response-guided therapy (RGT) regimes, additional side effects that may lead to therapy discontinuation, important drug–drug interactions and very high costs are the price to pay for this improvement in SVR. Furthermore, these two new compounds have very limited, if any, efficacy in HCV genotypes 2, 3, 4, 5 and 6. Consequently, novel antiviral substances that overcome all these limitations are eagerly awaited. This article reviews the next-generation of direct-acting antivirals (DAAs) as well as host-targeting agents against HCV that are currently being clinically developed.

HCV structure

The understanding of the HCV viral structure and its life cycle has been of vital importance for the identification of therapeutic targets for antiviral HCV drugs [10].

Similar to other members of the Flaviviridae family, HCV virions appear to be spherical, have a smooth outer coat (envelope) and a nucleocapsid that harbours the RNA genome. The envelope consists of a lipid bilayer in which the structural proteins E1 and E2 are embedded [11]. It surrounds a nucleocapsid composed of the HCV core protein [12,13]. The HCV genome, a 9.6-kb single-stranded RNA molecule, is sheltered in the nucleocapsid and contains only one open reading frame that yields a
large polyprotein precursor of 3,000 amino acid residues. This precursor is co- and post-translationally processed into at least 10 different proteins: 4 structural (core, E1, E2 and p7; an ionic channel involved in the formation of infectious virions) and 6 non-structural (NS; NS2, NS3, NS4A, NS4B, NS5A and NS5B) [14]. NS proteins have enzymatic activity critical in viral life cycle and thus represent potential targets for antiviral therapy. Their main characteristics are summarized in Table 1.

Direct-acting and host-targeting antiviral agents

The term ‘directly-acting antivirals’ (also known as specific targeted antiviral therapies or STAT-C) refers to the main quality that differentiates them from P/R; unlike P/R, DAAs aim for specific HCV components. Each step of the viral cycle is potentially a target for DAAs: the viral attachment and endocytosis in the hepatocytes, the translation of the RNA genome into the large polyprotein, the (auto)-cleavage of the resulting polyprotein into peptides, the viral assembly and the release from the cell.

Another type of antivirals are the host-targeting antivirals (HTAs). Two classes of HTAs can be distinguished: non-immunomodulatory and immunomodulatory. To the first, belong cyclophilin inhibitors, nita-zoxanide or the microRNA-122 inhibitors as well as host lipid synthesis inhibitors (the last two are currently in the preclinical stage of development). Non-immunomodulatory HTAs are directed against cellular components that are vital for the life cycle of HCV. Conversely, immunomodulatory HTAs do not target specific components of the HCV, but rather stimulate the host’s innate and adaptive immunity, which is partly suppressed by the infection itself. Both Toll-like receptor (TLR) agonists and interferons (IFNs) belong to this group. Figure 1 summarizes the main characteristics of DAAs and HTAs.

The new substances under development appear to be the long awaited solution for the treatment of chronic hepatitis C. However, not all that glitters is gold, these novel antivirals raise new questions that need to be carefully addressed.

Appearance of resistant mutants

The high replication rate of HCV (approximately one trillion virions per day) and the lack of proofreading of its polymerase \(10^{-3}\) errors per nucleotide per generation) result in the constant appearance of mutated variants in the viral population [15]. Most of these mutations can result in the removal of some of the qualities that permit HCV survival and these variants disappear rapidly. However, a small percentage of mutants do survive and coexist with the dominant strain or wild-type and they represent the quasispecies. Some of these quasispecies exhibit drug resistance; in fact, it is estimated that 1.4% of the patients infected with HCV G1b and 8.6% of HCV G1a carry at least one variant resistant to DAA therapies [16]. During antiviral monotherapy with DAAs, the wild-type virus is suppressed, providing selection advantage for the pre-existing resistant variants within the quasispecies [17]. Hence, some treatment failures can be explained by the emergence of pre-existing RAVs. Interestingly, no specific mutations conferring resistance against P/R have been identified yet [18]. Consequently, IFN and ribavirin are needed as a backbone for a successful therapy with DAAs.

Tolerability

The old P/R-based SOC is riddled with side effects and laboratory abnormalities that decrease the quality of

<table>
<thead>
<tr>
<th>Protein</th>
<th>AA, n</th>
<th>Localization</th>
<th>Function</th>
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<tr>
<td>NS2 217</td>
<td>Cytoplasm</td>
<td>Together with NS3: cysteine protease activity; autocleavage between NS2 and NS3 [123,124] Virion morphogenesis [125]</td>
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<tr>
<td>NS3 631</td>
<td>Cytoplasm</td>
<td>Together with NS2: autocleavage between NS2 and NS3 Together with NS4: serine protease; autocleavage of the remaining downstream proteins (NS4, NS4B, NS5A and NS5B) [24] Helicase activity: unwinding the viral RNA during replication [27,126] Innate immune response modulation (blocking of intracellular signal transduction pathways for interferon production [25,26]</td>
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<tr>
<td>NS4A 54</td>
<td>Cytoplasm</td>
<td>Cofactor and stabilization of NS3 serine protease [24,127]</td>
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<tr>
<td>NS4B 217</td>
<td>Membrane</td>
<td>Formation of the membranous web that serves as scaffold for HCV assembly NTPase and RNA binding Anti-apoptotic [128]</td>
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<tr>
<td>NS5A 458</td>
<td>Membrane</td>
<td>It seems to be essential for viral replication although its exact activity is still unknown [52]</td>
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<tr>
<td>NS5B 591</td>
<td>Membrane</td>
<td>Error prone RNA polymerase, synthesis of the minus strand of RNA [61]</td>
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AA, amino acid; NS, non-structural.
HCV’s days are numbered: next-generation DAAs and HTAs

