Hepatitis C virus resistance to the new direct-acting antivirals

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1. Introduction

Hepatitis C virus (HCV) is a leading cause of chronic liver disease which can progress to cirrhosis and hepatocellular carcinoma, and it is the most common indication for liver transplantation in Europe and the USA [1]. According to WHO reports, approximately 120 million people worldwide are infected with HCV, with an estimated global prevalence of 2–3% [2].

Until 2011, the combination of pegylated interferon (peg-IFN) and ribavirin (RBV) was the standard treatment for HCV infection, leading to sustained virological response (SVR) rates below 40% in HCV genotypes (GTs) 1 and 4. Besides its limited effectiveness, IFN-based therapy was associated with a long treatment duration and frequent and severe adverse effects, especially in cirrhotic patients.

The advent of direct-acting antivirals (DAAs) has revolutionized therapeutic options for patients with HCV. New oral IFN-free therapies provide cure rates above 90% in most patients, regardless GT/subtype, prior IFN experience, and fibrosis stage [3,4]. However, the effectiveness of new DAAs may be compromised by the rapid development of resistance-associated variants (RAVs) [5].

Currently available DAAs are classified into four categories on the basis of their molecular target in the viral lifecycle and mechanism of action: nonstructural protein 3/4A (NS3/4A) protease inhibitors (PIs), NS5A inhibitors, nucleotide analogue inhibitors of NS5B RNA-dependent RNA polymerase (RdRp), and non-nucleoside inhibitors of RdRp. The high specificity of DAAs against their viral targets makes them sensitive to small changes in the viral sequence, resulting in the emergence of antiviral resistance which plays a key role in IFN-free treatment failure. Given the large HCV genetic variability, the outcome of DAA-based therapies may be altered by the selection of mutations at different positions in the NS5 protease, NS5B polymerase, and NS5A protein, which affect viral susceptibility to the administered compounds. Each drug or class of DAAs is characterized by a specific resistance profile that influences the genetic barrier to resistance and differs between viral GTs/subtypes [6] as shown in Table 1. Currently, different IFN-free combination therapies with DAAs are approved (Table 2), and should provide additive or synergistic antiviral potency and prevent the emergence of DAAs resistance [7].

A sequence diversity analysis at drug resistance-associated amino acid positions is important to evaluate the risk of naturally occurring resistance-related variants present at baseline or the risk for the development of drug-resistant variants under drug-selective pressure [5]. The identification of treatment-emergent resistant variants as well as the impact of preexisting baseline mutations on treatment outcome in patients failing treatment with DAA therapy is essential to predict the rate of cure with distinct DAA combinations and to assess treatment and retreatment options.
2. HCV variability and emergence of RAVs

The combination of a high HCV replication rate, the low fidelity of HCV polymerase, and the selective pressures exerted by the host immune system has driven the evolution of HCV toward the development of a global diversity. Phylogenetic and sequence analysis of entire viral genomes split HCV into seven major distinct GTs and more than 60 subtypes [8].

HCV has a high rate of turnover with $10^{10}$–$10^{12}$ virions produced per day in an infected patient. The RdRp of HCV has a poor fidelity because it lacks an exonucleolytic proofreading activity. Therefore, HCV replication is error prone with an error rate of $10^{-3}$–$10^{-5}$ mutations per nucleotide per genomic replication cycle. Consequently, the virus population in a chronic HCV infection exists as a group of genetically distinct but closely related variants, termed ‘quasispecies’ [9]. While the majority of those variants are cleared by the host immune system or are unable to replicate as a result of mutations that confer loss of

Table 1. Direct-acting antiviral (DAA) agents approved.

| Class of DAA | Potency | Genotypic coverage | Barrier to resistance | Cross-resistance |
|--------------|---------|--------------------|-----------------------|------------------|
| NS5B nucleos(t)ide analogue inhibitors | Sofosbuvir | High | Pangenotypic | High | High |
| NS5A inhibitors | Dasabuvir | Low | Limited to GTs 1 (1b > 1a) | Low | Low |
| NS3/4A protease inhibitors | Telaprevir | Medium | Limited to GT1 (1b > 1a) | Low | High |
| | Boceprevir | Medium | Limited to GT1 (1b > 1a) | Low | Medium |
| | Paritaprevir | High | Across all but GT3 | Low | High |
| | Asunaprevir | High | Limited to GT1 (1b > 1a) | Medium | High |
| | Vaniprevir | High | Across all genotypes less effective for GT3 | Intermediate | High |
| Second generation | Grazoprevir | Very high | GT1, GT4, and GT6 | High | Low |
| | Voxilaprevir | Very high | Pangenotypic | High | Low |
| | ABT-493 | Very high | Pangenotypic | Intermediate | High |
| NS5A inhibitors | Daclatasvir | Very high | Pangenotypic | Low | High |
| | Ledipasvir | High | GT1, GT4, and GT5 | Low | High |
| | Ombitasvir | High | GT1 and GT4 | Medium | High |
| | Eliquis | Very high | GT1, GT4, and GT6 | Low | High |
| | Velpatasvir | Very high | Across all genotypes less effective for GT3 | Low | High |
| | ABT-530 | Very high | Pangenotypic | High | Low |
function to essential HCV-encoded proteins, some variants remain replication competent. Thus, within an HCV-infected individual, this heterogeneous pool of genetic variants consists of a dominant (or ‘wild-type’) HCV strain that replicates to high efficiency, within a background of less-fit HCV variants present at lower frequencies [10]. Moreover, some of these minor viral variants can carry amino acid substitutions which determine conformation changes of a drug–target binding site, and are therefore less susceptible to the drug’s inhibitory activity, subsequently leading to a virological breakthrough during treatment or a relapse after treatment cessation [11]. Although these drug-resistant variants represent only minor percentages of the total virus population (frequencies <1%), they can be selected and become the predominant viral species during drug exposure, as shown in Figure 1.

