

Directly acting antivirals against hepatitis C virus

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The approval of directly acting antivirals (DAA) for the treatment of chronic hepatitis C virus (HCV) infection will represent a major breakthrough for the 180 million persons infected worldwide. Paradoxically, hepatitis C is the only human chronic viral disease that can be cured, as all other pathogenic viruses infecting humans either display self-limited courses or establish non-eradicable persistent infections. Until now, treatment of chronic hepatitis C consisted of the combination of peginterferon- α plus ribavirin, which provided limited rates of cure and was associated with frequent side effects. Several DAA have been identified that inhibit the NS3 protease, the NS5B polymerase or the NS5A replication complex, and have entered the final steps of clinical development. These molecules, coupled with significant progress made in the recognition of more potent and safe interferon forms (e.g. interferon- λ) and host protein targets (e.g. alisporivir), are opening a new era in hepatitis C therapeutics. The expectations are so great that, to some extent, it is reminiscent of what happened in 1996 in the HIV field when the introduction of the first protease inhibitors as part of triple combinations revolutionized antiretroviral therapy. To maximize treatment success and reduce the likelihood of drug resistance selection, a proper individualization of hepatitis C therapy will be required, choosing the most convenient drugs and strategies according to distinct viral and host profiles. The complexity of HCV therapeutics has reached a point that presumably will lead to the birth of a new specialist, the HCV doctor.

Keywords: hepatitis C, antiviral therapy, DAA, protease inhibitors, polymerase inhibitors, telaprevir, boceprevir, drug resistance

The burden of HCV infection

Around 3% of the worldwide population is infected with the hepatitis C virus (HCV), which represents nearly 180 million people, with 3 million individuals newly infected each year.¹ Around 70% of acutely infected persons go on to develop chronic hepatitis C. In the absence of successful therapy, 25% of chronic HCV carriers will develop liver cirrhosis within an average of 25 years. Liver-related patient mortality once cirrhosis has developed is 3% per year, due to either complications of hepatic insufficiency or the development of hepatocellular carcinoma.¹ End-stage liver disease due to HCV currently represents the major indication for liver transplantation in the Western world.²

Achievements and drawbacks of peginterferon α /ribavirin (pegIFN- α /RBV) therapy

A protective vaccine against HCV does not exist and current standard therapy consists of the combination of pegIFN- α /RBV, given for 16–72 weeks, depending on HCV genotype, baseline viraemia and early viral response to therapy.³ However, less than half of individuals infected with HCV genotypes 1 or 4 obtain a sustained virological response (SVR) as compared with 85% of patients

infected with genotypes 2 or 3. Moreover, pegIFN- α /RBV therapy is frequently associated with serious unwanted side effects, including depression and anaemia.⁴

For the growing number of patients who already have failed current standard therapy for chronic hepatitis C, alternative options, such as maintenance therapy with pegIFN- α ,⁵ retreatment using induction doses of pegIFN- α ⁶ and/or extended duration of therapy, have failed to provide significant benefits in most cases.

A new era of antivirals for hepatitis C

New forms of IFN- α , such as albinterferon,⁷ have been explored without success, mainly due to side effects. More promising are other IFN molecules, such as IFN- λ , which is currently being evaluated and seems to be associated with increased efficacy and fewer adverse events than IFN- α .^{8,9} A prodrug of ribavirin, taribavirin (or viremagine), has been tested in randomized clinical trials, and although it causes significantly less anaemia than ribavirin, the overall efficacy is not increased.^{10,11}

Efforts in developing new compounds against HCV have been hampered by difficulties in replicating the virus in cell culture and the lack of suitable animal models. However, recent advances in the understanding of the HCV genomic organization and life

cycle, and the development of HCV replicons and infectious viral particles in tissue culture systems^{12–15} have enabled the rational design of agents that specifically inhibit HCV replication.¹⁶ The goal of future HCV treatment is to develop oral antiviral drugs that are less toxic, more potent and allow shorter duration of therapy than the current standard of care. Ideally, the aim is a regimen given for no longer than 24 weeks, including a combination of oral compounds and sparing IFN- α , that will result in most chronic hepatitis C patients being cured.

Three viral enzymes are currently the most promising targets for the design of specific anti-HCV inhibitors,¹⁷ and several compounds have already been tested against the NS3/4A protease, the NS5B polymerase and the NS5A protein. Table 1 records the list of those in clinical development that have entered and/or completed Phase II/III trials. It is anticipated that two protease inhibitors (PIs), telaprevir and boceprevir, will soon receive final approval for clinical use. Lastly, other compounds that inhibit cellular proteins critically involved in the HCV replication cycle are also showing promising results and may soon expand the spectrum of the HCV armamentarium. This is the case with alisporivir (DEB-025), a cyclophilin inhibitor with activity against all HCV genotypes.^{18,19}

HCV PIs

The HCV genome is a single positive-sense RNA strand that comprises nearly 10000 nucleotides. Both structural and non-structural proteins are released from an intermediate single transcribed polyprotein of 3300 amino acids. Together with its co-factor, NS4A, the amino-terminal domain of the HCV NS3 protein forms a heterodimeric serine protease that cleaves the downstream region of the HCV polyprotein into four functional non-structural proteins, including the HCV polymerase or NS5B. Viral replication can only initiate after all the individual proteins have been cleaved from the polyprotein. The importance of the NS3/4A serine protease for viral replication was first demonstrated using BILN2061 (ciluprevir), a PI that blocks the NS3/4A serine protease, preventing viral replication. This molecule established the first proof-of-concept, reducing serum HCV RNA up to 3 log₁₀ IU/mL within 2 days of monotherapy.^{20,21} Further clinical

development was stopped due to cardiotoxicity in animal models.

Two major classes of PI molecules against HCV genotype 1 have been developed so far. The first group is represented by covalent inhibitors, such as telaprevir and boceprevir, which are linear α -ketoamide derivatives. The second is constituted by non-covalent inhibitors, which are split out into two groups: the first group contain a carboxylic acid and are linear (e.g. BI-1335), while the second group are acid sulphonamide derivatives and can be either linear (e.g. BMS-032) or macrocyclic compounds (danoprevir, vaniprevir and TMC-435). Table 1 records the HCV PIs in the most advanced steps of clinical development.