Antiviral Therapy 17.6 Pt B

Life of HCV patients, compromises their compliance and may lead to dose reductions [19]. This results in a further decrease of the antiviral efficacy [20]. Moreover, the incorporation of the first wave of first-generation HCV PIs to the standard P/R combination has added new side effects, such as anaemia and rashes. So far, the new molecules under development have been shown to cause less anaemia and skin lesions than telaprevir or boceprevir. However, these novel substances are associated with other side effects, such as hyperbilirubinaemia, increased transaminases [21–23], gastrointestinal symptoms and photosensitivity [23]. One of the major goals to be achieved with next-generation antiviral therapies is a better safety profile.

Direct-acting antivirals

HCV NS 3/4A protease inhibitors: first- and second-generation

As shown in Table 1, the NS3/4A complex is a multifunctional heterodimeric protease that cleaves four of the six NS proteins contained in the HCV polypeptide (NS3-NS4A, NS4A-NS4B, NS4B-NS5A and NS5A-NS5B) [24]. It also functions as an immunomodulator, blocking the IFN-induced endogenous antiviral response [25,26], thus impairing the innate immune system. Furthermore, the NS3/4A protease has a C-terminal RNA-helicase/NTPase domain that is essential for viral replication [27]. Therefore, the inhibition of this enzymatic complex should not only hinder HCV replication, but also have a beneficial interference with the innate immune response.

The NS3/4A protease consists of two covalently bonded subunits: the NS3 or catalytic subunit, and the NS4A, an activation cofactor [28]. The substrate-binding site is shallow and very hydrophobic, which makes it a challenging target for antiviral drug development [29].

Two classes of NS3/4a PIs can be distinguished based on their molecular structure: linear tetra-peptide α-ketoamide derivatives (that is, telaprevir, boceprevir and BI201335) and macrocyclic inhibitors (that is, vaniprevir, danoprevir and TMC435). HCV PIs can also be divided into first-wave first-generation (telaprevir and boceprevir; the ones that have already reached the market in Europe and the US), second-wave first-generation (currently under development in Phase III, that is, BI201335 and TMC435) and second-generation PIs (that is, asunaprevir [ASV], ACH-1625 and vaniprevir). Second-wave PIs have once daily or twice daily dosing instead of three times daily dosing and have better safety and tolerability than first-wave PIs. However, they are rather specific for HCV G1 and have limited efficacy in other genotypes. Second-generation HCV PIs have similar antiviral activity and genetic barriers as those in the first wave, but offer a broader genotypic profile, some of them being pangenotypic.

Through a non-covalent reversible binding to the active site of the protease, PIs achieve a very effective blocking of the enzyme and thus a strong suppression of the generation of mature HCV proteins. This results in a robust inhibition of HCV replication in vitro and in vivo. Interestingly, amino acid substitutions close to the active enzymatic site alter the efficacy of these compounds without necessarily modifying the activity of the protease, which leads to breakthrough and resistance [30,31]. Furthermore, cross-resistance has been detected among first-generation PIs, and it seems that second-wave and second-generation HCV PIs also select variants bearing similar substitutions [32].

From all the DAA subclasses, NS3/4A inhibitors are probably the most dynamic ones with many molecules...
being presently under development. The competition is therefore significant. Consequently, the development of some substances must be halted, despite having proven favourable efficacy and safety, in order to make way for new molecules that suppress replication more effectively and that are not genotype-limited, namely the second-generation PIs. A good example for this is vaniprevir (MK-7009), whose clinical development has been halted (except for Japan, where clinical trials are still ongoing) to give way to MK-5172, despite showing appropriate antiviral activity in G1 and G2 and no safety/tolerability issues [33].

In this review, we will focus on the following compounds: TMC435, BI20133, danoprevir and MK-5172.

**Danoprevir**
The macrocyclic NS3/4A inhibitor danoprevir (RG-7227; ITMN-191) was specifically designed by Roche and InterMune Inc. to have milder side effects than first-wave PIs while keeping a maximal antiviral potency [34].

The first clinical trials evaluating triple therapy of danoprevir in combination with P/R showed rapid and persistent decreases in HCV viraemia [35]. However, alanine aminotransferase (ALT) increases were detected in some patients under high-dose danoprevir. For this reason, in subsequent clinical trials, lower doses of danoprevir were boosted with the CYP3A inhibitor, ritonavir, which increased the exposure of the PI without affecting ALT levels [22].