It is therefore not surprising that RAVs naturally occur in HCV-infected treatment-naive patients [12]. The frequency of baseline RAVs is extremely variable and depends on many factors, such as the replicative fitness of the natural variant, the characteristic of the drug administered, the drug-binding region in the HCV genome, and the viral GT/subtype.

The likelihood that a drug will select for and allow outgrowth of viral variants carrying resistance-associated mutations within the quasispecies depends on several factors including (i) the drug’s genetic barrier to resistance, (ii) the viral fitness of the resistant variant, and (iii) the drug-selective pressure [13].

(i) The genetic barrier to resistance refers to the number and type of nucleotide changes needed to result in amino acid substitutions required to acquire resistance to the antiviral drug and is different between GTs and also varies on the HCV subtype level. When a single amino acid substitution is sufficient to confer a high-level resistance, the drug is considered to have a low genetic barrier, whereas a drug with a high genetic barrier requires multiple mutations within the HCV genome to generate a resistant variant.

(ii) The replication fitness of a resistant variant is defined as its ability to survive and replicate in a highly mutagenic environment. A selected resistant variant must be able to replicate efficiently in order to fill in the replication space left vacant by the susceptible ‘wild-type’ virus during drug exposure. Therefore, a highly resistant but poorly fit variant may not emerge to become the dominant viral species under drug selection pressure and will be less clinically significant than a variant with a preserved replication fitness that can replicate efficiently in the presence of the drug.

(iii) The drug-selective pressure is influenced by the drug potency, the level of drug exposure, defined as the drug concentration achieved in vivo relative to the inhibitory concentration 50 (IC$_{50}$)–IC$_{90}$/effective concentration 50 (EC$_{50}$)–EC$_{90}$ values of resistant variants, and the patient adherence to therapy.

3. Major HCV resistance patterns and mutations

3.1. Resistance to NS3/4A PIs

The NS3/4A PIs bind to the catalytic site of the enzyme and block posttranslational processing of the viral polyprotein at cleavage sites, preventing the release of functional proteins necessary for the production of infectious viral particles.

First-generation HCV PIs telaprevir and boceprevir are no longer recommended, given their limited efficacy (restricted to GT1), troublesome toxicities, and low genetic barrier to resistance and considerable cross-resistance. The second wave of the first-generation PIs includes simeprevir (SMV), asunaprevir (ASV), paritaprevir, and vaniprevir. These drugs exhibit an improved safety profile, a higher genetic barrier to resistance, and a better antiviral activity against multiple GTs, except GT3.

A summary of NS3/4A PIs resistance-associated mutations is given in Table 3.

3.1.1. Simeprevir

SMV was approved to be used with peg-IFN/RBV or as a part of all-oral regimens in patients with HCV GT1 or GT4 who are treatment naïve and prior treatment failures.

![Figure 1. Emergence and selection of drug resistant variants.](image-url)
Resistance to SMV was reported in vitro for several amino acid changes at key position in NS3: 80, 122, 155, and 168 [14]. Results from QUEST-1 and QUEST-2 phase III trials assessing the efficacy and safety of the combination of SMV plus peg-IFN/RBV in treatment-naive GT1 patients confirmed the replicon studies [15,16]. Most patients with treatment failure had emerging mutations in the HCV NS3 protease domain, which were mainly D168V in patients with GT1b or R155K alone or in combination with amino acid substitutions at positions 80 or 168 in those with GT1a.

Pretreatment natural resistance to SMV is rare among HCV GT1-infected patients [17]; however, Q80K variant is associated with a much higher natural prevalence in HCV subtype 1a isolates, leading to reduced susceptibility to SMV when combined with peg-IFN/RBV [15,16], but not in association with the nucleos(t)ide NS5B polymerase inhibitor sofosbuvir (SOF) as pointed out in the COSMOS randomized study [18].

The OPTIMIST-1 and -2 studies showed that the effect of Q80K on clinical outcome to SMV plus SOF seems to be substantially attenuated or possibly eliminated. High SVR12 rates were achieved, including in subjects with baseline Q80K, showing the strength of combinatorial treatment [19,20].

No data is available for GT4, the other indication of this combination.

### 3.1.2. Asunaprevir

ASV has been approved in Japan in combination with the NS5A inhibitor daclatasvir (DCV) in patients chronically infected with HCV GT1b.

In HCV replicon systems and in short-term ASV monotherapy studies [21], the most common NS5 substitutions identified were R155K and D168E, which conferred low- to moderate-level ASV resistance in GT1a, and D168V associated with high-level ASV resistance in GT1b.

The impact of preexisting drug-resistant substitutions on clinical outcome of the combination treatment with DCV and ASV was studied in GT1b-infected patients [22,23]. While preexisting DCV-resistant variants at positions 31 or 93 might compromise the response to this regimen, no ASV-resistant variants were detected at baseline. However, treatment failure was associated with the emergence of both NS5A-L31/Y93 and NS3-D168 variants. While ASV-resistant variants that emerged during therapy returned to wild type, DCV-resistant variants tended to persist in the absence of the drug, suggesting a higher relative fitness of NS5A variants [24].

### 3.1.3. Paritaprevir

Paritaprevir is coadministered with the pharmacokinetic enhancer ritonavir (r) (paritaprevir/r) and approved in combination with the non-nucleoside NS5B inhibitor dasabuvir (DSB) and the NS5A inhibitor ombitasvir ± RBV for the treatment of GT1-infected patients. This IFN-free regimen is referred to as three-drug combination (3D).

Amino acid variants conferring resistance to paritaprevir were detected in NS3 at positions 155 and 168 in GT1a and at positions 156 and 168 in GT1b, in vitro or following combination therapy, with the D168V variant conferring the highest level of resistance to paritaprevir in both subtypes [25].

