# New Horizons in Hepatitis C Antiviral Therapy with Direct-Acting Antivirals

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**List of abbreviations:**

DAA: direct-acting antiviral; HCV: hepatitis C virus; NS: non-structural protein; PEG-IFN: pegylated interferon-α; RBV: ribavirin; PI: protease inhibitors; BOC: boceprevir; TVR: telaprevir; DNV: danoprevir; DCV: daclatasvir; NI: nucleoside/-tide inhibitor; NNI: non-nucleoside inhibitor; SOF: sofosbuvir; SIL: silibinin; FQ: ferroquine; SVR: sustained viral response.

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**Abstract**

Most direct-acting antivirals (DAAs) being developed against the hepatitis C virus target the NS3/4A protease, the NS5A protein and the NS5B polymerase. The latter enzyme offers different target sites: the catalytic domain for nucleoside/-tide analogues as well as a number of allosteric sites for non-nucleoside inhibitors. Two NS3/4A protease inhibitors have recently been approved and more than 40 new NS3/4A, NS5A or NS5B inhibitors are in the development pipeline. These agents can achieve very high cure rates when combined with pegylated-interferon-$\alpha$ and ribavirin and show promising clinical results when combined in all-oral combinations. In addition to the more canonical drug targets, new alternative viral targets for small-molecule drug development are emerging, such as p7 or NS4B and viral entry. Future research will need to define well-tolerated and cost effective DAA combinations, providing the highest rates of viral eradication in all patients, including those with advanced liver disease, with broadest spectrum of action on viral genotypes, showing minimal or no clinical resistance and with the shortest treatment duration.

## Introduction

For more than a decade, the standard treatment of chronic hepatitis C has been based on the combination of ***pegylated interferon-α*** (PEG-IFN) and ***ribavirin*** (RBV), administered for 24 or 48 weeks. Depending on the viral genotypes, these regimens yielded eradication of the infection in a fraction of the treated individuals that could vary between 40-50% in genotype 1 (HCV-1) and 80% in genotype 2 and 3 HCV infections (HCV-2 and HCV-3, respectively). In addition, PEG-IFN/RBV regimens are poorly tolerated and contraindicated in a high number of patients. In order to address the shortcomings of PEG-IFN/RBV therapy, new direct-acting antivirals (DAAs) are being developed that target specific HCV functions. The recent approval of the first two NS3/4A oral protease inhibitors (PIs) ***boceprevir*** (BOC) and ***telaprevir*** (TVR) for use in combination with the PEG-IFN/RBV backbone has represented a dramatic advance in the efficacy of pharmacotherapy against chronic hepatitis C. With this new triple therapy regimen, the patient cure rates for chronic HCV-1 infection have increased to around 70-80%, while significantly reducing treatment duration. These recently-approved DAA-containing regimens, however, are poorly tolerated, associated with a high pill-burden and an inconvenient dosing frequency, and are not indicated for viral genotypes other than HCV-1. Moreover, selection of DAA-resistant viral variants does occur in patients who respond poorly to the PEG-IFN/RBV component of combination therapy. In light of these limitations, newer DAAs are being developed with the objective to identify more convenient and efficacious regimens, better tolerated, pan-genotypic and with a low propensity to develop viral resistance. These are primarily targeted at the NS3/4A protease, NS5A protein, or NS5B RNA-dependent RNA polymerase. In addition, other less-studied viral proteins, such as NS4B or p7, or the virus entry steps have been recently demonstrated to be "druggable". The ultimate goal in HCV research is to develop a broadly efficacious, IFN-free all-oral therapy. Toward this aim, several clinical trials, combining only oral antivirals, have started to show very promising results. In this review, we summarize the progress toward the development of DAA-based therapies, with particular emphasis on those compound classes/combinations that have shown the most encouraging antiviral activity in the clinic.

## Inhibitors of the NS3/4A serine protease

The HCV NS3/4A protease is a heterodimeric serine protease composed of the NS3 180 N-terminal amino acids and a short central hydrophobic domain of the NS4A protein, a protease co-activator (1) (**Figure 1A**). The protease activity of the NS3/4A complex is responsible for the proteolytic maturation of a large portion of the non-structural region of the HCV polyprotein, NS3 to NS5B. A

number of peptidomimetic active-site inhibitors have now been developed. From a chemical point of view, these can be divided into three main categories: i) covalent linear PIs, , ii) non-covalent linear PIs, and iii) non-covalent macrocyclic PIs. Macrocyclic PIs can be further classified as $P_3$-$P_1$ macrocycles or $P_4$-$P_2$ macrocycles (**Figure 1B**).