The ATLAS study compared 24/48 weeks of RGT (depending on undetectable HCV RNA at week 4) versus 48 weeks of fixed duration therapy (FDT) with danoprevir in combination with P/R versus 48 weeks of P/R alone. Danoprevir was given through week 24. The final results of this Phase II clinical trial were recently presented by Terrault et al. [36]. At 24 weeks after the end of therapy, 85% of the patients treated with danoprevir and just 42% of the patients receiving P/R-only remained with undetectable HCV RNA levels (SVR24). Importantly, nearly 5% of resistance-related breakthrough was detected and the relapse rate was 7.6% [36], which suggests that danoprevir should be administered in combination with other DAAs in development, such as nucleoside/nucleotide analogue (NA) HCV NS5B polymerase inhibitors.

INFORM-1 was the first clinical trial exploring the possibility of an IFN-free therapy, based on the combination of DAAs with different mechanisms of action. The efficacy results demonstrated the proof-of-concept, with HCV RNA decreases of approximately 5 log_{10} IU/ml after 2 weeks of treatment with danoprevir and mericitabine (a first-generation NS5B nucleoside analogue polymerase inhibitor; see NS5B polymerase inhibitors) in combination. Of note, all patients subsequently received P/R combination therapy [37]. Further studies are being conducted in this line, evaluating the IFN-free combinations of danoprevir with other DAAs.

**TMC435**
TMC435 (TMC435350) is also a small molecule macrocyclic inhibitor of the NS3/4A serine protease of HCV. Four features differentiate this PI from all other second-wave NS3/4A inhibitors. Firstly, it has a particularly long half-life: 24 h post-dosing the liver concentrations remained above 10^9, which supports once-daily dosing instead of two or three times daily dosing like other DAAs [38,39]. Secondly, with the exception of G3, it has shown to be highly effective against all other HCV genotypes (1, 2, 4, 5, 6 and 7) with half maximal inhibitory concentration values as low as 13 nmol/l. The reduced activity against G3 can be explained by the presence in this subtype of variations at D168, which is an essential amino acid for the non-covalent binding of TMC435 [40]. Thirdly, other NS3/4A inhibitors rarely have any immunomodulatory activity at therapeutic doses, yet it appears that TMC435 does restore the innate immune system at non-toxic doses [41]. Finally, in addition to its high antiviral potency, TMC435 has shown a very favourable tolerability profile with only mild and reversible bilirubin increases in Phase IIa clinical trials.

The PILLAR study is the Phase II clinical trial that aimed to assess the efficacy of a RGT with TMC435 once daily in combination with P/R in treatment-naive HCV G1 patients. The combination TMC435 plus P/R proved to be highly effective: 75–86% of the patients in the TMC435 arms achieved SVR. Additionally, 79–86% of the patients were eligible for a 24-week shortened therapy without decreasing their chances of SVR. Finally, apart from mild bilirubin increases, TMC435 in combination with P/R showed no additional side effects to this antiviral backbone [42]. However, relapse and breakthrough rates under triple therapy were quite similar to those in patients on P/R. Most of the treatment failures were associated with the development of RAVs, particularly frequent were those with mutations at NS3 positions 80, 155 and/or 188 [42]. Fortunately, these resistant variants remain fully susceptible to NS5A and NS5B inhibitors [32]; thus, combinations of TMC435 with IFN-α and NS5A and NS5B HCV polymerase inhibitors should be considered in order to obtain successful antiviral treatments.

TMC435 in combination with P/R has also been evaluated in patients who had already received a prior course of P/R in the ASPIRE study. The final results of this Phase Ib clinical trial are outstanding: 85% of the prior relapers, 75% of the prior partial responders and 51% of the prior null-responders remained with undetectable HCV RNA in serum 24 weeks after the end of treatment consisting of P/R plus different doses.
and durations of TMC435 (control group [treated with 48 weeks P/R] SVR rates: 37% for relapers, 9% in partial responders and 19% in null-responders) [43]. TMC435 is currently in Phase III clinical development.

**BI201335**

BI201335 is a highly selective linear tri-peptide PI that non-covalently binds to the NS3/4A complex. Its high selectivity relies on the presence of a carboxylic acid group, which specifically interacts with the NS3/4a active site, avoiding the interaction with host proteases, therefore reducing the toxicity of this substance [44].

Preclinical studies showed potent antiviral activity against genotypes 1, 4a, 5a and 6a, and a slightly weaker efficacy against HCV G2a and G3a [44]. Early clinical studies confirmed this high potency and showed a long elimination half-life that allows a more convenient once-daily dosing schedule [45]. This fact was of particular importance in the subcohort of patients with cirrhosis, who experienced less adverse events when BI201335 was administered once daily in combination with P/R [46].