These findings are consistent with resistance analyses conducted in the AVIATOR phase II trial in which the most prevalent NS3 treatment-emergent variants among patients with virologic failure were D168V and R155K [26].

The phase III SAPPHIRE-I and SAPPHIRE-II trials evaluating the safety and efficacy of the combination of paritaprevir/r with ombitasvir and DSB with RBV in HCV GT1-infected patients naive or previously treated with peg-IFN/RBV, respectively [27,28], showed that the patients who experienced virologic failure during treatment or relapse had at least one amino acid variant that was known to confer resistance to one of the three DAA agents included in the regimen. The most frequently detected variants in patients with GT1a infection who did not achieve SVR were D168V in the protein NS3, M28T/V and Q30R in the protein NS5A, and S556G/R and M414T in the protein NS5B. The GT1b infected with virologic failure had Y56H and D168A/V in NS3, L31M and Y93H in NS5A, and C316N and S556G in NS5B.

No data are available for the 2D regimens of ombitasvir and paritaprevir/r (without DSB) in patients infected with HCV GT4.

### 3.1.4. Vaniprevir

Vaniprevir is an investigational PI currently approved only in Japan, which exhibits potent antiviral activity in GT1-infected patients when added to peg-IFN/RBV.

In vitro resistance selection experiments and sequence data from phase I and II clinical studies have identified several NS3 variants at positions R155, A156, and D168 associated with decreased susceptibility to vaniprevir [29].

RAVs from patients failing to achieve SVR on vaniprevir-containing regimens from a trial of triple-combination therapy were R155K and D168T/V/Y in GT1a patients, and D168H/T/V

| NS3/4A inhibitors | GT1a | GT1b | GT1b | GT1a | GT1b |
|-------------------|------|------|------|------|------|
| Simeprevir        | V36  | T54  | V55  | Y56  | Q80  |
|                   | Y56  | Q80  | Y56  | Q80  | Q80  |
|                   | R155K | S122G/R | S122G/R | S122G/R | S122G/R |
|                   | D168V/E/A/H | D168V/E/A/F/H/T | D168V/E/A/T/Y | D168V/E/A/F/H/TV | D168V/E/A/F/H/T |
| Asunaprevir       | V36L/M | V55A | Y56H/L | Q80R/K | S122D/G/I/N/T |
|                   | Y56H/L | Q80R/K | S122D/G/I/N/T | S122D/G/I/N/T | S122D/G/I/N/T |
|                   | R155K | S122G/R | S122G/R | S122G/R | S122G/R |
|                   | D168V/E/A/H | D168V/E/A/F/H/T | D168V/E/A/T/Y | D168V/E/A/F/H/TV | D168V/E/A/F/H/T |
| Paritaprevir      | V36A/M | V55I | Y56H | D168V | A156T |
|                   | Y56H | D168V | A156T | A156T | A156T |
|                   | R155K | S122G/R | S122G/R | S122G/R | S122G/R |
|                   | D168V/E/A/H | D168V/E/A/F/H/T | D168V/E/A/T/Y | D168V/E/A/F/H/TV | D168V/E/A/F/H/T |
| Vaniprevir        | V36L/M | V55A | Y56H | Q80K | S122T |
|                   | Y56H | Q80K | S122T | S122T | S122T |
|                   | R155K | S122T | S122T | S122T | S122T |
|                   | D168V/E/A/H | D168V/E/A/F/H/T | D168V/E/A/T/Y | D168V/E/A/F/H/TV | D168V/E/A/F/H/T |
| Grazoprevir       | V36L/M | V55A | Y56H | Q80K | S122T |
|                   | Y56H | Q80K | S122T | S122T | S122T |
|                   | R155K | S122T | S122T | S122T | S122T |
|                   | D168V/E/A/H | D168V/E/A/F/H/T | D168V/E/A/T/Y | D168V/E/A/F/H/TV | D168V/E/A/F/H/T |

*The amino acid substitutions most commonly observed in patients who did not achieve SVR are visualized in bold.

Table 3. Main resistance mutations associated with HCV NS3/4A protease inhibitors in genotypes (GTs) 1a and 1b. |
in GT1b-infected patients. Moreover, R155K variants were observed at baseline in two naive patients who subsequently experienced virologic failure [30]. It is difficult to draw general conclusions from this observation, however, due to the limited data set.

3.2. Resistance to NS5B polymerase inhibitors

The NS5B polymerase inhibitors which interfere with viral replication by binding to the NS5B RdRp can be divided into two distinct categories [31].

Nucleos(t)ide analogue inhibitors (NIs) mimic the natural substrates of the polymerase and are incorporated into the nascent RNA chain causing direct chain termination. This class of DAA shows a high potency, a pan-genotypic activity, and a high genetic barrier to resistance because the active site of the HCV NS5B polymerase is strongly conserved among all HCV GTs.

Non-nucleoside inhibitors (NNIs) usually bind to several discrete sites on the HCV polymerase, which results in conformational protein changes before the elongation complex is formed. A limitation of this mechanism of action is that these allosteric binding sites are less conserved among GTs compared to the active site. As a consequence, lower cross-genotypic activity and higher probability of resistance development is observed.

A summary of NS5B polymerase inhibitors resistance-associated mutations is given in Table 4.

3.2.1. Sofosbuvir

SOF is the first nucleos(t)ide NS5B polymerase inhibitor approved for the treatment of HCV infection as part of IFN-based and IFN-free regimens.

Using HCV replicon systems, the S282T mutation was most commonly selected. The S282T known as signature NS5B mutation associated with resistance to SOF from in vitro studies has been rarely detected at baseline in phase II or III SOF-containing clinical trials; this could be explained by the low replicative fitness of this variant [32]. However, in the ELECTRON trial, the S282T substitution was detected in a patient infected with HCV GT2 who suffered a virologic relapse after 12 weeks of SOF monotherapy [33].