Telaprevir

It is a peptidomimetic NS3/4A serine PI that was discovered using structure-based drug design techniques.²² The drug binds covalently but reversibly to the NS3/4A protease with slow binding and dissociation kinetics.²³ In the subgenomic HCV replicon system, telaprevir produces a dramatic reduction in viral RNA, eventually resulting in the elimination of HCV RNA from the replicon cells.²⁴

The Phase II PROVE 1 trial enrolled IFN- α -naive HCV genotype 1-infected patients into four treatment groups.²⁵ The rate of SVR ranged from 35% to 67% in telaprevir arms compared with 41% in controls. The SVR rate in patients who completed 24 weeks of treatment was close (61%) to that seen in subjects treated for 48 weeks (67%). Overall, 12 out of 175 patients treated with telaprevir experienced viral breakthrough with telaprevir-resistant strains, most of which harboured the R155T/K mutations. Interestingly, resistance was selected more frequently in HCV-1a than in HCV-1b. A single nucleotide change is needed to produce the amino acid change at codon 155 in HCV-1a, while two nucleotide changes are required in HCV-1b.²⁶ Rash, gastrointestinal events and anaemia were the main side effects of telaprevir.²⁵

The Phase II PROVE 2 trial was conducted in Europe and had a similar design to PROVE 1, with an extra arm evaluating pegIFN- α /telaprevir without ribavirin.²⁷ Triple therapy for 12 weeks provided significantly more antiviral efficacy at weeks 4 and 12 than standard pegIFN- α /RBV therapy. Moreover, 62% of patients reached SVR with only 12 weeks of triple therapy. The trial also highlighted the importance of ribavirin co-administration; only 36% of patients treated with pegIFN- α /telaprevir for 12 weeks achieved SVR. Moreover, ribavirin reduced the risk of selecting telaprevir resistance. As in PROVE-1, skin rashes, nausea and anaemia were the main side effects of telaprevir. It must be acknowledged that in contrast with other common drug hypersensitivity reactions, which tend to occur within the first days or weeks of therapy, the incidence of rash associated with telaprevir increases with time and may affect more than one-third of patients beyond 12 weeks of therapy. Viral breakthroughs and selection of resistance were observed in subjects during the first 12 weeks of telaprevir therapy, especially in those not taking RBV.

More recently, results from the Phase III trials with telaprevir have been released (Figure 1). In the ADVANCE trial,²⁸ 1088 HCV genotype 1 patients were randomized to triple therapy or standard of care, with two arms of telaprevir, one giving the drug for 8 weeks and the other for 12 weeks. The length of therapy was dependent of the achievement of extended rapid virological

Table 1. DAA against HCV in more advanced stages of clinical development

Protease inhibitors	Polymerase inhibitors		NS5A inhibitors
	nucleos(t)ide analogues	non-nucleoside analogues	
Telaprevir	mericitabine	tegobuvir	BMS-052
Boceprevir	PSI-7977	filibuvir	IDX-184
Danoprevir	PSI-938	BI-7127	
Vaniprevir		ANA-598	
BI-1335		VX-222	
TMC-435		VCH-759	
GS-9256		ABT-072	
ABT-450			

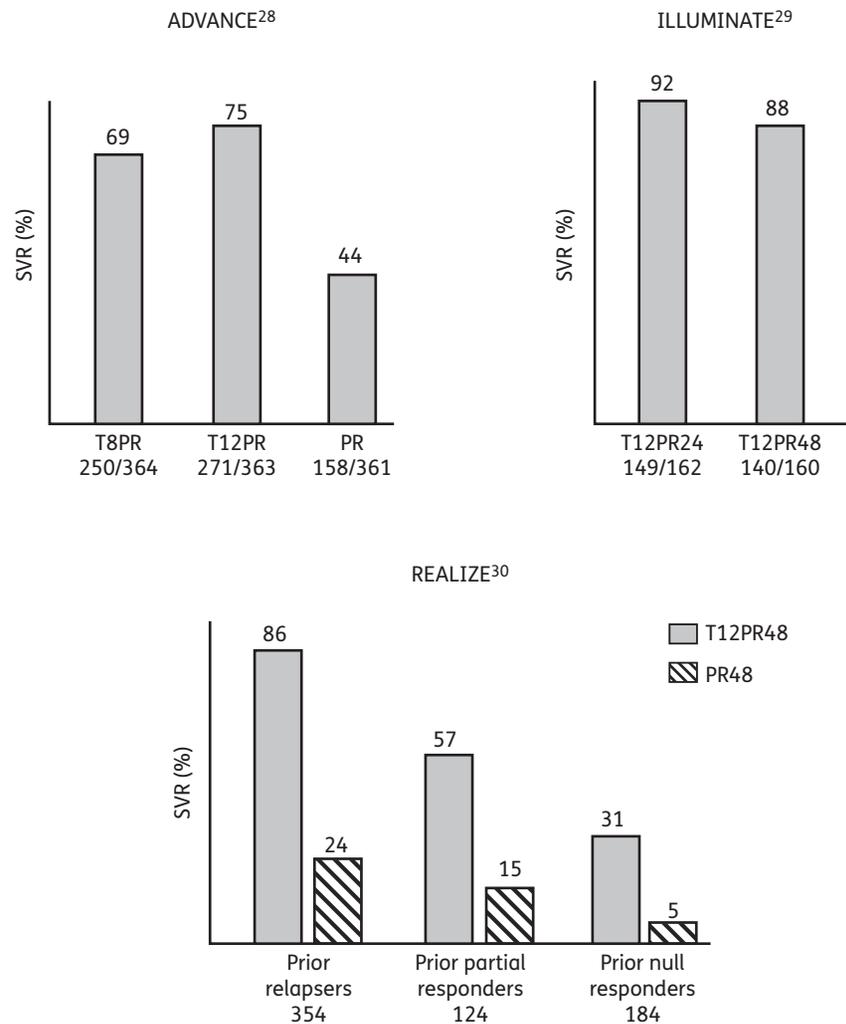


Figure 1. Phase III trials with telaprevir in interferon-naive (ADVANCE and ILLUMINATE) and interferon-experienced patients (REALIZE). T, telaprevir; R, peginterferon α ; R, ribavirin; SVR, sustained virological response. The numbers following 'T' or 'PR' indicate weeks of therapy.

response (eRVR), defined as viral undetectability from weeks 4 to 12. Then, duration of therapy was 24 weeks for patients with eRVR and 48 weeks for the rest. Patients with triple therapy for 12 weeks achieved 75% SVR, while it was significantly lower (69%) in the 8 weeks triple arm (Figure 1). The ILLUMINATE trial²⁹ was designed to specifically demonstrate that treating HCV genotype 1 IFN- α -naive patients for 24 weeks was not inferior compared with treating them for 48 weeks if they achieved eRVR. A total of 540 patients were recruited in the trial, of whom 65% achieved eRVR and were randomized to complete 24 or 48 weeks of pegIFN- α /RBV therapy. Using this response-guided therapy (RGT) approach, no significant differences were seen comparing the two arms (92% versus 88%, respectively) (Figure 1).