SOUND-C1 is the Phase Ib clinical trial that set the proof-of-concept that an IFN-free therapy based on the combination of BI201335 (120 mg once daily) with the non-nucleosidic NS5B inhibitor BI207127 (600 mg three times daily) and ribavirin could strongly suppress HCV replication. Over 80% of the treated patients had undetectable HCV viraemia at week 4 of treatment without any significant difference between subgenotypes 1a and 1b [47]. Accordingly, the currently ongoing SOUND-2 is the Phase Iib clinical trial evaluating efficacy and safety of different treatment durations of this IFN-free antiviral combination (with and without ribavirin). In this study, the overall viral response at week 12 was 75%, comparable to that achieved under triple therapy with P/R and other PIs. Furthermore, ribavirin proved to be of vital importance because the response rate of the ribavirin-free treatment arm was substantially lower (57% versus 70–76% at week 12). Breakthrough rates were high, particularly in the group without ribavirin (13–21% with ribavirin versus 33% without ribavirin). Moreover, discontinuation rates of 6.4–12.2% were observed. Treatment was interrupted primarily due to skin changes (rash and photosensitivity) and gastrointestinal adverse events (vomiting). However, this clinical trial has set an important milestone because it reports the highest SVR12 rates with an IFN-free therapy thus far [23].

**MK-5172**

MK-5172 is a second-generation, potent, macrocyclic PI with a broad HCV genotypic spectrum [48]. It is currently undergoing Phase II of clinical trials. Importantly, it is active against resistant mutations associated with other PIs, for example, R155K, D168V and A156T, which makes MK-5172 a good candidate to treat patients who have failed to respond to the currently available NS3/4A inhibitors [49,50]. In addition, its favourable pharmacokinetic profile supports once-daily dosing [51]. The efficacy of MK-5172 is presently being assessed in several clinical trials in combination with P/R (for G2 and G3) and boceprevir plus P/R (for G1).

Inhibitors of the HCV non-structural protein NS5A

The HCV NS5A is a zinc metal protein associated with the cellular membrane with no known intrinsic enzymatic function [52]. Three domains have been identified, yet their precise role in HCV replication remains unclear. Domains I and II seem to be essential for HCV RNA replication; the function of the third domain is still unknown [53]. It has been hypothesized that NS5A could also have an immunomodulator role in HCV infection through an interaction with IFN mechanisms [54].

Despite the poor understanding of this HCV protein, a few molecules inhibiting NS5A have been developed in the past years. For example, ACH-2928 recently entered Phase I clinical development [55] after demonstrating potent and pan-genotypic antiviral activity against HCV [56]. However, the most advanced NS5A inhibitor in clinical development is daclatasvir (DCV; BMS790052) [57].

**Daclatasvir**

DCV is the product of a high-throughput screening effort (over one million substances were tested) and optimization process by Bristol–Myers Squibb. It is a small molecule inhibitor that specifically binds to the domain I of the NS5A [58]. The precise mechanism of action of DCV remains unclear; however, it has proven to alter the subcellular localization and biochemical fractionation of NS5A into functional replication complexes. DCV inhibits viral replication at picomolar half-maximum effective concentrations, which makes it the most potent anti-HCV compound reported to date, and it has shown a broad genotypic coverage [58]. However, DCV has a low genetic barrier to resistance when administrated as monotherapy [58]. Resistant mutants appear after a single amino acid change in HCV G1a virus, significantly decreasing susceptibility to DCV, thus leading to breakthrough. In HCV G1b, at least two mutations are needed to confer resistance. Importantly, all the observed RAVs remained fully sensitive to IFN-α, as well as to HCV protease and polymerase inhibitors [59]. Current clinical trials have reported a good safety and tolerability profile along with very promising SVR rates using DCV with or without P/R and/or in combination with other DAAs [60]. Of particular importance are the results recently published by Lok et al. [60], which have set the proof-of-concept that even the most difficult to
treat patients – the ones who have already undergone antiviral therapy without success – could potentially benefit from IFN-free regimes. From the 11 therapy-experienced patients treated with just DCV and ASV (BMS650032; an NS3/4A PI), 4 (36%) achieved SVR24. Breakthrough occurred in 6 (55%) patients relapse in 1 (9%) and 2 (18%) of the 11 patients never achieved undetectable HCV RNA. Moreover, the SVR rates of the patients treated with DCV and ASV plus P/R reached 90–100%. Strangely, several patients with undetectable HCV RNA had RNA levels of <25 IU/ml at some point after the end of therapy. Although this did not translate to a relapse of the virus, this event should be further studied and understood [60].

NS5B polymerase inhibitors
The RNA-dependent RNA polymerase (RdRp) NS5B represents the heart of HCV’s replication machinery and is therefore an important target for antiviral therapies. NS5B initiates viral RNA de novo synthesis and then switches to a fast RNA elongation mode [61]. Its three-dimensional structure resembles a right hand with three main domains: fingers, palm and thumb. The active site of the polymerase is located in the central part of the enzyme and is the target of NAs. The NS5B has four additional allosteric sites situated in the fingers (2×) and thumb (2×) domains, which are targets for non-nucleoside inhibitors.