This polymorphism was also found in two GT1-infected patients who relapsed after treatment with SOF/RBV for 24 weeks [34] and with SOF plus the NS5A inhibitor ledipasvir (LDV) for 8 weeks [35], respectively.

In a pooled analysis of SOF phase III clinical trials for which drug resistance analyses were performed, low-frequency treatment-emergent NS5B substitutions including L159F and V321A were associated with virological failure in some SOF-treated subjects [36]. In a pooled analysis of SOF phase III clinical trials (FISSION, POSITRON, FUSION, and NEUTRINO) for which drug resistance analyses were performed, low-frequency treatment-emergent NS5B substitutions including L159F and V321A emerged in several patients infected with HCV GT3 who experienced post-treatment relapse.

3.2.2. Dasabuvir

DSB currently is the only non-nucleoside NS5B inhibitor binding to the palm I site approved as a component of the 3D combination.

A number of RAVs have been selected in HCV replicon or monotherapy studies at several amino acid positions in the NS5B protein: S556G and C316Y in GT1a, while C316Y and M414T in GT1b. The C316Y variant in both subtypes conferred >900-fold resistance to DSB [37].

3.3. Resistance to NS5A inhibitors

The NS5A inhibitors target the domain I of NS5A protein and block the phosphorylation of NS5A, which is important for viral replication assembly and release of HCV particles.

HCV NS5A inhibitors are likely to be a component of any multidrug combination regimens with pangenotypic activity potent enough to prevent the emergence of resistance mutations. Currently available NS5A inhibitors are DCV, LDV, and ombitasvir. Although NS5A inhibitors are quite potent and have a broad genotypic coverage (which is explained by a more conserved interaction site within the NS5A protein), they are also characterized by their relatively low viral barrier to resistance and long-time persistence of RAVs, as viral fitness seems not to be impaired [24]. All resistance mutations to this class of inhibitors were mapped to the N-terminal region of NS5A (domain I).

A summary of NS5A inhibitors resistance-associated mutations is given in Table 5.

3.3.1. Daclatasvir

DCV shows a very potent antiviral effect on several HCV GTs. NS5A mutations emerged in vivo and associated with failure of DCV mono- or combination-therapy are similar to those selected in the HCV replicon system or with the infectious clone [38].

The primary resistance conferring mutations observed in vivo for GT1a-infected patients who did not achieve SVR were M28T, Q30E/H/R, L31M/V, P32L, H58D, and Y93H/N, and

### Table 4. Main resistance mutations associated with HCV NS5B polymerase inhibitors in genotypes (GTs) 1a and 1b.

| NS5B inhibitors | Genotype (GT) | L159 | S282 | V321A | C316 | M414 | S556 |
|-----------------|--------------|------|------|-------|------|------|------|
| Sofosbuvir      | GT1a         | L159F| S282T/R| V321A | C316 | C316N|      |
|                 | GT1b         | L159F| S262T|       | C316 | C316N|      |
|                 | GT1a         | C316Y|       |       | C316Y| M414T/| S556G/R|
|                 | GT1b         | C316Y|       |       | C316Y| M414T/| S556G/R|

*The amino acid substitutions most commonly observed in patients who did not achieve SVR are visualized in bold.*
for GT1b the major resistance substitutions were L31M/V, P32L, and Y93H/N.

NS5A residues 30, 31, and 93 were the major sites associated with resistance to DCV in most of the GTs [39], suggesting that the location of the DCV binding site is conserved among diverse HCV strains.

Due to a relatively low genetic barrier, DCV was developed as part of an IFN-free dual therapy in combination with ASV. Subsequently, the impact of baseline polymorphisms associated with loss of susceptibility to NS5A inhibitors was evaluated in an open-label phase III clinical trial of DCV plus ASV in GT1-infected patients. This study highlighted that the presence of mutations at amino acids L31 and Y93 may reduce the barrier to resistance and influence virologic outcome for those patients who carry these polymorphisms at baseline [40].

More recently, the results of safety and efficacy for the combination DCV plus SOF for previously treated or untreated chronic HCV GT1-infected patients have been published. In this study, although the prevalence of baseline polymorphisms associated with DVC resistance was around 8%, all but one patient achieved SVR [41].

### 3.3.2. Ledipasvir

LDV was approved in combination with SOF against HCV GTs 1a and 1b.

The NS5A amino acid substitutions Q30E/R, L31M, and Y93C/H/N in GT1a and Y93H in GT1b, both in cell culture and in clinical trials, have been associated with high levels of reduced susceptibility to LDV [42,43].

In phase II and III clinical trials, the combination of LDV/SOF ± RBV resulted in high rates of SVR among untreated and previously treated patients with HCV GT1 infection, including those with compensated cirrhosis [44–46]. Although virological failure was rare using this DAA regimen, NS5A-resistant variants have been found in half of the patients who relapsed both at baseline and at the time of relapse, without NS5B RAVs.

### 3.3.3. Ombitasvir

Ombitasvir is an HCV NS5A inhibitor with pan-genotypic efficacy, co-formulated as a single tablet with the PI paritaprevir/r and administered along with the NNI DSB in the 3D combination regimen.

The in vitro profile of ombitasvir and the results in the 3-day monotherapy study identified variants conferring resistance at amino acid position 28, 30, 31, 58, and 93 in the NS5A gene across GTS 1–6; however, the resistance conferred by variants at these amino acid positions to ombitasvir varied by GT [47].

### 3.4. Next-generation DAA therapies

As the availability of broad pangenotypic DAAs remains scarce and the emergence of resistance remains challenging, there is still a search for more potent DAA combination therapies with increasing SVR rates and shorter treatment duration. The second-generation DAAs exhibit improved barrier to resistance as they aim to overcome restrictions in terms of resistance profile of the previous drug classes as well as concerning the coverage of distinct HCV GTs and subtypes. In the coming years, 2016/17, hopefully at least three other DAA-based combinations could be approved: grazoprevir (GZR) in co-formulation with elbasvir (EBR), SOF with velpatasvir (VEL) ± voxilaprevir (VOX), ABT-493 plus ABT-530 combination therapy.