In the REALIZE study, patients with prior null response, partial response or relapse to pegIFN- α /RBV received triple therapy for the first 12 weeks followed by 36 weeks of pegIFN- α /RBV.³⁰ The final results are shown in Figure 1. Significantly higher SVR rates were obtained with triple therapy compared with standard of care, especially in prior relapsers (86% versus 24%). More

recently, the results from another trial have shown the feasibility of twice-daily instead of thrice-daily telaprevir administration.³¹

Boceprevir

This is another covalent, linear PI with activity against HCV genotype 1. It binds reversibly to the NS3 protease active site and shows potent activity in the HCV replicon system (EC_{50} 0.3–0.4 μ M), with additive potency when combined with pegIFN- α .³² The initial results in Phase II trials were somewhat discouraging, most likely due to limitations in study design and dosing. However, in the Phase II SPRINT-1 trial, higher doses of boceprevir (800 mg thrice daily) were tested in combination with pegIFN- α /RBV in 595 IFN-naive HCV genotype 1 patients. A subset received a lead-in phase of 4 weeks with pegIFN- α /RBV alone before adding boceprevir. Results were significantly better than in prior studies, with SVR ranging from 54% to 75% in the triple therapy arms.³³

More recently, the results of the Phase III trials were released. The SPRINT-2 study examined triple therapy in IFN- α -naive HCV

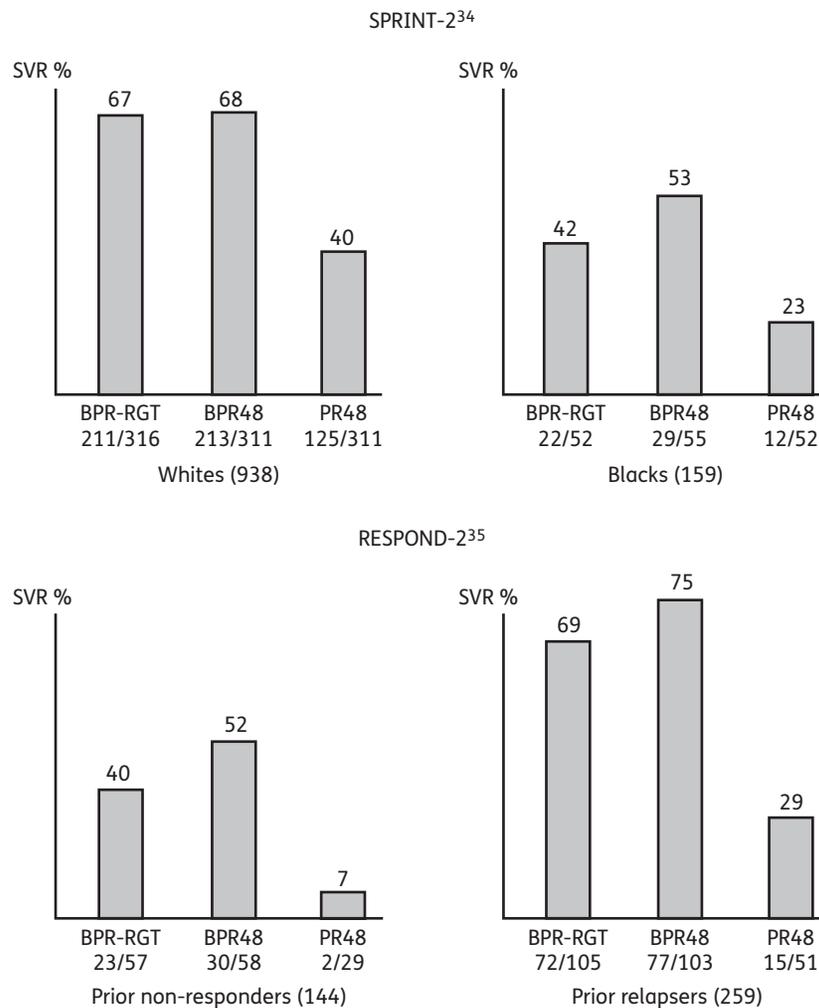


Figure 2. Phase III trials with boceprevir in interferon-naive (SPRINT-2) and interferon-experienced patients (RESPOND-2). B, boceprevir; P, peginterferon α ; R, ribavirin; RGT, response-guided therapy; SVR, sustained virological response. The numbers following 'PR' or 'BPR' indicate weeks of therapy.

genotype 1 patients,³⁴ while the RESPOND-2 trial tested triple therapy in patients with prior failure to IFN- α .³⁵ Figure 2 summarizes the main results of these studies. In all cases, boceprevir was preceded by a lead-in period of 4 weeks of pegIFN- α /RBV alone, with the aim of reducing the viral load and the likelihood of selecting boceprevir resistance. In the SPRINT-2 trial, the overall SVR was 66%, with no significant differences between patients treated for 1 year and those treated according to RGT. In the last group, patients were treated for only 24 weeks if they had achieved and sustained undetectability from week 4 of triple therapy. Overall, whites responded better than blacks. Anaemia and gastrointestinal side effects are the major dose-limiting side effects of boceprevir. However, only 15% of patients discontinued therapy prematurely due to serious adverse events.³⁴

In the RESPOND-2 study, prior IFN- α -experienced patients were treated with triple therapy.³⁵ Of note, prior null responders, such as individuals who had not shown a decline in serum HCV RNA $>2 \log_{10}$ at week 12, were not included in this study. Overall, the SVR rate ranged from 59% to 66% in the triple

arms, depending on whether patients were prior relapsers or non-responders. Interestingly, up to 40% of patients who did not achieve a negative serum HCV RNA at week 4 on triple therapy achieved SVR. This figure rose to 86%–88% in patients with undetectable viraemia at that time.

While telaprevir will be administered for only 12 weeks (and perhaps soon after first approval using twice-daily dosing), boceprevir will be given for the whole length of therapy (24 or 48 weeks) thrice daily. Both drugs will use the RGT principle, adapting the whole duration of therapy (24 versus 48 weeks) to early viral kinetics (week 4 response to triple therapy). A lead-in phase of pegIFN- α /RBV therapy alone will be used with boceprevir, but not with telaprevir. While this strategy may increase the complexity of treatment, it may allow the identification of a subset of patients with unresponsiveness to IFN- α ($<1 \log_{10}$ HCV RNA decline at week 4), in whom therapy might be deferred. If therapy is otherwise indicated, as may occur in patients with advanced liver fibrosis, selection of drug resistance is the major concern in this group. On the other hand, achievement of undetectable viraemia at week 4

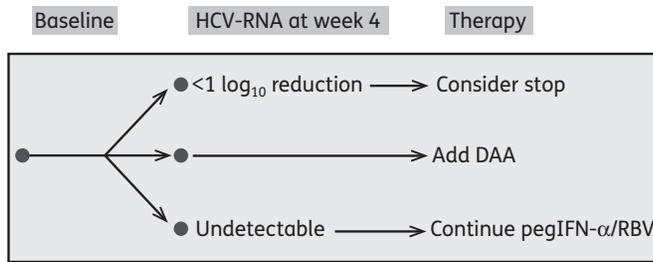


Figure 3. Proposed treatment strategy with a lead-in phase of pegIFN- α /RBV therapy. DAA, directly acting antivirals.

with pegIFN- α /RBV therapy only may identify a subset of individuals in whom adding boceprevir may not be required.³⁶ This treatment strategy is summarized in Figure 3.