Nucleoside and nucleotide inhibitors
NAs have long been a central part of the antiviral therapy of HBV, HSV [62] and HIV [63] infections. The key to their mechanism of action is that they mimic the structure of these glycosamines. This way, the NS5B polymerase recognizes and incorporates them into the complementary RNA chain during the transcription process of the HCV genome; however, because they act as chain terminators, the synthesis and elongation of new HCV RNA molecules is inhibited [64].

The active site of the NS5B is one of the most conserved regions of the HCV genome intra- and intergenotypically; mutations of this site imply an alteration of the activity of the RNA polymerase, which automatically hinders the HCV replication [61]. This way, very few resistant variations have been reported (for example, NS5B-S282T mutant) and also for this reason, NAs are highly effective against all HCV genotypes [64].

Nucleoside inhibitors: mericitabine
Nucleoside analogues are non-phosphorylated charged molecules, which facilitates their cell membrane crossing, allowing a better distribution in all tissues [65].

Mericitabine (R7218) is the prodrug of PSI-6130, a cytosidine analogue. For its activation, mericitabine requires intracellular phosphorylation and conversion into two active triphosphate molecules that resemble cytidine and uridine, which block the synthesis of HCV RNA as they are incorporated into the new RNA chain [66]. Mericitabine has a particular pharmacokinetic profile. Compared with other antiviral compounds, the viral load decrease observed under mericitabine therapy is initially slow, gradually accelerating afterwards, to finally achieve a highly effective blockage of the viral replication cycle. This probably occurs because the hepatocytes first need to accumulate the intracellular phosphate required to activate this prodrug [67].

Mericitabine has shown to be active against G1–G4 [68–70]. No RAVs have been detected so far in finished or ongoing clinical trials; only the S282T mutant seems to be selected during mericitabine therapy; however, this quasispecies has a very low replication capacity and would need to be the predominant variant in vivo really to have any clinical significance [71–73].

Unlike most of the other DAAs, mericitabine is not metabolized in the liver and its secretion is mainly renal, which reduces importantly the risk of drug–drug interactions.

The PROPEL study could prove the efficacy and the lack of development of resistance of mericitabine in combination with P/R (8/12 weeks) followed by P/R (through week 24 in patients in the RGT arms with undetectable RVR at week 4 and up to 48 weeks otherwise) in G1/G4 HCV treatment-naive patients. The higher SVR rates observed in patients who were given mericitabine for 12 weeks – as compared with those who only received 8 weeks – and the similar adverse event profile between all groups treated with this substance [74], supported the inclusion of longer mericitabine regimes in further clinical trial designs. At the same time, another Phase IIb clinical trial, JUMP-C, compared RGT of mericitabine in combination with P/R with a 48-week P/R FDT in G1 and G4 HCV previously untreated patients. Preliminary results of JUMP-C confirmed appropriate safety and tolerability, and the lack of resistance-related breakthrough. Over 90% of the patients treated with mericitabine had undetectable HCV viraemia at the end of therapy, which indicates a strong suppression of the viral replication. Surprisingly, HCV relapsed in 24% of the patients with extended RVR (undetectable HCV RNA from week 4 to week 22), as only 37/49 (76%) remained with undetectable HCV RNA 12 weeks after the end of therapy [75]. The reasons behind these unusually high relapse rates are not known, and although they do not seem to be class-dependent, the recently reported high recurrence of the virus after a combination treatment of ribavirin and GS-7977 – another nucleoside and nucleotide inhibitor (NI; see Nucleotide inhibitors: GS-7977) – in null-responders [76] could suggest an association between this antiviral class and post-therapy relapse
of the virus. An understanding of this phenomenon is of vital importance, and will allow optimal choice of antiviral substances in the future.

Valopicitabine (NM283) and MK-0608 are two other nucleoside inhibitors. However, due to safety issues, their clinical development has been terminated.

**Nucleotide inhibitors: GS-7977**

Pharmasset has developed a series of very potent NS5B polymerase inhibitors with good tolerability and the possibility of once-daily dosing. GS-7977 (PSI-7977) is one of the products of the metabolism of PSI-7851, a second-generation uridine nucleotide [77,78]. For its favourable in vitro potency and manufacturing characteristics, GS-7977 was selected for further clinical development and is currently undergoing Phase III clinical trials.

The striking preliminary results of the PROTON study, a Phase II clinical trial evaluating the combination of GS-7977 with P/R, revealed RVR rates of 98% in G1 patients [79] and 95% in G2 and G3 [80]. SVR12 rates in G1 patients reached 88–91% after 12 weeks of triple therapy plus 12 or 36 weeks of P/R in an RGT approach depending on presence/absence of RVR. Neither viral breakthrough nor associated RAVs were detected and a very promising safety profile was reported [79,80].