#### 3.4.1. Grazoprevir/elbasvir

GZR, an HCV PI has been approved in the USA in 2016 in combination with the NS5A inhibitor EBR either ± RBV for the treatment of chronic HCV GT 1 or 4 infection. Due to their improved structure, GZR and EBR showed, in vitro, increased potency against some common clinical NS3 and NS5A RAVs selected by previous first-generation compounds.

Virologic findings in patients treated with GZR/EBR from phase II and III clinical studies were consistent with the preclinical observations. This therapy combines two DAA agents with distinct action mechanisms and nonoverlapping resistance profiles to target HCV at multiple steps in the viral lifecycle, resulting in SVR12 rate up to 95%, even in difficult-to-treat patients such as cirrhotic, human immunodeficiency virus (HIV) coinfected, or those who previously failed antiviral therapy [48].

The C-SALVAGE study demonstrated that GZR/EBR combination plus RBV for 12 weeks provides a promising retreatment option for HCV-infected patients with GT 1 with a history of failure on a triple regimen containing earlier-generation PI [49].

SVR12 was attained in 91% of the patients with prior virologic failure harboring virus with documented NS3 RAVs conferring decreased susceptibility to boceprevir, telaprevir, and/or SMV at baseline. This new regimen exerts a potent effect on HCV RNA replication and presents a high genetic barrier to resistance and not cross-resistance to the failed PI.

The presence of NS3 RAVs at baseline did not significantly affect the efficacy of GZR/EBR ± RBV, although NS5A baseline

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**Table 5. Main resistance mutations associated with HCV NS5A inhibitors in genotypes (GTs) 1a and 1b.**

| NS5A inhibitors       | HCV NS5A wild-type amino acid, position and resistance-associated substitution(s) |
|-----------------------|----------------------------------------------------------------------------------|
| Daclatasvir GT1a      | M28T/A/S/V, Q30E/R, L31M/V, Y93H/N                                               |
| Daclatasvir GT1b      | L28M/T, R30G/H/P/Q, L31M/V/F/I, Y93H/N                                           |
| Ledipasvir GT1a       | M28T/A, Q30E/R, L31M/V, Y93H/N                                                   |
| Ledipasvir GT1b       | L31M/V, L31I/M/V, Y93H/N                                                         |
| Ombitasvir GT1a       | M28T/V/A, Q30R/H/Y, L31M/V                                                       |
| Ombitasvir GT1b       | L31M/V, L31I/M/V, Y93H/N                                                         |
| Elbasvir GT1a         | M28T/G/A, Q30R/H/Y, L31M/V                                                       |
| Elbasvir GT1b         | L31M/F, L31I/M/V, Y93H/N                                                         |

*The amino acid substitutions most commonly observed in patients who did not achieve SVR are visualized in bold.*
RAVs had some effect on SVR12. Results from the C-WORTHY trial suggest that preexisting NS5A RAVs pose a bigger clinical problem than NS3/4A RAVs (SVR12 82% vs. 92%) [50]. Although EBR shows a higher barrier of resistance, NS5A polymorphisms at the same positions as for first-generation HCV NS5A inhibitors were observed in patients with treatment failure (M28, Q30, L31, Y93), especially in GT 1a patients.

### 3.4.2. SOF/velpatasvir ± voxilaprevir

Promising pangenotypic regimens in development are the co-formulation of SOF with the second-generation NS5A inhibitor, VEL and SOF/VEL plus VOX, an experimental macrocyclic HCV NS5/4A PI.

In ASTRAL phase III clinical trials, a fixed-dose combination of SOF/VEL for 12 weeks was highly effective in both treatment-naïve and -experienced patients, infected with GTs 1–6, including those with compensated and decompensated cirrhosis and those who did not achieve SVR after prior treatment with other DAA regimens. At baseline, the presence of NS5A RAVs had no impact on SVR (99%) in patients infected with GTs 1a, 1b, 2, 4, 5, and 6 in whom only two virologic failures occurred, both in patients with HCV GT 1 infection. Those two patients who had a relapse, had NS5A-resistant variants at baseline (Q3OR and L31M) and at the time of relapse (Y93H) [51].

Otherwise, among patients with HCV GT 3, the rate of SVR was 88% in patients who had NS5A RAVs at baseline and 97% among those who did not, with the lowest rate (84%) observed among patients with the Y93H variant at baseline [52].

Data from a phase II clinical trial demonstrated that the combination SOF/VEL plus VOX for 8 weeks was effective, achieving SVR rates over 95% across different patient populations including previously difficult-to-treat patients with cirrhosis, GT 3 HCV infection, and previous nonresponse to treatment. In addition, VOX and VEL retain potent activity in the presence of most commonly detected NS3 and NS5A RAVs, respectively. No specific baseline NS3, NS5A, or NS5B RAV alone or in combination predicted virologic failure, even for those patients with prior treatment experience [53]. Baseline RAVs, including Y93H, the only NS5A substitution which confers high-level resistance to VEL, did not appear to affect response to short durations of this treatment, confirming a very high barrier to resistance of this regimen and further suggesting its potential as a salvage regimen for DAA-experienced patient with longer treatment duration.

### 3.4.3. ABT-493/ABT-530

One of the exciting new combinations with several phase II clinical trials results being presented is the new second-generation HCV PI ABT-493 and the NS5A inhibitor ABT-530. In vitro, both compounds demonstrated potent pangenotypic antiviral activity, with a high barrier to resistance and maintained potent antiviral activity against key RAVs that often negatively affect the potency of other DAs.