In the scientific community, the terms "first-generation" or "second-generation" NS3/4A inhibitors are also used to define subsequent PIs generations. First generation NS3/4A PIs are defined as agents that display potent activity on HCV-1, but oppose a low barrier to selection of resistant viral variants and are not effective on all viral genotypes. First-generation NS3/4A PIs are in turn distinct in "first-wave" (*i.e.*, BOC and TVR, both covalent linear inhibitors,) and "second wave" (non-covalent linear or macrocyclic inhibitors). Conversely, second-generation NS3/4A PIs are defined as agents that pose a high barrier to the development of viral resistance, retain activity against the viral variants that are resistant to first-generation compounds and are active across all HCV genotypes.

*First generation NS3/4A HCV protease inhibitors*

The currently approved NS3/4A inhibitors, BOC (2) and TVR (3), contain a α-ketoamide warhead that form covalent reversible bond with the protease catalytic serine (**Figure 1B**). Mutations associated with resistance to TVR or BOC readily occur at several positions close to the protease active site and are selected within a few days of monotherapy (**Figure 1A**). These include V36A/M/L, T54A/S, R155K/M/S/T, A156S (conferring low- to medium-level resistance) and A156T/Y (conferring high-level resistance) (4).

A number of second-wave, first-generation NS3/4A protease inhibitors are in advanced clinical development. They include: non-covalent linear PIs ***faldaprevir/BI 201335*** (5), ***asunaprevir/BMS-650032*** (ASV)(6), ***sovaprevir /ACH-1625*** (7) and ***GS-9451*** (8); non-covalent $P_3$-$P_1$ macrocyclic PIs ***simeprevir/TMC435*** (9), ***danoprevir/RG7227/ITMN-191*** (DNV) (10), ***ABT-450*** (11), and ***GS-9256*** (12); non-covalent $P_4$-$P_2$ macrocyclic PI ***vaniprevir/MK-7009*** (13) (**Figure 1B**). These agents are characterized by potent activity on genotype 1 HCV replicons (typically, low-nM $EC_{50}$). This translates into clinical efficacy on HCV-1 patients similar to that of BOC or TVR, leading to a decrease in circulating viral RNA of 3.5 to 4.5 Log IU/ml when administered as monotherapy for a few days. Different from their first-wave counterpart, second-wave PIs do not have the chemical reactivity needed to covalenty attack their target, leading to generally better tolerability. In addition, these agents have pharmacokinetic profiles compatible with once or once daily dosing (low-dose ritonavir boosting is used with DNV and ABT-450 in order to decrease dosing frequency).

Although some second-wave NS3/4A PIs have a significantly broader spectrum of action on the different HCV genotypes compared to their predecessors, including activity on HCV-4, these agents are not pan-genotypic, being invariably inactive on genotype 3 (14).

Along with the restricted genotype coverage, the genetic barrier posed to resistance by first generation NS3/4 protease inhibitors is low, with extensive cross-resistance is observed between the different compound classes. In particular, mutations of Arg155 have been shown to confer broad cross-resistance to all first generation inhibitors. Conversely, mutations of Val36 or Thr54 have been exclusively observed in association with covalent linear inhibitors (first- wave) and mutations of Asp168 are specifically found to confer mutation to non-covalent peptidomimetic inhibitors (second-wave, either linear or macrocyclic) (14).

*Second-generation active site and allosteric NS3/4A protease inhibitors*

***MK-5172*** (**Figure 2B**) is a second-generation NS3/4A PI with pan-genotype antiviral activity and improved resistance profile (15). Importantly, this agent maintains antiviral activity against most mutations that confer resistance to first-generation PIs, such as the two multi-drug-resistant variants R155K and D168A. A recent crystallographic study analyzing the molecular basis of drug resistance against NS3/4A protease inhibitors revealed that TVR, DNV and vaniprevir interact directly with the residues that confer resistance upon mutation, whereas MK-5172 interacts in a unique conformation with the catalytic triad, avoiding direct contact with R155 and D168 (16). No viral breakthrough has been observed in HCV-1 patients who received this drug alone for 7 days (17), suggesting a higher barrier to resistance compared to first-generation inhibitors. Morevoer, HCV-3 patients responded with a robust decline in viral RNA at the higher drug doses. ***ACH-2684***, a $P_3$-$P_1$ macrocyclic inhibitor, is another second-generation HCV PI, currently in Phase I clinical trial. ACH-2684 has potent biochemical activity against genotype 1-6 viruses and against known resistant variants (18). Worthy of note is the recent discovery of a new class of allosteric NS4/4A PI that binds at the interface between the NS3 protease and helicase domains (19). These agents exhibit a unique and novel resistance profile in vitro, implicating mutations of amino acids located at the allosteric drug binding site (M485 and V630). Comparable inhibition against genotypes 1, 3a, 5 or 6, but loss of activity against HCV-2a and 4 were reported for these compounds.