These extraordinary data led to the design of ELECTRON, a clinical trial to determine the need of PEG-IFN to achieve SVR in patients infected with chronic hepatitis C treated with GS-7977. Patients were randomized in four arms and received 12, 8 and 4 weeks of PEG-IFN in combination with GS-7977+ ribavirin; arm four was IFN-free. In the first place, the ELECTRON study only included G2 and G3 previously untreated patients, thus initial results only applied to this subcohort. Remarkably, 100% of the patients in all arms achieved RVR, and undetectable HCV RNA was maintained through week 24 after the end of therapy (SVR24). As expected, patients in the IFN-free arm experienced significantly less adverse events than those in arms 1–3. Moreover, no breakthrough or resistance to therapy was observed [81]. In view of these remarkable results, the original protocol of ELECTRON was expanded: untreated G1 patients and prior null-responders with G1/G2/G3 were included, and an additional arm exploring 12 weeks of IFN-free therapy (GS-7977/R) was incorporated to the study design. In February 2012, Gilead announced that six of the eight G1 prior null-responders to P/R therapies who had been treated with GS-7977/R for 12 weeks had experienced viral relapse 4 weeks after the end of therapy – SVR24 data on the remaining two patients is not available thus far [76].

In parallel, and in another attempt to achieve HCV treatment that is free of IFN, the NUCLEAR study found the combination of GS-7977 and PSI-938 to be highly effective and well-tolerated over 14 days in HCV G1 patients [82]. PSI-938 is a purine nucleotide analogue with a resistance profile complementary to that of GS-7977 and otherwise similar pharmacological features. Further evaluation of this promising combination was planned in the QUANTUM study. Unfortunately, significant liver abnormalities were observed in patients receiving regimens containing PSI-938 and Pharmasset announced in December 2011 that all treatment arms containing the purine analogue PSI-938 would be discontinued [83].

Taken together, GS-7977 is a very promising compound and has set an important proof-of-concept: a highly effective antiviral therapy without IFN is possible through the combination of different NIs. However, difficult-to-treat patients remain difficult to treat, and require longer regimes and possibly more compounds in combination than other populations. A deeper and more thorough understanding of all these new antiviral substances is required to find the ideal combinations that effectively eradicate HCV without IFN.

Other NAs, such as IDX184, are currently in Phase II of clinical development.

**Non-nucleoside polymerase inhibitors**

Non-nucleoside polymerase inhibitors (NNIs) target one of the four allosteric sites situated in the ‘th Mb’ and ‘fingers’ of the NS5B polymerase and not the enzymatic active site. Their binding produces a change in the conformation of the NS5B RdRp, which no longer has affinity for nucleotides and thus becomes inactive, hindering the generation of new viral genomes [64].

The four allosteric targets are relatively far from the active site, which facilitates the appearance of mutant variants resistant to NNIs without an impairment of the RdRp’s activity. NNIs that share the same binding site produce overlapping RAVs. Surprisingly, cross-resistance has also been observed among NNIs targeting different allosteric sites, probably due to conformational changes of the enzyme associated with these amino acid variations [64]. Additionally, enzymatic polymorphisms may be detrimental to the efficacy of NNIs. Altogether, NNIs should not be administered alone but in combination with potent antiviral drugs with a different mechanism of action. Given their good tolerability they could be used as co-adjuvants in triple or quadruple DAA combinations to achieve maximal efficacy and prevent the appearance of RAVs.

**Non-nucleoside polymerase inhibitor: filibuvir**

Filibuvir (PF-00868554) targets the thumb 2 allosteric site of the RdRp and is mainly active against G1a. It has proved to be safe and well-tolerated in Phase I clinical trials. In Phase II studies, high efficacy was shown...
in combination with P/R: >65% of the treated patients achieved RVR under triple therapy [84]. Mutations at position 423 are the most frequent resistant variants observed under therapy with filibuvir. However, M423 mutants have a limited replicative capacity [85].

**Non-nucleoside polymerase inhibitor: VX-222**

VX-222 is another small molecule NNI currently in Phase II of clinical development that selectively targets the thumb 2 allosteric site of the NS5B polymerase. It has high efficacy against G1a and G1b [86,87], very good tolerability [88] and an excellent pharmacokinetic profile, it does not inhibit nor induce CYP or Pgp [87]. This is of particular importance given that if an IFN-free therapy is to be achieved, it will be through the combination of several DAAs, lack of interference with CYP and Pgp means easier management of drug–drug interactions.

ZENITH is the clinical trial assessing efficacy, tolerability and safety of VX-222 plus telaprevir alone or in combination with P/R in G1 previously untreated HCV patients. Importantly, all arms containing only VX-222 plus telaprevir had to be stopped due to the emergence of viral breakthrough. Nevertheless, 86–87% of the patients receiving VX-222, telaprevir and P/R achieved undetectable HCV RNA after just 2 weeks of treatment [89]. Consequently, VX-222 and telaprevir alone do not offer a strong enough suppression of HCV and should therefore be combined with either P/R or other DAAs.