Moreover, the presence of baseline NS3 and NS5A RAVs did not appear to affect viral load declines during ABT-493 and ABT-530 monotherapy, respectively, in treatment-naïve adults with HCV GT 1 infection, with or without compensated cirrhosis [54]. Taken together, these results suggest that the combination of these next-generation DAs holds promise for more difficult-to-treat patients who harbor NS5A RAVs that are known to confer resistance to currently approved NS5A inhibitors. The combination of ABT-493 and ABT-530 has been advanced into phase II clinical studies in both treatment-naïve and previously treated HCV patients with GT 1–6 infections, including patients with compensated cirrhosis, achieving encouraging SVR rates between 97% and 100%.

### 4. RAVs before and after treatment failure

#### 4.1. Clinical significance of baseline RAVs

The error-prone nature of HCV polymerase determines that pretreatment RAVs are likely to occur. Indeed, standard population sequencing and next-generation sequencing (NGS) technologies have described the natural existence of RAVs for all DAs classes [55–57].

RAVs may be present in the inherent sequence of some GTs and subtypes which could explain the reduced activity of certain DAs to different HCV GTs. For example, S556G which confers resistance to DSB is present in 97–100% of HCV GTs 2, 3, 4, and 5 isolates. The high frequency of this natural variant, together with RAVs at other positions within the NS5B polymerase (M289I/L, C316N), could explain the lack of antiviral activity of this DAA in non-HCV GT 1-infected patients [57].

It is currently unclear which frequency of RAVs is clinically relevant for the prediction of virologic treatment failure as the impact of pretreatment RAVs on therapy efficacy is also variable. Several clinical studies have revealed that in most cases, the preexistence of an RAV is not always related to treatment failure, thus suggesting that many other factors are also implicated. First, the level of resistance of a certain RAV is not necessarily related to treatment failure in a DAA-based antiviral therapy. For example, Q80K, a low-level RAV to NS3 PIs, significantly influences virologic treatment outcome in a triple therapy with SMV/peg-IFN/RBV; whereas for the combination therapy of SMV/SOF, the baseline presence of Q80K seems to be less relevant [18,58,59]. Second, in some cases, viral and host negative predictors of virologic treatment response together with baseline RAVs seem to be of clinical relevance. It has been reported that for the combination therapy of SOF/LED, response to antiviral therapy depends not only on the preexistence of high-level resistant NS5A variants (for example, Y93H with a baseline frequency of 3.8–14.1% in HCV GT1b patients), but also on other predictive factors, such as treatment duration and the stage of liver fibrosis [60]. Finally, the antiviral activity and the genetic barrier to resistance of the chosen DAA or combination of different drug classes influence therapy response. In fact, it has been reported that the preexistence of RAVs seems to have a greater impact on treatment schemes that include DAs with low barrier to resistance; whereas for regimens with DAs with high antiviral activities and high genetic barrier to resistance the presence of baseline resistance leads only to a small reduction of SVR rates [5].
4.2. Persistence of RAVs

In HIV and hepatitis B virus (HBV) infections, resistant variants are archived for prolonged periods after virologic failure and reselection during retreatment with the same type of drug [61,62] based on their replication with stable DNA intermediates. In contrast, in the case of HCV, studies are contradictory. While in some reports, no evidence for long-term persistence and reselection of isolates with RAVs during retreatment with the same drug was observed; in others indirect evidence pointed to the possibility of persistence and reselection [63–65].

After stopping DAA treatment, the frequency of many RAVs with impaired replicative fitness within the HCV quasispecies rapidly decline to levels undetectable by population and clonal sequencing [66,67]. For example, immediately after treatment failure with telaprevir and boceprevir, 82% of patients exhibited RAVs, but persistent variants were detected 1 year later in 18% of HCV GT1a-infected patients [68]. In the case of patients with repeated PI-based therapy, clonal and deep sequencing analysis revealed a continuous evolution of the NS3 genomic region with no clear evidence of persistence and reselection of RAVs but strong signs of independent de novo generation of resistance [65].

In contrast, RAVs to NS5A inhibitors are associated with high replicative fitness and frequently additional compensatory mutations. Indeed, in clinical studies it has been reported persistence of NS5A RAVs over 1–2 years after treatment failure in over 85% of patients [69,70]. After 24 weeks of retreatment with SOF plus LDV, in patients with detectable NS5A RAVs at baseline, the overall SVR rate was 60% which highlights the importance of persistent NS5A RAVs for the selection of effective retreatment options [35].

Regarding the long-time persistence of NS5B RAVs, preliminary data suggest that at least some RAVs (M414T, S556G) may tend to persist during long-term follow-up for at least 1 year after treatment failure. Interestingly, the persistence rate of NS5B RAVs which occur together with NS5A RAVs appears to be higher in comparison to isolated NS5B RAVs [71].

Although RAVs are almost always observed in patients with virologic breakthrough during treatment, in relapse patients the detection rate of RAVs varies between 53% and 91% depending on the duration of treatment, the DAA class, and regimen [72–76]. This is most likely explained by the low sensitivity of the DNA sequencing method used, potential rapid reversion to wild type between end-of-treatment and the day of blood sampling for sequence analysis, and a very low frequency of isolates containing RAVs within HCV quasispecies. Moreover, in patients with short duration of DAA-based antiviral therapies, wild-type virus may not be completely eradicated yet which also justifies relapse with a predominantly wild-type variant.

All oral DAA combination therapies have exhibited high efficacy in the majority of patients with chronic hepatitis C. However, given the large number of infected individuals around the world, treatment failure is still expected as a consequence, in most cases, of the combined presence of RAVs and negative predictive host or viral factors, reduced susceptibility to additional antiviral agents, or suboptimal treatment duration. Therefore, the problem of persistence, transmission, and reselection of RAVs will be more relevant in the near future [77].