**NS5A Inhibitors**

HCV NS5A is a multifunctional, dimeric protein essential for HCV RNA replication and virion assembly (1). The NS5A protein structure consists of three domains: Domain I (aa 1-213), Domain II (aa 250-342) and Domain III (aa 356-447). The crystal structure of Domain I has been

crystallized in a dimeric form containing zinc- and an RNA-binding motif (20) (**Figure 2A**). NS5A inhibitors, initially discovered by replicon screening (21, 22), are believed to bind to domain I of NS5A and result in the suppression of viral RNA synthesis. Subsequent medicinal chemistry efforts led to the identification of extremely potent compounds characterized by a peculiar dimer-like structure (**Figure 2B**). The most advanced of this "palindromic" NS5A inhibitor class is ***daclatasvir /BMS-790052*** (DCV) (23), a compound with picomolar activity against a broad range of HCV genotypes. Clinically, single doses of DCV have been associated with a sharp and long-lived reduction in viremia (23). In spite of the potent antiviral activity, the genetic barrier to resistance for DCV is low, especially for genotype 1a. Thus, resistant variants emerge readily, with the more relevant substitutions found at NS5A residues 28, 30, 31, and 93 for genotype 1a and residues 31 and 93 for genotype 1b (24). DCV is currently being evaluated combination with PEG-IFN/RBV as well as in IFN-free regimens, in combination with SOF (polymerase nucleotide inhibitor), ASV (protease inhibitor) and/or BMS791325 (polymerase non-nucleoside inhibitor).

Other NS5A inhibitors in clinical development include ***GS-5885***, ***ABT-267, PPI-461, ACH-3102*** and ***MK-8742***. The latter two are early-stage agents with a higher barrier to resistance and which retain substantial levels of potency against resistance mutations selected by early NS5A inhibitors. These novel agents can thus be viewed as "second-generation" NS5A inhibitors (25).

**NS5B Polymerase Inhibitors**

The NS5B RNA-dependent RNA polymerase (RdRp) is the enzyme directly responsible for the synthesis of the HCV RNA genome (1). Similar to other nuclei acid polymerases, NS5B has the typical right-hand polymerase structure, consisting of a thumb-domain and a fingers-domain encircling the enzyme active site located within the palm-domain (**Figure 3A**). Inhibitors of the NS5B RdRp are classified into nucleoside/-tide (NI) and non-nucleoside (NNI) inhibitors (**Figure 3B**).

*Nucleoside/-tide analogues*

NIs target HCV RNA synthesis at the catalytic site of the NS5B enzyme. They are mimics of the natural polymerase substrates and are incorporated by the polymerase in the nascent RNA, leading to premature chain termination. Nucleoside inhibitors need three phosphorylation steps by cellular kinases to be converted to the active 5'-triphosphate form. Conversely, nucleotide polymerase inhibitors are prodrugs of nucleoside 5'- monophosphates, thus bypassing the rate-limiting step represented by the first phosphorylation step. Because of the active site conservation, NIs have

similar efficacy across all HCV genotypes/isolates (26). For the same reason, nucleoside/-tide inhibitors pose a high barrier to development of drug-resistance (27). Virtually all NIs in development contain 2'-*C*-methyl and 2'-fluoro groups at the sugar 2'-position. The primary mutation associated with decreased susceptibility to these drugs is NS5B S282T (28) (**Figure 3A**). The S282T mutation severely reduces HCV replication capacity, explaining the high-barrier to resistance posed by 2'-modified NIs (27). ***Sofosbuvir/GS-7977*** (SOF) **(Figure 3B)** is currently the most advanced NI NS5B polymerase inhibitor in clinical development (Phase III). SOF is a uridine nucleotide monophosphate analogue (beta-D-2'-deoxy-2'-fluoro-2'-C-methyluridine monophosphate). The S282T mutation is the common SOF resistance mutation emerging during resistance selection in vitro (29). While this mutation conferred resistance to SOF in genotype 1 replicons, it only caused a very minor shift in potency in genotype 2a, thus suggesting that additional mutations in genotype 2a NS5B are required for the resistant phenotype (29). Most importantly, to date, viral resistance has been hardly observed in any SOF-based clinical study, regardless of the viral genotype (30). SOF is currently studied in IFN-free combinations with a number of other DAAs, including NS3/4A protease inhibitors (GS-938, simeprevir) and NS5A inhibitor (DCV or GS-5885). Other nucleoside/-tide polymerase inhibitors in active clinical development include ***mericitabine/RG7128*** *(prodrug of* 2'-deoxy-2'-fluoro-2'-*C*-methylcytidine; Phase II), and ***ALS-2200/VX-135*** (structure undisclosed, Phase I).