**New host-targeting agents**

**Non-immunomodulatory agents**

**Cyclophilin inhibitors**

Cyclophilins are proteins involved in the folding and isomerization of peptides and can be found in the cytosol, endoplasmic reticulum and mitochondria of vertebrates and other organisms. They received their name because of their high affinity to the immunosuppressive drug cyclosporin A (CsA) [90].

The role of cyclophilins in the cycle of HCV is not yet fully understood. It has been proven that they are essential cofactors in the replication of the HCV and they have been associated with several viral ligands including NS2, NS5A [91] and NS5B [92–94]. This way, CsA analogues lacking the immunomodulatory activity of cyclosporin have shown to efficiently suppress the replication of HCV in vitro and in vivo [95,96].

Host proteins are, by definition, well-conserved. Thus, the inhibition of ‘essential for viral amplification’ host proteins results in improved resistance profiles. Accordingly, the development of resistance against cyclophilin inhibitors has proven to occur slower (taking up to 20 weeks [97]) and less frequently than with other DAAs [97]. In this line, since the interaction between cyclophilin and HCV is conserved across genotypes, cyclophilin inhibitors are effective against all HCV genotypes [98].

Three cyclophilin inhibitors have shown promising results for the treatment of HCV infection in early clinical trials: alisporivir (DEBIO-025), SCY635 and NIM811 (currently on hold). Despite belonging to the same class of antiviral substances, these molecules have shown varied safety profiles and antiviral activities, which suggests they could have different pharmacology, metabolism and cellular distribution mechanisms [99].

Alisporivir is a Phase III non-immunosuppressive cyclophilin inhibitor that exerts potent suppression of the replication of HCV alone and in combination with P/R [100]. Furthermore, the encouraging results of RGT consisting of alisporivir plus P/R indicate that G1 patients with undetectable HCV viraemia after 4 weeks of treatment can reduce the total duration to 24 weeks without decreasing their chances of achieving SVR [101].

Recently, a Phase II clinical trial evaluated the efficacy of IFN-free therapies based on alisporivir compared with the P/R combination in G2/G3 HCV patients. Although the observed RVR rates in the IFN-free arms were only modest (28–42%), patients with detectable HCV RNA at week 4 received additional P/R, and finally >85% of the patients achieved undetectable HCV RNA at week 6 (after 2 weeks of additional P/R). Thus, although promising, alisporivir alone may not be enough for IFN-free therapies, and further clinical trials exploring combinations with other DAAs are needed [102].

Importantly, alisporivir features a high barrier to the development of viral resistance. Resistance can only occur through a reduction of the dependence of HCV to the host’s cyclophilins, and requires multiple mutations mainly at NS5A but also at NS2 and other regions [103]. Alisporivir is well-tolerated and has repeatedly shown a safe profile. The most frequently observed adverse event was transient hyperbilirubinaemia, which resolved after discontinuation of therapy [21]. Finally, alisporivir has proven to not only inhibit HCV replication, but also to exert a potent anti-HIV activity, which is of particular interest given the high number of HCV patients coinfected with HIV [100].

**MicroRNA inhibitors: anti-miR oligonucleotides**

MicroRNAs (miRs) are very small non-coding RNA molecules (approximately 22 nucleotides) whose main function is to repress translation of specific genes by binding to their post-transcriptional messenger RNA products [104]. MicroRNA-122 (miR-122) is very abundant in the liver – it constitutes 70% of all miRs [105] – and although its precise function in the liver is not clear yet, it has shown to be of vital importance for HCV’s infectivity [106]. In fact, the blocking of miR-122...
with complementary oligonucleotides has proven to effectively inhibit HCV replication [107]. Miravirsen (MIR) is one of such specific oligonucleotides that specifically targets miR-122. It has already been through Phase I and IIa clinical trials showing that MIR is very well-tolerated (no limiting toxicity was identified), and that it efficiently inhibits HCV replication (mean HCV RNA reductions of $-2.7 \log_{10} \text{IU/ml}$ after 10 weeks of monotherapy) [108]. Although rather innocuous, the inhibition of miR-122 is associated with several laboratory changes such as reduction of cholesterol, apoA and apoB levels or increase of alkaline phosphatase [108].

Importantly, the two target sites where miR-122 binds to the HCV genome are highly conserved among and within HCV genotypes, which provides MIR with high barrier to resistance and with pan-genotypic efficacy [109].

In conclusion, MIR opens a completely new front in the battle against HCV and additional clinical trials are needed to allocate it in its ideal position.

**Immunomodulators**

**Toll-like-receptor agonists**

TLR are a subclass of pattern recognition receptors, the immune system molecules that trigger acute inflammatory responses and initiate development of an antigen-specific adaptive immunity when they detect the presence of foreign microbial products. Eleven TLRs recognizing specific and conserved components of different microorganisms have been identified in mammals [110].