5. HCV drug resistance testing

HCV resistance testing is fundamental to understand the clinical impact of drug resistance, optimize treatment schemes, increase SVR rates, and reduce treatment failure. In clinical research or in the clinical setting, the available tools could be used either to determine the individual variant pattern of a patient’s quasispecies (genotypic analysis) or to characterize resistance substitutions (phenotypic analysis) in samples collected at baseline (pretreatment), in case of virological breakthrough or relapse (virologic failure), and after treatment cessation (follow-up period).

The genotypic analysis is based on sequencing technologies which include population sequencing (also called direct sequencing) [78,79], clonal sequencing [80,81], and NGS [82–84]. The direct sequencing of the HCV genome only exhibits appropriate sensitivity to determine those dominant HCV variants that are present in the sample’s quasispecies with a frequency ≥20%; thus, it is a method useful for generating a consensus sequence. For many years, the only alternative method to population sequencing was genetic cloning followed by Sanger sequencing. Due to the fact that each clonal sequence represents a single variant present in the viral population, this technique shows high sensitivity to detect minor viral variants. However, the number of clones that can be analyzed is limited and the method is time consuming and laborious. As a consequence, clonal sequencing is now being replaced by deep sequencing technologies, as they allow reliable and fast detection of numerous viral variants with a frequency down to 0.5–1% [85].

The choice of one method over the other mainly depends on the aim of research and time point of sample collection. For example, pretreatment samples are analyzed to detect the preexistence of known or unknown resistance substitutions and/or provide a comparator for on- and posttreatment changes. Because of its high sensitivity, baseline resistant variants are more frequently revealed with NGS targeting short regions of a specific gene for quasispecies analysis [86,87]. On the other hand, at the time of virological breakthrough or relapse, it is important to identify amino acid changes relative to baseline that confer resistance to the administered drugs. This type of analysis is best performed by NGS or clonal sequencing to describe quasispecies changes. Finally, in the follow-up period, population sequencing would be useful if the resistant variant is present as a dominant viral population, but more sensitive techniques (i.e., clonal sequencing or NGS) are required to fully characterize the dynamics of RAV decay after treatment cessation.

Due to the error-prone activity of the HCV polymerase, all possible variants are continuously generated in the mixture of viral populations [88]. Therefore, all abovementioned sequencing methods may miss some RAVs as a result of their low frequencies within HCV quasispecies below the detection
limits of available assays. In addition, other methodological restrictions such as non-amplification based on HCV RNA secondary structures and primer selection may increase the lack of detection of RAVs.

In some cases, the inability to explain virologic failure requires the selection of candidate resistance substitutions detected in treated patients to perform phenotypic assays. In order to ensure an accurate assessment of the level of reduced susceptibility conferred by the selected substitution and a correct evaluation of its viral fitness cost, it is recommended to carry out this analysis by using site-directed mutagenesis or viral sequence insertion on a chimeric replicon backbone with the same GT as the original isolate. In addition, to take into account the quasispecies distribution of HCV populations and the fact that resistant substitutions are present in mixtures of viral variant populations, phenotypic assays are best performed on a mixture of isolated clones [89].

The usefulness of performing HCV resistance testing before starting a DAA treatment scheme is still under debate [90]. However, in some situations, resistance testing can be suitable in the clinical practice to decide which DAA is the best treatment option for a given patient.

In the case of treatment-naive patients and patients after failure with peg-IFN/RBV treatment, DAA resistance testing is not justified as it has been reported that treatment success with these new drugs occurs in high rates independently of the preexistence of RAVs. For regimens that include DAAAs with high antiviral activities and high genetic barrier to resistance, the presence of pretreatment RAVs is related to a small reduction of SVR rates. In these cases, it is important to take into consideration additional predictors of response such as the stage of liver fibrosis or baseline viral load.

However, a well-known exception is the Q80K substitution which confers resistance to NS3 PIs. Unlike other RAVs to this group of DAAs, Q80K exhibits no loss of replicative fitness in the majority of patients and a high probability of preexistence in HCV GT1a. International guidelines recommend testing for the presence of Q80K in DAA-naive patients infected with HCV subtype 1a who are being considered for treatment with SMV plus peg-IFN/RBV in cirrhotic patients due to the relative high frequency of preexisting Q80K variants in GT1a in South American (9%), European (18%), and North American populations (48%) [91]. It has been reported that SVR rates in treatment-naive HCV GT1a-infected patients with and without Q80K were 58% and 84%, respectively. Therefore, SMV-based triple therapy is not recommended in patients with detectable Q80K substitution at baseline.

In regions with economic limitations, pretreatment resistance testing may be cost effective in order to avoid virologic failure and the need of retreatment due to the high costs of these new drugs. In addition, HCV resistance testing may also be useful to select optimal treatment schemes in patients with shortened treatment duration, in those with liver cirrhosis, or in patients experiencing virological breakthrough or posttreatment relapse [92], particularly when their treatment comprises NSSA inhibitors. Indeed, in contrast to NS3 protease variants, NSSA RAVs can remain detectable several years after treatment withdrawal in 85% of patients [93], as viral fitness seems not to be impaired. The AASLD/IDSA guidelines 2015 recommend testing for RAVs that confer decreased susceptibility to NS3 protease and NSSA inhibitors, for retreatment of cirrhotic patients, or other patients who require retreatment urgently when these patients have history of failure to NSSA inhibitor-containing regimen [94].

Currently, there is no available recommendation for patients who failed to all oral DAA regimens. A proposed approach is longer retreatment with the same class of drugs although lower SVR rates are expected. Due to the high probability of the presence of multiple RAVs as well as the accumulation of negative predictive factors in these patients, monitoring resistance for the persistence of RAVs may be useful to determine the most appropriate and effective DAAs for second-line therapy, thus reinforcing the need of resistance testing in the context of virological failure in the clinical setting. Up to now, it is suitable to wait for the results of clinical studies or in case of urgent need of retreatment, select a different class of DAAs and take data obtained from resistance analysis into thorough consideration [95].