*Non-nucleoside inhibitors*

Different from NIs, NNIs bind to less conserved allosteric sites found on the enzyme surface (**Figure 3A**). In contrast to NIs, these molecules have shown a restricted spectrum of activity against the various HCV genotypes (26) and present a very low barrier to emergence of resistance (27). The hallmark of all allosteric HCV NNIs described so far is that, in contrast to active site NIs, they are non-competitive with NTP substrates and inhibit the polymerase at a stage preceding the elongation reaction (1). DIfferent NNI binding sites are illustrated in **Figure 3B**. These include "thumb site I" (benzimidazole-binding site). "thumb site II" (thiophene-binding site), "palm site I" (benzothiadiazine-binding site); and "palm site II" (benzofuran-binding site). Significant variability in the amino acid sequence is observed at these sites, making it difficult to achieve antiviral efficacy against different genotypes or even HCV isolates within the same genotype. As a result, most reported NNIs are rather specific for genotype 1 or 1b (14).

Several structurally related NNIs have been shown to bind to the thumb I site (31). This class of inhibitors interrupts the intramolecular contacts between the thumb and the finger-tip loop, thereby preventing the formation of a productive enzyme/RNA complex (32). These agents are also known

as “finger-loop” inhibitors and are characterized by having a common benzimidazole or indole chemical core (**Figure 3B**). HCV variants resistant to these agents carry mutations at positions P495, P496 and T389 (33, 34) (**Figure 3A**). Clinically, agents belonging to this class of NNIs display reduced activity against genotype 1a HCV compared to genotype 1b (35). Several thumb I NNIs are currently being investigated in Phase II clinical trials, including ***BI 207127, TMC647055, BMS791325***.

Thumb II NNIs bind to a cavity located at the base of the thumb domain of NS5B (**Figure 3A**). Mutations at positions L419, M423, and I482 in the viral polymerase have been shown to confer resistance to this class of compounds (36). ***Lomibuvir/VX-222*** (**Figure 3B**), a tiophene carboxylic acid, and ***filibuvir/PF-868554,*** a dihydropyranone derivative, are currently in Phase II clinical trials.

Palm I NNI-binding site is located at the junction of the palm and the thumb domain of NS5B, in proximity to the catalytic site. Benzothiadiazine compounds, such as ***setrobuvir/RG7790*** (formerly ANA598; **Figure 3B**), ***ABT-333*** and ***ABT-072*** bind to this NNI site. The most frequently resistance mutations selected by these agents are C316Y, M414T, Y448H/C, or S556G (**Figure 3A**) (37). These compounds are currently in Phase II clinical trials. Acylpyrrolidines are yet another class of palm I-binding compounds (38). In this class, ***GSK625433*** was advanced into phase I clinical trials, but this study was halted because of adverse effects noted in pre-clinical carcinogenicity studies (39).

The palm II NNI-binding site partially overlaps with the palm I site and is located proximal to the junction between the palm and thumb domain. A class of benzofurans was originally identified as an NNI class binding to this site. These molecules select for resistant mutants at residues L314, C316, I363, S365 and M414 (40) (**Figure 3A**). ***HCV-796*** showed significant activity in early stage clinical trials (41), but was discontinued because of adverse side effects. HCV variants resistant to imidazopyridines**,** another HCV NNI chemoptype, carry mutations in the same site (C316Y) as well as mutations in a β-hairpin loop (C445F, Y448H, Y452H) located in close proximity of the catalytic active site (**Figure 3A**). In contrast to other NNIs, imidazopyridines do not inhibit the enzymatic activity of the purified RdRp, suggesting that these molecules target the enzyme via a unique mechanism. Recent data indeed revealed that imidazopyridine NNIs require metabolic activation by CYP1A for its activity. The resulting metabolite, after forming a conjugate with glutathione, directly and specifically interacts with NS5B (42). Within this class of imidazopyridine NNIs, ***tegobuvir/GS-9190*** (**Figure 3B**) is in Phase II clinical trials.