Unmethylated CpG oligonucleotides (CpG ODNs) are found very often in microbial genomes. TLR7 and TLR9 specifically recognize these motifs and activate the secretion of IFN and cytokines to initiate the innate immune response [111,112]. Taking advantage of these properties, antiviral molecules that mimic these CpG ODNs are currently at very early stages of clinical development: ANA773, an agonist of the TLR7, and IMO-2125 and SD-101, agonists of the TLR9. So far, preclinical and Phase I studies have demonstrated that these molecules induce the endogenous production of IFN-α and other cytokines, and that their antiviral potency is dose-dependent. Their side effects resemble those of PEG-IFN-α: flu-like symptoms and injection site pruritus, which makes perfect sense with the mechanism of action of these compounds [113,114]. Additional clinical trials with larger number of patients are needed, to confirm these promising results.

**IFN-λ1**

IFNs are a large family of proteins released in response to pathogens with antiviral, immunomodulatory and cytostatic activities. Three subtypes can be distinguished depending on the different receptors they bind to, yet, they all share similar biological activities [115].

The key to the abundant and varied side effects of IFN-α lies in the wide distribution of its receptors. The IFN-λ1 (interleukin-29) receptors are specifically located in epithelial cells – including hepatocytes – and only in a small subset of marrow-derived cells [116,117], which suggests a more favourable safety and tolerability profile. Phase Ia and Ib studies have evaluated this aspect with encouraging results: indeed PEG-IFN-λ1 seems to have similar antiviral activity to IFN-α and produces only minimal constitutional symptoms and haematological alterations [118].

The efficiency of different doses of PEG-IFN-λ and ribavirin against 180 μg P/R for 24 (in G2 and G3 HCV) and 48 (in G1 and G4) weeks is currently being studied in a Phase Ib clinical trial. Thus far, IFN-λ has proven to have superior RVR and cEVR – rates than PEG-IFN-α2a in both cirrhotic and non-cirrhotic patients [119,120]. Furthermore, patients treated with IFN-λ experienced fewer adverse events and needed less IFN dose reductions. However, patients taking PEG-IFN-λ experienced hyperbilirubinaemia and ALT or AST increases much more frequently than the patients treated with PEG-IFN-α2a. These parameters returned to acceptable levels after 1–2 weeks; nevertheless, 4 of 393 patients receiving the study medication had to discontinue therapy for this reason [119]. Phase III clinical trials are presently on-going.

**Conclusions**

Until 2011, the SOC for chronic hepatitis C has been rather unsatisfying because of its limited antiviral efficacy and abundant side effects. The present triple therapies with first-generation, first-wave HCV PIs plus P/R have only partly overcome these problems. On the one hand, SVRs have been improved and therapy duration may be reduced to 24–28 weeks in rapid responders. On the other, adverse events, three times daily administration and poor efficacy against genotypes other than 1 remain a burden. A better safety profile, once (or twice) a day dosing and more patients eligible for shorter treatment duration – with an RGT approach – can be expected for upcoming triple therapies with second-wave, second-generation HCV PIs. In addition to once-daily dosing and a better safety profile, second-generation HCV PIs are expected to offer broader genotype specificity and some of them are pan-genotypic.

The next goal certainly is to have all oral IFN-free regimens effective against all genotypes, safe and suitable for all populations of HCV-infected patients – also those with decompensated cirrhosis or with severe cardiac conditions. At the moment, there is an impressive list of antiviral substances at different stages of preclinical and clinical development.
Table 2. DAAs in current* clinical development

<table>
<thead>
<tr>
<th>Clinical development</th>
<th>Drug category</th>
<th>Drug name</th>
<th>Company</th>
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<tbody>
<tr>
<td>Phase I</td>
<td>Entry inhibitor</td>
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<td>iTherX</td>
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<tr>
<td></td>
<td>NSSB polymerase inhibitor</td>
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<td>Phase II</td>
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<td>Telaprevir</td>
<td>Telaprevir</td>
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*January 2012. DAA, direct-acting antiviral; NS, non-structural; PI, protease inhibitor.

(Table 2), tackling various steps of the HCV life cycle to inhibit replication. Due to their significantly higher antiviral potency and their favourable safety and tolerability profiles this goal finally appears to be possible. The combination of a first-generation HCV PI and an HCV NS5A inhibitor has already set the proof-of-concept for HCV G1b patients – including null-responders to previous therapies [60,121]. Furthermore, the combination of a nucleoside HCV NS5B polymerase inhibitor (NNI), PSI 7977, plus ribavirin has shown that an all oral therapy is also possible for G3 [81]. Thus, it seems clear that IFN-free therapies are feasible and will soon be available. Yet, the present burning question is: will we be able to get rid of ribavirin [122]?

Disclosure statement

MPM is on the speaker’s bureau of Schering Plough, Roche, Bristol–Myers Squibb, GlaxoSmithKline and Gilead, is a consultant for Schering Plough, Roche, Bristol–Myers Squibb, Gilead, Valeant, Boehringer Ingelheim, Novartis, Idenix, Tibotec, Vertex, GlaxoSmithKline, Merck and Astra/Arrows, and has received a grant/
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