Danoprevir (RG-7227/ITMN-191)

Data from *in vitro* biochemical assays point to this drug as a potent antiviral agent against HCV-1b, with IC₅₀ values of 0.8 nM. In contrast with telaprevir and boceprevir, this drug is a non-covalent, macrocyclic PI that selectively blocks the HCV NS3/4A protease.³⁷ Pre-clinical trials at doses higher than those intended for humans showed a favourable safety and toxicological profile, with pharmacokinetics supporting twice-daily dosing. In the replicon system, treatment with danoprevir plus pegIFN- α gave peak synergy at predicted therapeutically relevant concentrations of each drug. More recently, the activity of this molecule has been examined against HCV genotypes other than HCV-1. As such, HCV-2 and HCV-3 showed 3- and 19-fold reduced susceptibility to the drug, respectively.³⁷

The results of a planned interim analysis of the Phase II ATLAS trial have recently been released.³⁸ At 12 weeks of triple therapy with danoprevir, pegIFN- α and RBV, the rate of RVR was significantly higher (73%–86%) in the arms receiving danoprevir at distinct doses than in controls treated with only pegIFN- α /RBV. Similarly, rates of undetectability at week 12 were higher in patients who received triple therapy rather than standard therapy (88%–92% versus 43%, respectively).

Given that danoprevir is metabolized in the liver by the cytochrome P450 3A, pharmacokinetic enhancement with low-dose ritonavir has proven to allow once- or twice-daily dosing of the drug. Moreover, this boosting seems to reduce peak drug exposures, which have been associated with elevations in liver enzymes. Thus, further clinical development of danoprevir will use ritonavir boosting.³⁹ Preliminary results in 24 prior null responders to pegIFN- α /RBV have shown that the use of 100/100 mg of danoprevir/ritonavir twice a day plus pegIFN- α /RBV is safe and provides robust antiviral activity in patients harbouring subtype 1b during the first 12 weeks of therapy. In contrast, half of patients infected with subtype 1a developed drug resistance.⁴⁰

BI-1335

This is a non-covalent, linear HCV PI that has recently entered Phase III clinical development. Its mean elimination half-life ranges from 20 to 30 h, supporting once-daily dosing. It is

metabolized by glucuronidation with marginal involvement of the cytochrome P450 complex, meaning reduced risk for drug interactions, a major concern with most other PIs. However, unconjugated hyperbilirubinaemia is not uncommon, particularly in those with Gilbert's syndrome. The drug exhibits potent dose-dependent antiviral activity, with median serum HCV RNA reductions of 4.4 log₁₀ within 15 days of monotherapy with the 240 mg once-daily dose. It has a strong protein binding and the estimated targeted C_{min} in plasma is 17 ng/mL, which ensures ~100-fold IC₅₀ exposure within the liver.⁴¹

In SILEN-C1, a Phase II trial, 427 HCV genotype 1 IFN-naïve patients were randomized to receive various doses of BI-1335 or placebo for 24 weeks. A 3 day lead-in phase of pegIFN- α /RBV was found to reduce SVR rates compared with triple therapy from the first day (71%–73% versus 83%, respectively). A dose of 240 mg once daily provided the best results. Jaundice developed in one-third of patients and rashes in 20%–30%. Responses were higher in subtype 1b than in 1a.⁴²

In the SILEN-C2 trial, 288 HCV genotype 1 patients with prior pegIFN- α /RBV experience (excluding relapsers) were randomized to receive BI-1335 once or twice daily with or without a 3 day lead-in phase of pegIFN- α /RBV.⁴³ Premature discontinuations due to adverse events (including jaundice and rash) occurred in 8% of patients. Overall, SVR was achieved by 47% of patients in the 240 mg once-daily arm. Relapses were fewer in patients who extended pegIFN- α /RBV to 48 weeks.

TMC-435

This is a non-covalent, macrocyclic HCV NS3/4A PI that displays potent activity in the replicon system (EC₅₀ 8 nM). *In vitro* studies have demonstrated additional synergic effects when TMC-435 is given in combination with other anti-HCV compounds. It can be administered orally once a day, and provides good tissue distribution and potent antiviral effect.⁴⁴ In the Phase IIa OPERA 1 trial,⁴⁵ TMC-435 was given for 28 days along with pegIFN- α /RBV in HCV genotype 1 patients and showed strong antiviral activity.

In the PILLAR study, a Phase IIb trial that enrolled 386 HCV genotype 1 patients naïve to IFN- α , the interim analysis at week 24 showed undetectability in 68%–79% of patients at week 4, in 91%–97% at week 12 and in 94%–97% at week 24, with no remarkable serious side effects.⁴⁶ Lastly, in the ASPIRE study,⁴⁷ a Phase IIb trial that enrolled 396 HCV genotype 1 patients with prior failure on pegIFN- α /RBV therapy, patients received 100 or 150 mg of TMC-435 once a day. At week 24, 69%–93% of patients on triple therapy had undetectable viraemia, while this was 52% in the control arm treated with pegIFN- α /RBV therapy. As expected, the best results were seen in prior relapsers and the worst in prior null responders. In this population, the 150 mg dosing outperformed 100 mg once-daily dosing.

Vaniprevir (MK-7009)

This is a non-covalent, macrocyclic, competitive inhibitor of the NS3/4A HCV protease. Phase IIa studies showed that vaniprevir produced a synergistic inhibitory effect on HCV genotype 1 when given along with pegIFN- α . In a trial conducted in 94 HCV genotype 1, IFN-naïve patients who were treated for

28 days with vaniprevir in combination with pegIFN- α /RBV followed by pegIFN- α /RBV alone for up to 48 weeks, the rate of RVR ranged from 67% to 84% in the vaniprevir arms and was only 5% in the control arm. The rate of SVR was 78%–84% with vaniprevir and 63% in controls.⁴⁸

HCV polymerase inhibitors

Many antiviral drugs against herpesviruses, hepatitis B virus (HBV) or HIV have focused on the viral polymerase, and advances in the treatment of chronic HCV infection are no exception. The HCV RNA-dependent RNA polymerase NS5B is the key viral enzyme responsible for HCV replication. It has been extensively characterized at the biochemical and structural levels.⁴⁹ The NS5B enzyme forms a replicase complex with other viral and cellular proteins in the perinuclear region.⁵⁰ Two types of HCV polymerase inhibitors are in development: nucleos(t)ide and non-nucleoside analogues (Table 1). It is noteworthy that nucleos(t)ide analogues are the only directly acting antivirals (DAA) in clinical development exhibiting broad activity against all HCV genotypes, the remaining inhibitors being mainly or exclusively active against HCV genotype 1.