Presumably due to their barrier to resistance and restricted genotype/subtype coverage, HCV polymerase NNIs, such as tegobuvir or filibuvir, have provided disappointing clinical results when combined with PEG/RBV. Several NNIs, including setrobuvir, lomibuvir, BI207127, BMS791325, ABT-333 and ABT-072 are now being tested with more promising results in all-oral, IFN-free combination regimens (see below).

**Emerging targets for DAA development**

While many inhibitors of HCV protease, polymerase or NS5A are at an advanced development stage, new classes of direct-acting antivirals targeting less explored viral functions have started to appear on the horizon.

NS4B is an RNA-binding integral membrane protein required for the biogenesis of the membranous web required for the formation of HCV RNA replication complex (1). Several classes of NS4B inhibitors have been recently identified (43). ***Clemizole***, a first generation antihistamine, inhibits NS4B RNA-binding thereby preventing HCV RNA replication (44). Clemizole resistant variants carry mutations at position W55 and R214 in the NS4B protein. An ongoing proof-of-concept study is evaluating the safety and efficacy of clemizole monotherapy in treatment-naïve HCV-infected patients. Preliminary data reveal that clemizole, while inactive as a single agent, when combined with PEG-IFN and RBV may result in a more efficacious reduction in viral load than PEG-IFN/RBV alone (45).

Unexpectedly, very recent data point to NS4B as a candidate target for the anti-HCV action of silibinin (SIL) (46). SIL is an intravenous drug that has recently been administered to HCV(+) liver transplant recipients, leading in some instances to eradication of the infection. In cell culture, SIL potently inhibits HCV RNA replication for genotype 1a and genotype 1b, but not for genotype 2a. Mutations in NS4B, obtained either by selection in cell culture (Q203R) or observed in a liver transplant recipient experiencing viral breakthrough under SIL monotherapy (F98L+D228N), were found to confer in vitro resistance to SIL. These new exciting data point to the possibility that an already approved agent may be added to the growing armamentarium of HCV DAAs. Unfortunately, the intravenous mode of administration will ultimately limit the use of SIL in all-oral DAA combinations.

The HCV p7 protein is a viroporin (1) critical for the release of infectious virions. When its cation channel activity is pharmacologically blocked, virus production is significantly reduced (47). A number of HCV p7 inhibitors have been identified, such as ***amantadine***, ***rimantadine, long-alkylated iminosugasr*** and amiloride derivatives. The in vitro sensitivity to HCV to these drugs is highly genotype dependent, presumably because of the high sequence variability associated with the

p7 genetic region. To date, none of these p7-directed agents has demonstrated any significant clinical activity.

Another way to potentially limit acute as well as chronic HCV infection would be to prevent virus entry into the non-infected cells. ***Ferroquine*** (FQ), a novel antimalarial currently undergoing clinical evaluation, has been recently reported to inhibit HCV entry in cell culture at the membrane fusion step (48). FQ-resistant HCV was selected with a single resistance-conferring mutation in the E1 envelope protein (S327A). FQ may therefore represent a novel direct antiviral agent ready to be combined with other DAAs for all-oral therapy.

**Future Treatment strategies**

Although there are still some concerns on how many of the anti-HCV drugs currently in development will actually hit the market, it is clear we are on the verge of a revolution in the treatment of chronic hepatitis C. This revolution, at least for the hard-to-cure HCV genotypes 1 and 4, is likely going to be made of a 2-3 step process that will ultimately lead us to the holy-grail of an all-oral pan-genotypic IFN-free therapy. The first step forward in anti-HCV therapy will be the introduction of the second-wave PI to be used in combination with PEG-IFN/RBV. This will be followed by NS5A and NS5B inhibitors to be used with PEG-IFN/RBV in triple therapy regimens or in quadruple therapy regimens in combination with a second-wave PI. Finally, several all-oral combinations will enter the market, likely becoming the standard of care first therapeutic option for all HCV genotypes.