Nucleoside analogue HCV polymerase inhibitors

Mericitabine (RG-7128)

Mericitabine is an oral prodrug of PSI-6130, a cytidine analogue. Pre-clinical observations demonstrated that PSI-6130 had an EC₉₀ value of $4.6 \pm 2 \mu\text{M}$ in the HCV replicon assay. The drug showed high specificity for HCV, minimal cytotoxicity and did not affect mitochondrial DNA. PSI-6130 is converted through phosphorylation by cellular kinases to an active 5'-triphosphate metabolite, which inhibits the NS5B RNA polymerase of HCV. Mericitabine demonstrated a relatively good safety profile and significant potency against HCV-1 in a multiple ascending dose trial conducted in 24 patients exposed to monotherapy for 2 weeks. Mericitabine produced serum HCV RNA declines ranging from 0.7 to 2.9 log₁₀ in a dose-dependent manner. The mean reduction in serum HCV RNA using the 1500 mg twice-daily dose was 2.7 log₁₀ IU/mL at day 15. Twice-daily administration of mericitabine was superior to once-daily administration, the half-life being 5 h. No viral breakthroughs were seen with mericitabine, suggesting a higher genetic barrier for resistance when compared with HCV protease or non-nucleoside polymerase inhibitors.^{51,52} Furthermore, no serious adverse effects were recorded, with headache and dry mouth being the most common. The drug is renally excreted with no hepatic metabolism involved, which may reduce drug–drug interactions.

Preliminary results of the Phase IIB PROPEL trial were recently released.⁵³ A total of 408 HCV genotype 1/4 patients were enrolled and received RG-7128 along with pegIFN- α /RBV for 8–12 weeks followed by pegIFN- α /RBV until completion of 24–48 weeks of therapy. The rate of RVR was 62% in the 1000 mg twice-daily triple arm compared with 18% in the standard-of-care arm. At week 12, undetectability was seen in 80%–87% of patients who received triple therapy for 12 weeks and was 49% in the control group.

The JUMP-C trial⁵⁴ is an ongoing Phase IIB study comparing triple therapy with mericitabine 1000 mg twice daily and

standard of care in IFN- α -naive patients infected with HCV genotypes 1 or 4. Interim analysis showed that up to 76% of patients in the triple arm achieved SVR with 24 weeks of therapy. Interestingly, no evidence of drug resistance selection was noticed, although up to 24% of patients relapsed after drug discontinuation.

The INFORM-1 study was the first trial that examined the efficacy of an IFN- α -sparing regimen in HCV genotype 1 patients, testing a combination of two experimental oral antivirals, mericitabine (nucleoside analogue) and vaniprevir (PI).⁵⁵ After 14 days of treatment, most patients showed a continuous decline in serum HCV RNA, with an average reduction of 5.1 log₁₀ in the higher dose arm. This study was the first proof of concept that IFN- α /RBV-sparing regimens may work against HCV.

Nucleotide analogue HCV inhibitors

Pharmasset has developed several nucleotide compounds that inhibit the HCV polymerase by acting as chain terminators. PSI-7977 is a pyrimidine analogue and PSI-938 is a purine analogue. These drugs show robust activity against all HCV genotypes and a high barrier to resistance. In the PROTON study, a Phase IIB trial, all 24 individuals with genotypes 2 or 3 that received PSI-7977 400 mg once daily for 12 weeks along with pegIFN- α /RBV achieved undetectable viraemia at weeks 4 and 12 of therapy.⁵⁶ Similar results were seen in 125 genotype 1 carriers.⁵⁷ No serious limiting side effects were recognized.

Non-nucleoside analogue HCV polymerase inhibitors

While HCV nucleos(t)ide analogues block HCV replication by acting as chain terminators and therefore stopping further elongation of the nascent RNA strand, non-nucleoside inhibitors interact with the polymerase outside the catalytic site and produce allosteric changes that critically compromise its function. The HCV polymerase structure shares the same general right-handed configuration as that of HIV reverse transcriptase (RT), consisting of finger, thumb and palm domains.^{49,58} While the polymerase active site is located in the palm domain, to date, five different sites (named 1–5) have been shown to be targeted by non-nucleoside inhibitors (Table 2). It is remarkable and unprecedented that more than one of these pockets have been found in the HCV polymerase, while in contrast only one has

Table 2. Non-nucleoside analogue HCV polymerase inhibitors

Pocket	Location	Main biochemical compounds	Drugs	Main drug resistance codons
1	thumb 1	benzimidazoles	BI-7127	495, 496, 499
2	thumb 2	thiophenes and others	filibuvir, VX-222, VCH-759	419, 423, 482, 494
3	palm 1	benzothiazidines	ANA-598, ABT-333, ABT-072	316, 414, 448, 554, 559
4	palm 2	benzofurans	HCV-796	314, 316, 363, 365, 414
5	B-hairpin	imidazopyridines	tegobuvir	445, 448, 452

Table 3. Main differential features of new DAA against HCV

Parameter	NS3 protease inhibitors	NS5B polymerase nucleos(t)ide analogues	NS5B polymerase non-nucleoside analogues	NS5A inhibitors
Mechanism of inhibition	inhibitory competition	inhibitory competition	Allosteric	?
Genotype activity	G1 (G1b>G1a)	across all	G1 (G1b>G1a)	across all
Resistance barrier	low	high	low	low
Cross-resistance	high	low	split out in four families	?
Drug interactions	pharmacokinetic	pharmacodynamic	pharmacokinetic	?

been identified in the HIV RT. Mutations conferring resistance to drugs binding at different sites do not cause cross-resistance. In contrast with HIV and HBV polymerases, which carry an RNA-dependent DNA polymerase (or RT), the HCV polymerase only works with RNA as template and product. This explains the lack of cross-inhibition of the HCV polymerase using HIV and/or HBV polymerase inhibitors.^{16,59} Table 3 summarizes the main differential features of the major DAA drug classes.

Filibuvir (PF-00868554)

This is a potent and selective non-nucleoside inhibitor of the HCV RNA polymerase (EC₅₀ of 9.6 nM against HCV-1), which is rapidly progressing through clinical development steps. In a Phase II trial, the drug was given for 28 days at doses of 200 or 500 mg twice daily along with pegIFN- α /RBV to 26 HCV genotype 1 patients.⁶⁰ Selection of mutation M423I/V in the NS5B polymerase significantly impairs the activity of the drug.

Tegobuvir (GS-9190)

Tegobuvir is a novel imidazopyridine acting as a non-nucleoside HCV polymerase inhibitor. It shows more activity against HCV-1 than other HCV genotypes. It binds to the β -hairpin of NS5B and resistance mutations arising around this pocket cause loss of susceptibility to the drug. Phase I trials in humans testing escalating doses of the drug showed a potent dose-dependent inhibitory effect.⁶¹ Mutation Y448H, either alone or together with Y452H, was selected in patients failing this drug.