*Protease Inhibitors in combination with PEG-IFN/RBV*

One of the main limitations of the first-wave PIs BOC and TVR is tolerability when they are used with PEG-IFN/RBV. This stems both from the induction of specific side effects as well as from the rather impractical assumption mode that both compounds require (49). These first-generation, first-wave PIs need to be taken every 7-9 hours with food, causing a significant pill burden that may lead to sub-optimal adherence and sub-optimal efficacy. First-generation second-wave PIs, such as simeprevir, faldaprevir and ritonavir-boosted DNV, will be able to bypass this issue as they are being studied in Phase III trials with a once daily dosing (50). For HCV-1 patients, dosing convenience is likely to be one of the main advantage of these PIs as Phase IIb SVR rates of second-wave PI in combination with PEG-IFN/RBV do not exceed those seen with first-wave PIs. Second-wave PI triple therapies, in fact, achieve sub-optimal response rates in poor responders to PEG-IFN/RBV, in patients with cirrhosis or in HCV-1a patients (51, 52). Moreover their resistance profile is largely similar to that of BOC or TVR, meaning that second-wave PIs can't be considered

as a rescue therapy. These drugs, however, can be a clinical breakthrough for patients with non genotype-1 infection, as they are active against genotype 2,4,5 and 6 (53). This is especially significant for HCV-4 patients that not only are on the rise in many countries due to immigration from endemic areas but also currently represent a large unsatisfied medical need, given that TVR and BOC show little efficacy and are not reimbursed in this patient population. Importantly, in a Phase IIb study of HCV-4 patients receiving PEG-IFN/RBV and ritonavir-boosted DNV, 100% achieved an SVR following a course of 24 weeks of triple therapy (54).

*NS5A or NS5B inhibitors in combination with PEG-IFN/RBV*

NS5A inhibitors and NS5B polymerase inhibitors will enter the HCV market in a second phase and will probably be, at least for a short time period, associated with PEG-IFN/RBV therapy in substitution of first-wave PIs and in competition with second-wave PIs. Whether they will provide a true innovation in terms of viral cure rates, safety profile or patient tolerability is still to be demonstrated. A 24-week treatment of PEG-IFN/RBV plus DCV in HCV-1 naïve patients has been shown to attain SVR rates that range from 87% for HCV-1b patients to 58% for HCV-1a. These rates are similar to TVR or BOC triple-combination regimens, and also confirm the low barrier to resistance of first-generation NS5A inhibitors in the 1a subtype (14, 55). NS5A inhibitors seem better fit as partners of other DAAs (56)as shown by the very promising data obtained by a 24-week quadruple regimen of PEG-IFN/RBV plus DCV and ASV (PI) in HCV-1 patients with a previous null response to PEG-IFN/RBV. This regimen was associated with a 100% SVR rates in a small pilot-study and is now being explored in phase 3 studies (57). Although this is an impressive performance gain compared to TVR/BOC, which reach sub-par SVR rates (30-35%) in this population, this quadruple regimen is still relatively complex for patients, has a largely unknown safety profile and still needs to be explored in cirrhotics.

Equally impressive SVR rates have been seen with a 12-week regimen of PEG-IFN/RBV plus the NS5B nucleotide inhibitor SOF, as 90% of 51 HCV-1 (77% HCV-1a) naïve patients achieved SVR12 in the Phase II ATOMIC study (58). This regimen will improve SVR rates in HCV-1a patients, as NS5B NI activity is not influenced by HCV-1 subtype, but is unlikely to revolutionize the field in HCV-1b patients. In fact, when comparing the SVR rates of the ATOMIC study to Phase III trials of BOC/TVR in HCV-1b patients with similar viral and disease characteristics, the actual SVR gain quantifiable in the 10-15% range. Importantly, equally high SVR rates have been achieved by the PEG-IFN/RBV plus SOF combination in HCV-4 patients (82%).

*All-oral IFN-free regimens*

The first all oral anti-HCV regimen will be likely available in 2014 for HCV-2 and 3 patients. Phase III Studies investigating a 12-week course of the NS5B inhibitor SOF in combination with RBV are already fully enrolled and completed, and final results are expected for the second semester of 2013. This regimen has proven to be particularly effective in the Phase II Electron study, where 100% rates were obtained by this combination in HCV-2 and HCV-3 patients (59). For HCV-1 patients in the Electron trial this regimen turned out less effective, as SVR rates have ranged from 84% (naïve patients) to a disappointing 10% in the treatment-experienced patients (59). In the NIH-sponsored SPARE study 25 HCV-1 naïve patients were treated with SOF and RBV for 24 weeks. The SVR12 rate was 72% (60), not dissimilar from the current TVR/BOC-based standard of care. This study should not be overlooked as it was obtained in a cohort of patients enriched in known predictors of treatment failure such as advanced fibrosis (24% of patients), African American ethnicity (72%) and Il28B CT/TT (84%). Altogether, these data may indicate this regimen might be an effective treatment option only for easier-to-cure patients, including those infected with HCV-1b, IL28B CC and patients with mild disease, while probably being suboptimal in patients with harder to cure HCV disease, especially those who have failed previous PEG/IFN therapy.

The combination of two or more DAA is fundamental to achieve more potent and broad HCV RNA suppression and avoid IFN in HCV-1 patients. Several regimens meeting these requirements are in advanced phase of development (61). The optimal regimen should combine a drug with potent antiviral activity (PI or NS5A inhibitor) with a drug with high genetic barrier to resistance (NS5B NI), however high SVR rates have been achieved by regimens that are driven more by the drug portfolio of the various pharmaceutical companies than by rational mixing and matching of DAAs. A quadruple therapy regimen consisting of 12 weeks of a ritonavir boosted PI (ABT-450/r) plus a NNI NS5B (ABT-333) and an NS5A inhibitor (ABT-267) obtained SVR rates of 97.5% in 79 HCV-1 naive patients and 93.3% in 45 previous null-responders to PEG-IFN/RBV, with no significant differences in HCV-1a or HCV-1b patients (62). A similar 12-week regimen of ASV (PI) plus DCV (NS5A inhibitor) plus BMS791325 (NS5B NNI) reached 94% SVR rates in 16 HCV-1 naïve patients (63). These impressive numbers compare well with what today could be considered the optimal IFN-free regimen, *i.e.*, the combination of the NS5A inhibitor DCV and the NI NS5B inhibitor SOF. This regimen given for 12 weeks achieved an SVR4 of 98% in 41 HCV-1 naïve patients (64).

**Remaining issues**

Although treatment of chronic HCV infection is advancing at such a furious pace that keeping a strong grip on drug development can be problematic at times even for experts, still some common themes surface from most if not all ongoing studies (Table I). First of all there is a wide spread perception that most investigational agents for the treatment of chronic hepatitis C are being explored in "easy to cure" populations, at least partially devoid of negative prognostic factors (65). The lack of consistent safety and efficacy data in patients with advanced fibrosis/cirrhosis represents a major drawback of most, if not all, regimens. Although cirrhosis is likely going to lose its negative predictive power as a response moderator once potent anti-HCV regimens will be available, still it might remain a key determinant of reduced safety with some regimens. Indeed some drugs have side effects that might be worrisome in patients with cirrhosis, such as increased bilirubin with simeprevir (66), while others (such as ASV) show significant pharmacokinetic modifications in patients with impaired liver function (67), and thus need to be managed with caution in this group of patients. The same safety questions need to be ascertained in post-liver transplant (LT) patients and in those on the LT waiting list. To date we only have two very preliminary case reports of post-LT fibrosing cholestatic hepatitis C patients who reached an SVR with either a triple therapy regimen of PEG-IFN/ RBV and DCV (68) or an IFN-free regimen of DCV plus SOF (69), in each case without any significant safety issue and without any clinically relevant drug-drug interaction with the ongoing immunosuppressive regimen. . Real life studies of IFN free regimens, have shown surprising low rates of adherence to the correct treatment schedule and lower SVR rates compared to sponsored studies, meaning that once we move drugs into more "difficult to cure" patients, we might not completely replicate the amazing data obtained by Phase II trials (60, 70).

Affordability of some of these innovative regimens will also be an issue. Whether anti-HCV regimens that provide small benefits in terms of SVR but radically improve patient tolerability will be deemed cost/effective by national health systems and hence be reimbursed universally is unclear. Given that cost-effective drugs, such as TVR/BOC(71, 72), are still not reimbursable in many countries, it is possible that these innovative regimens will be confined to groups of patients where TVR/BOC or PEG-IFN/RBV are either ineffective or unsafe. This might create a paradox where pharmaceutical innovation might not translate into clinical innovation, with some patients receiving marginally less effective and less tolerable drugs for cost containing issues (73). This might reduce the penetration of treatment into key cohorts of infected patients, preventing anti-HCV therapy from playing a major role in containing the spread of the disease and in reducing the rate of progression to cirrhosis in resource-constrained countries.