ANA-598

This is a potent oral inhibitor of the NS5B polymerase of HCV-1 (EC₅₀ of 51 nM against HCV-1a and 3 nM against HCV-1b). It accumulates in the liver with a ratio to plasma ~20-fold higher. In a Phase II trial that investigated the efficacy of triple therapy for 12 weeks followed by pegIFN- α /RBV for an additional 24 or 48 weeks in HCV genotype 1 patients, 42%–56% of 63 subjects receiving triple therapy with two distinct doses of ANA-598 achieved RVR compared with 13% in the control group treated with pegIFN- α /RBV alone. At 12 weeks, the proportion of patients with undetectable viraemia was 73%–75% versus 63%, respectively.⁶²

VCH-759

This is an orally bioavailable non-nucleoside inhibitor of the HCV polymerase, which has demonstrated submicromolar IC₅₀

against HCV-1 in the replicon system. In a multiple ascending dose study, the efficacy and safety of VCH-759 were assessed during 10 days of monotherapy in 32 patients with chronic hepatitis C due to HCV-1. Patients received either placebo, 400 mg thrice daily, 800 mg thrice daily or 800 mg twice daily. All patients exposed to the drug experienced viral load reductions of between 1.2 and 3.3 log₁₀. Doses of 800 mg either twice or thrice daily will be used in further steps of clinical development in combination with other agents.⁶³

BI-7127

BI-7127 is a thumb pocket 1 inhibitor. BI-7127 is metabolized by glucuronidation and is particularly active against subtype 1b. The drug showed potent antiviral activity in a Phase I trial that enrolled 60 HCV genotype 1 patients. They were randomized to different doses given for 5 days. A reduction of >3 log₁₀ in serum HCV RNA was seen with the highest doses (800–1200 mg thrice daily). Rash was the most common side effect and is dose dependent.⁶⁴ In a more recent trial, HCV genotype 1 patients were treated for 28 days with triple therapy, confirming the potency of the combination.⁶⁵ The 600 mg thrice daily dose has been chosen for further clinical development. Boehringer is conducting trials combining BI-1335 (a PI) with BI-7127, with and without ribavirin and sparing IFN- α . In the SOUND-C1 study, a Phase Ib trial, 32 genotype 1 IFN- α -naive patients received this double or triple therapy, with impressive results at week 4.⁶⁶ Based on these results, the SOUND-C2 study was initiated. This is an ongoing Phase IIb trial that has enrolled 360 genotype 1 IFN- α -naive patients. Therapy is given for 16, 28 or 40 weeks with BI-7127, BI-1335 and/or ribavirin.

MK-3281

This is an oral compound that has successfully completed Phase I trials. In a study conducted in chimpanzees infected with HCV genotype 1, in which the drug was given in different doses as monotherapy for 5 days, a mean reduction in serum HCV RNA of 3.8 log₁₀ was seen with the highest dose. More recently, 22 humans were treated for 7 days with MK-3281 and the drug was well tolerated, although antiviral activity was limited to HCV subtype 1b.⁶⁷

NS5A inhibitors

A new family of antivirals against HCV was recently found to inhibit the NS5A replication complex by unclear mechanisms

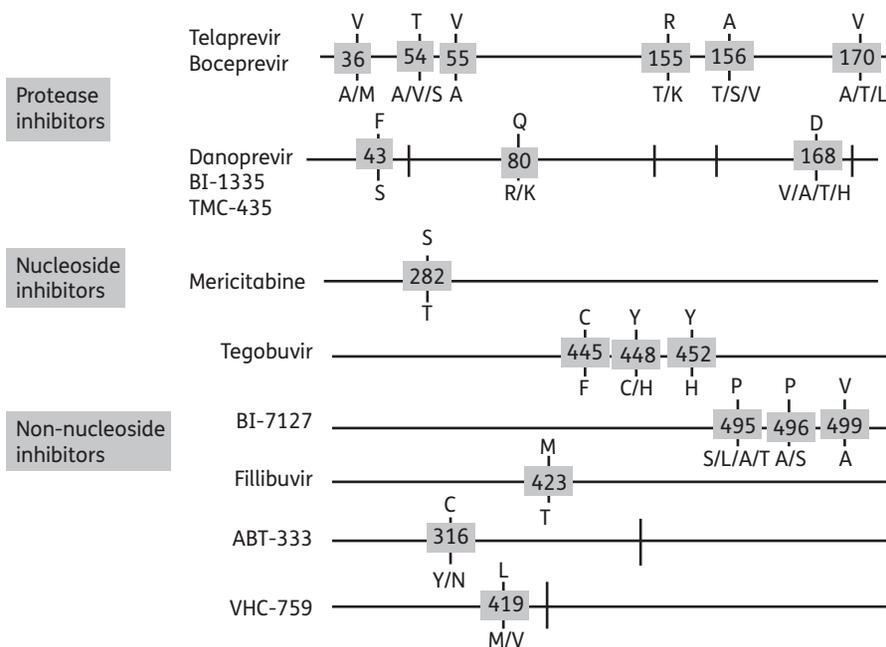


Figure 4. Main mutations conferring resistance to DAA in HCV.

interacting with the NS5A viral protein.⁶⁸ The first compound in this class is BMS-052, which exhibits picomolar antiviral activity against all HCV genotypes, although subtype 1b seems to be the most susceptible.⁶⁹ It is metabolized by cytochrome P450 and can be administered once daily (60 mg). Selection of drug resistance may occur rapidly. In a trial that enrolled 48 IFN- α -naive genotype 1 patients, triple therapy for 48 weeks provided rates of SVR of 83%–92% in the BMS-052 arms at doses of 10–60 mg/day, while it was 25% in the placebo control group. Overall, the drug displayed good tolerance.⁷⁰

HCV drug resistance

Antiviral potency, safety issues, posology (number of pills and how often they must be taken per day), drug interactions and resistance are the major determinants of success for any of the new DAA against HCV. Of these aspects, drug resistance has emerged as a major threat against using almost all of these compounds. Because of the error-prone nature of the HCV RNA-dependent RNA polymerase, drug resistance inevitably occurs in patients treated with antiviral drugs targeting distinct HCV specific enzymes and therefore limits their efficacy.^{71,72} Various *in vitro* studies using the HCV subgenomic replicon system have characterized resistance mutations against HCV protease and polymerase inhibitors. Mutations have been identified by *in vitro* selection and, less frequently, after patients fail drug therapy in trials. Discordances between *in vitro* and *in vivo* patterns of resistance mutations are largely dependent on the level of loss of susceptibility and the fitness of mutants.^{71,72}

Resistance to HCV PIs

Selection of resistance mutations to HCV PIs depends on both HCV genotype 1 subtypes and the specific drugs given. *In vivo*, HCV

subtype 1a predominantly selects R155K/T along with V36M and T54A/S, while in HCV subtype 1b the most frequent changes are D168A/V, A156S, T54A/S and I170A.^{73–79} Broad cross-resistance exists between all known PIs. However, changes selected by α -ketoamide compounds (telaprevir and boceprevir) do not overlap completely with those selected by other PIs (danoprevir, vaniprevir, TMC-435 and BI-1335) (Figure 4).