The last open issue that often goes unnoticed when discussing future anti-HCV drugs is whether drug resistance will emerge as a clinical problem with all oral IFN free regimens (4). Resistance to TVR/BOC has limited clinical significance as HCV quasispecies reverts to wild type virus in a relatively short period of time (74). This is explained by the lack of a stable genetic reservoir for HCV and by the replication unfitness of most resistant variants to TVR/BOC. Whether this last point holds true for other classes of DAA needs to be discussed. NS5B NI are characterized by high barrier to resistance as the S282T mutation associated with decreased susceptibility to this class of compounds dramatically reduces HCV replication capacity. This means that this mutation is very rarely found as a pre-treatment naturally occurring variant and is also seldom found at the time of relapse (30). However NS5B NIs require compounds from other classes to achieve maximal SVR rates. Resistance to first generation NS5A inhibitors, ideal partners for an NS5B NI, have been shown to naturally occur and in some cases to persist as the dominant viral strain for at least 48 weeks following treatment failure (75, 76). In a Japanese study on HCV-1 patients treated with 24 weeks of ASV and DCV, DCV-resistant variants were found in 20% of patients at baseline. In virological failures, when NS3 and NS5A resistance associated variants were detected together (NS3: D168A/V; NS5A: L31M/V-Y93H), DCV-resistant substitutions persisted through 48 weeks whereas ASV-resistant substitutions were no longer detectable (77). It is too early to tell if this finding should alarm us, since the ASV-DCV combination is considered suboptimal in terms of genetic barrier to resistance, but it shows that not all we have learned from TVR/BOC can be safely translated to future anti-HCV drugs.

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**Table I**

| Therapeutic Regimen | Advantages over SOC 2013 | Open Issues |
|---|---|---|
| **IFN containing regimens** | | |
| PEG-IFN+RBV+2nd wave PI | Improved tolerability | Cost-effectiveness |
| | Reduced pill burden | Specific side effects |
| | Effective in non HCV-1 patients | Limited efficacy in HCV-1a |
| | | Drug-drug interactions |
| PEG-IFN+RBV+NS5A inhibitor | Improved tolerability | Cost-effectiveness |
| | Reduced pill burden | No data in cirrhotics |
| | | Limited efficacy in HCV-1a |
| | | Drug-drug interactions |
| PEG-IFN+RBV+NS5B NI | Improved tolerability | Cost-effectiveness |
| | Reduced pill burden | No data in cirrhotics |

| | | |
|---|---|---|
| | Effective in HCV-1a patients | |
| | Effective in non HCV-1 patients | |
| PEG-IFN+RBV+2nd wave PI + NS5A inhibitor | Effective in HCV-1a patients | Tolerability |
| | Effective in non HCV-1 patients | Pill burden |
| | | No data in cirrhotics |
| | | DDI |
| **IFN free regimens** | | |
| NS5B NI + RBV | Improved tolerability | Cost-effectiveness HCV-2/3 |
| | Effective in HCV-2 and 3 | Pill burden (RBV component) |
| | Effective in HCV-1a patients | Limited efficacy in difficult to cure patients |
| 2nd wave PI + NS5A inhibitor | Reduced pill burden | Drug-drug interactions |
| | Improved tolerability | Limited efficacy in HCV-1a |

| | | |
|---|---|---|
| 2nd wave PI + NS5A inhibitor + NS5B NNI ± RBV | Improved tolerability<br>Effective in HCV-1a patients | Pill burden<br>Drug-drug interactions<br>Limited data in cirrhotics |
| NS5B NI + NS5A inhibitor | Improved tolerability<br>Reduced pill burden<br>Fixed-dose combination<br>Effective in HCV-1a and in non HCV-1 patients | Limited data in cirrhotics |

## Figure Legends

**Figure 1 –Structure of the NS3/4A protease and resistance to protease inhibitors**

a) Three-dimensional structure of an NS3/4A protease domain – inhibitor comple
3SU3]. NS3 is depicted in green, NS4A in red. The structural $Zn^{++}$ ion is in gre
acids corresponding to the main resistance mutations are evidenced and numbe
b) Chemical structures of selected protease inhibitors.

**Figure 2 - Structure of the NS5A protein and resistance to NS5A inhibitors**

a) Three-dimensional structure of NS5A protein Domain I [PDB entry: 1ZH1]. Th
$Zn^{++}$ ion is in grey. Amino acids corresponding to main resistance mutations ar
and numbered. Substitions of Y93 represent primary mutations, whereas mutat
position P58 act as a secondary mutation (23). Other positions that are mutated
resistant HCV variants are not visible in the Domain I crystallographic structur
b) Chemical structures of a selected NS5A inhibitor.

**Figure 3 - Structure of the NS5B RdRp and resistance to polymerase inhibitors**

a) Structure of the HCV NS5B polymerase and binding sites for nucleoside/-tide and
nucleoside inhibitors. The palm, fingers and thumb domains are colored green, red
respectively. $Mn^{++}$ ions in the active site in grey. The amino acids corresponding t
resistance mutations for different inhibitor classes are evidenced and numbered.
b) Chemical structures of selected NS5B polymerase inhibitors.