HCV displays a large genetic variability, which is even more pronounced than in HIV. HCV subtypes can vary by up to 25% at the nucleotide level. Accordingly, differences between HCV genotypes and subtypes are large in the polymerase and protease genes. The presence of natural polymorphisms that may influence the susceptibility to antivirals are present at different rates according to genotypes or subtypes.⁸⁰ Two separate studies conducted in >500 drug-naïve subjects each have shown that changes associated with resistance to HCV PIs can be recognized in a minority of subjects.^{81,82} However, overall changes are seen in 8%–9% of subtype 1a and in 1%–2% of subtype 1b, with R155K being the most frequent change in subtype 1a. These results have also been confirmed by examining the Los Alamos database for HCV as well as in other studies,^{83,84} including one conducted in HIV–HCV co-infected patients.⁸⁵ In one of these studies,⁸⁴ the NS3 polymorphism Q80K, which confers a reduced susceptibility to macrocyclic PIs, was found in nearly half of subtype 1a isolates. Table 4 records the rate of naturally occurring HCV resistance mutations for PIs in different studies. It is noteworthy that HCV subtype 1a is the one harbouring more frequent changes that may compromise the activity of PIs and non-nucleoside polymerase inhibitors.

Resistance to HCV polymerase inhibitors

HCV nucleos(t)ide analogue inhibitors display a greater genetic barrier to resistance than other DAA. They select drug resistance

Table 4. Naturally occurring polymorphisms at positions associated with drug resistance to protease inhibitors and polymerase inhibitors in HCV genotype 1 patients

	GenBank database	Bartels <i>et al.</i> ⁸¹	Kuntzen <i>et al.</i> ⁸²	Gaudieri <i>et al.</i> ⁸³	Sun <i>et al.</i> ⁹²	Bae <i>et al.</i> ⁸⁴	Treviño <i>et al.</i> ⁸⁵
NS3 protease, no. tested	3197–3328	570	507 ^a	192 ^b		39	55
V36A/M/L/G	0.03%	0.9%	1.6% ^c	1%		0	0
T54S/A	1.4%	—	1.8%	3% ^c		5%	0
V55A	—	—	—	—		0	3.6%
Q80R/K	4.7% ^d	—	—	—		28%	16% ^c
R109K	0.21%	0.2%	0	1%		0	0
R155K/T/I/M/G/L/S	0.03%	0.7%	0.6% ^c	0.5%		0	0
A156S/T/V/I	—	—	0	0		0	0
D168A/V/E	—	—	0.2%	1% ^c		0	0
V170A	0.12%	0.2%	0	5% ^c		0	0
NS5B polymerase, no. tested	—	—	—	212 ^b	55		47
S282T/R				0.4%	0		2%
L314F				0	—		0
C316Y/F/S				2%	2%		11%
M411S				0	0		0
M423T/I				1.4%	0		0
V499A				75% ^c	—		51% ^c
S556N/G				0	6%		0

^aOverall, 28% co-infected with HIV.

^bOverall, 42% co-infected with HIV.

^cSignificantly more frequent in HCV subtype 1a than 1b.

^dCombined Los Alamos and Gilead databases for HCV subtype 1a.

mutations within or near to the polymerase active site. As a result of critically compromising the enzymatic activity, the emergence of resistance mutations tends to be costly, with significant impairment of viral fitness. Changes at codon 282, with S282T being the most common, are characteristically selected *in vitro* by mericitabine.^{86–88} No resistance mutations have been reported so far for PSI-7977 or PSI-938.

Along with the active site, the HCV polymerase shows four and potentially five distinct binding sites for compounds that may block its activity allosterically. These pockets are located at the thumb (sites 1 and 2) and palm (sites 3 and 4) of a right hand-modelled HCV polymerase enzyme. The fifth site (E) is the β -hairpin region. Non-nucleoside inhibitors are non-competitive blockers and accordingly can be grouped into distinct families (Table 2), which generally select for different resistance changes.^{89–91} At least two mutations, however, may compromise the activity of more than one drug class. This is the case for C316Y/N, which reduces the activity of site 3 and 4 compounds. Likewise, Y448H reduces the activity of site 3 and 5 inhibitors. Overall, non-nucleoside inhibitors display the lowest genetic barrier for resistance among the DAA drugs. As shown in Table 4, natural polymorphisms in some HCV variants may compromise the activity of some of these compounds in subjects not previously exposed to any of these drugs.^{83,85,92} If confirmed, baseline drug resistance testing, as in HIV, might be considered before prescription of non-nucleoside HCV inhibitors. Figure 4 records the most frequent resistance mutations arising upon failure under HCV polymerase inhibitors.

Resistance to NS5A inhibitors

This new class of antiviral agents does not exhibit cross-resistance with either protease or polymerase inhibitors. Changes at positions Q30R, L31V/M and Y93C/N are the most frequently selected in patients failing NS5A replication complex inhibitors.⁹³ As with PI and non-nucleoside polymerase inhibitors, the barrier for resistance to BMS-052 is lower in HCV subtype 1a than in 1b.

HCV combination therapy

All DAA except nucleos(t)ide analogues uniformly show rapid selection of resistance mutations in most patients when given as monotherapy. It is noteworthy that resistance mutations to nucleos(t)ide inhibitors have been selected *in vitro* but as yet not shown *in vivo*, most likely reflecting their dramatic impact on viral replication. In the NUCLEAR study, PSI-7977 (pyrimidine) and PSI-938 (purine) were given alone or in combination for 14 days to 32 HCV genotype 1 patients. Mean viral load decline was 5 log₁₀ in the combination arms and none of the patients selected drug resistance mutations.⁹⁴

The use of combination therapy may increase the barrier for drug resistance and maximize antiviral activity, similar to what has been demonstrated in the HIV field. It is striking, however, that drug resistance mutations emerge even more rapidly in HCV than HIV, most likely due to the biological characteristics of the HCV life cycle. Viral turnover involves rapidly vanishing

Table 5. Ongoing trials using DAA combination therapy against HCV

Company	DAA-1 (protease inhibitor)	DAA-2 (polymerase or NS5A inhibitors)
Roche	danoprevir	mericitabine ^a
Boehringer	BI-1335	BI-7127 ^b
Vertex	VX-950	VX-222 ^b
Gilead	GS-9256	tegobuvir ^b
BMS	BMS-65032	BMS-790052 ^c
Idenix	IDX-320	IDX-184 ^c
Abbott	ABT-450	ABT-072 ^b

^aNucleoside analogue.

^bNon-nucleoside analogue.

^cNS5A inhibitor.

RNA molecules in the cytosol of infected cells and HCV does not involve a lifelong genetic reservoir, such as the proviral DNA in HIV or the cccDNA in HBV. In these other viruses, the lifespan of genomes depends on the infected cell survival time, since the genetic material is either integrated into the chromosomes (HIV) or maintained as episomes within the hepatocyte nucleus (HBV). The direct consequence of these biological features is that any drug pressure rapidly drives the selection of pre-existing resistant variants in HCV, but this is less rapid in HIV and even slower in HBV.⁹⁵

Another striking observation from Phase II trials using DAA against HCV is the recognition of a significant additive role for ribavirin, acting as a true antiviral agent.^{96–98} The best demonstration of this beneficial interaction so far comes from the PROVE-2 trial.²⁷ In that study, the arm with a triple drug combination (pegIFN- α /RBV/telaprevir) significantly outperformed the arm with dual pegIFN- α /telaprevir, with SVR rates of 69% versus 29%. Clearly, ribavirin should no longer be considered as a modest accompanying agent and its mechanism of action should definitively be elucidated. A hypermutagenic effect is currently the most plausible explanation.

Given that HCV infection can be eradicated from chronic carriers with treatment, it was initially thought that ‘the first will be the winner’, meaning that the first anti-HCV drug to gain approval would decrease the marketing opportunities for any other drugs arriving later. However, as growing evidence suggests that the selection of drug resistance will be an important challenge and that multiple drug combinations will be needed, it is clear that there will be room for several of these compounds. In fact, multiple drugs from distinct drug families will likely be needed, especially if therapy without pegIFN- α is desired. These drug combinations should ideally be constructed with compounds exhibiting complementary features, such as having distinct drug resistance profiles, targeting either the same enzyme (convergent therapy) or different HCV enzymes (divergent therapy). To some extent, we are currently experiencing in hepatitis C what happened in 1996 in the HIV field, when the introduction of triple combination antiretroviral therapy revolutionized HIV care.

Table 5 records some of the ongoing studies that aim to explore the efficacy and safety of IFN- α -sparing, DAA combinations against HCV.^{19,55,66,94,99,100} The results of the INFORM-1

trial were the first proof of concept that a combination of a PI (danoprevir) and a polymerase inhibitor (mericitabine) administered for 14 days may induce complete viral suppression in most treated patients.⁵⁵ Similar trials have been conducted, are ongoing or are planned using other compounds, with or without the addition of ribavirin.^{19,66,100} At this time, it seems that the addition of ribavirin reduces viral breakthroughs and/or relapses using these regimens, at least when PIs are combined with non-nucleoside polymerase inhibitors or NS5A inhibitors. This is particularly true in patients harbouring HCV subtype 1a. In contrast, the robustness of nucleos(t)ide analogues in terms of resistance selection might not require ribavirin to be added to IFN- α -sparing combination therapies.

Conclusions

The great expectation created with the arrival of DAA must be balanced with the consideration of potential concerns. While the antiviral potency of most DAA is impressive, complete suppression of viral replication is not obtained in most cases using monotherapy. Dose-limiting side effects preclude the use of higher doses of most molecules and, therefore, combination therapy will almost always be required. On the other hand, the low barrier for resistance, perhaps with the only exception of nucleos(t)ide analogues, will similarly push the use of combinations that ensure rapid viral suppression on therapy. At this time, pegIFN- α /RBV will be the forced marriage in most cases, with the DAA being the third agent. This requirement may limit both the patient’s tolerance (because of the well-known side effects of pegIFN- α /RBV) and efficacy (because of minimal or null response to IFN- α in a subset of patients). However, short courses of pegIFN- α /RBV (~24 weeks) driven by RGT will make triple combination treatment more affordable.

The emergence of drug resistance in HCV must be considered as a major threat. Many resistance mutations will result in broad cross-resistance to other compounds within the same drug family, which may complicate rescue options, at least in the short- or mid-term. The only exceptions are non-nucleoside analogues, given that distinct resistance profiles may allow the combination or sequential use of some of these compounds. The length of persistence of HCV resistance mutations upon drug discontinuation must be established, although recent data from the EXTEND study suggest that nearly 90% of drug resistance mutations selected failing on telaprevir may vanish within the first 2 years following discontinuation of therapy.¹⁰¹ However, the method used for testing was ‘bulk’ sequencing and minority variants present in a proportion <20% might have been missed. Currently, no threshold in drug-resistant mutant populations has been defined that may impact on antiviral activity in hepatitis C.

In contrast with HIV infection, in which the recognition of rates of transmission of drug resistance >10% has prompted the recommendation of drug resistance testing before initiating drug therapy, it is very unlikely that similar rates of transmission of drug-resistant HCV strains will occur. However, the finding of natural polymorphisms at positions that compromise the susceptibility to DAA in a substantial proportion of chronic hepatitis C patients,^{81–86} which is very much dependent on HCV subtypes, may support drug resistance testing in HCV before DAA prescription.

The majority of trials have tested new anti-HCV inhibitors against HCV genotype 1, and so the efficacy of these drugs against other genotypes remains largely unknown. The largest genetic diversity is observed between HCV genotypes 1 and 3, and explains that the activity of PIs and non-nucleosides designed against genotype 1 will be only residual or null against HCV genotype 3 viruses. The activity against HCV genotypes 2 or 4 might be intermediate for some of these compounds.^{102,103}

On the other hand, the influence of interleukin-28B (IL-28B) gene polymorphisms on the response to DAA, the risk for selection of drug resistance or the opportunity for shortening the length of therapy have not been established yet and should be clarified by the time the first DAA are approved. Controversial results have been reported regarding the impact of IL-28B polymorphisms on the response to telaprevir and boceprevir.^{104,105} Given that the impact of IL-28B polymorphisms would mainly depend on the relative contribution of pegIFN- α as part of the triple combination regimen, it seems worth elucidating to what extent the presence of good IL-28B variants may help overcome the deleterious impact of baseline predictors of poor response, such as high viraemia, advanced liver fibrosis, infection with HCV subtype 1a etc. In these patients, closer follow-up of the viral response and the selection of drug resistance as well as the benefit of longer duration of therapy are generally worth considering. On the other hand, patients with predictors of good treatment response, including good IL-28B variants, might benefit from shorter treatment duration with triple therapy or just be treated with standard of care. In this regard, the Prometheus index, which freely provides an estimation of the likelihood of response to pegIFN- α /RBV therapy according to four baseline variables (HCV genotype, liver fibrosis staging, serum HCV RNA and IL-28B alleles),¹⁰⁶ may help to identify the best candidates for DAA therapy.

Finally, the role of new DAA in the treatment of special populations must be established clearly. This is particularly true for prior non-responders to IFN- α -based regimens, since this subset of patients is generally in most need of successful therapy. Likewise, the efficacy of DAA must be defined for decompensated cirrhotics and for HIV-co-infected patients, given that these groups of patients represent a relatively large population in urgent need of therapy.¹⁰⁷ In the future, it is clear that the search for IFN- α - and/or ribavirin-sparing regimens must be pursued, using several oral DAA in proper combinations.

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