6. Expert opinion

The confluence of high viral replication turnover and the error-prone nature of the virus polymerase accounts for the large genetic variability displayed by HCV. Within each single infected person, the dynamic quasispecies nature of the viral population explains that mutations causing reduced susceptibility to antivirals are constantly been produced and that they would be selected under drug pressure. Combination antiviral therapy may overcome viral escape due to drug resistance in most instances and halting viral replication for time enough would lead to HCV elimination. This is in contrast with HIV or HBV, for which there is no stable cellular reservoir for the HCV genomic material, namely proviral DNA in HIV and covalently closed circular DNA (cccDNA) in HBV [96]. In the latest, antiviral treatment generally must be keep forever.

Viral gene sequencing may recognize drug resistance substitutions for almost all DAA, with rates depending on HCV GT/subtype [12] and sensitivity of methods used. The choice of the methods for RAV testing depends on the research subject and on the sensitivity expected from the sequencing technique, and on the financial supports of each clinical laboratory. As the investigation of RAVs has to be performed on several genomic regions, with the use of different classes of DAAs in combination, could therefore gain from complete genome sequencing techniques [97]. In our opinion, the use of NGS in the clinical laboratory and thus the implementation of HCV whole-genome sequencing in clinical practice could help in identifying compensatory mutations located on the outside of the usually investigated regions, directly targeted by antiviral drugs, and recombinant or rare viral types. Besides expanding genomic region coverage, deep sequencing methods may provide unique information on the impact of minor quasispecies that otherwise would be missed using crude population sequencing. The study of both the natural history of HCV infection and drug resistance could therefore benefit from the advantages of new molecular tools. However, the rapid and constant evolution of assays and their original high costs tend to slow down as NGS is steadily entering clinical practice.
Drug resistance in HCV has reached enough maturity to be considered a key factor in hepatitis C therapeutics. A reduced susceptibility to antiviral agents may be present in both drug-naive and treatment-experienced patients. The rate of natural polymorphisms at positions associated with drug resistance varies across HCV GTs/subtypes and each antiviral agent [98]. In contrast, selection of drug resistance following treatment failure occurs in most instances, although long-term persistence is mainly a concern for NS5A inhibitors. Considerable progress is being made and next-generation DAAs are coming with activity against drug-resistant viruses to either NS5A or PI. Moreover, these new agents are pangenotypic and exhibit higher resistance barrier [98].

The robustness of SOF against DAA resistance largely accounts for its pivotal inclusion within most current DAA regimen combinations, being taken along with NS5A inhibitors, PIs, and/or non-nucleoside polymerase inhibitors. All of the latest exhibit low resistance barrier. Besides the well-characterized S282T mutation, two additional changes (C316N and L159F) have recently been shown to confer reduced susceptibility to SOF. Whereas codon 282 changes dramatically impair viral fitness, C316N and L159F do not. Accordingly, they are recognized as naturally occurring polymorphisms in never treated patients, especially in persons infected with HCV GT1b [99].

Newer DAA are being designed that display greater resistance barrier and could allow building soon SOF-free, alternative therapeutic options, following the path of 3D for HCV GT1b. As example, phase III trials are ongoing with ABT-530 plus ABT-493, drugs that are pangenotypic NS5A, and PIs, respectively [98].

Although DAA failures generally occur in less than 5–10% of treated chronic hepatitis C patients, selection of drug resistance is the rule in most cases. Of note, most treatment failures are relapses rather than viral breakthroughs on therapy. HCV retreatment options are available, but first-line therapeutic strategies should be optimized to efficiently prevent DAA failure due to baseline HCV resistance [100]. For patients with cirrhosis or in whom previous treatment with any HCV NS5A inhibitors has failed and require retreatment urgently, testing for RAVs that confer decreased susceptibility to NS3 PIs (e.g. Q80K) and to NS5A inhibitors should be performed using commercially available assays prior to selecting HCV treatment regimen. Given that baseline NS5A RAVs are one of the strongest pretreatment predictors of treatment outcome with certain regimens, testing for these RAVs should be considered prior to the use of LDV/SOF. If LDV-associated RAVs are detected, consideration should be given to adding RBV to the regimen and extending therapy to 24 weeks; otherwise treatment with SMV, SOF, and RBV for 24 weeks is recommended. For patients who have both NS3 and NS5A inhibitor RAVs detected, limited data suggest a retreatment approach based on SOF combined with either GZR/EBR may be efficacious.

Besides compromising therapeutic options, drug-resistant viruses may also be transmitted. This caveat is of particular concern for NS5A inhibitor resistance-associated mutations that once selected may persist for years. Transmission of DAA-resistant viruses may occur from patients that have failed drugs within this family and are engaged in high-risk practices, that is, needle sharing among injection drug users or promiscuous sex among men who have sex with men. As proof of concept, sexual transmission of PI-resistant HCV has already being reported [77]. However, the major concern is for NS5A inhibitors, given that resistance mutations to these compounds generally persist for years and produce wide cross-resistance to most agents within this family.

Implementation of HCV drug resistance testing is challenged by the lack of commercial assays, difficult interpretation rules, and the rapid progress of the HCV armamentarium with next-generation DAA that would overcome the impaired response to current DAA driven by resistance-associated mutations, present either at baseline or following prior treatment failure. The usefulness of resistance testing in the clinical setting requires continued scrutiny as the use of different classes of DAAs for chronic HCV infection becomes increasingly widespread. Not all baseline and emergent RAVs will actually confer clinically significant drug resistance. HCV drug resistance testing prior to first-line therapy currently is not recommended [95]. Indeed, the SVR rates are very high both in patients without and with detectable amounts of preexisting RAVs; therefore, the detection of RAVs will not influence the treatment decision. Resistance testing may be useful in patients experiencing virological breakthrough or posttreatment relapse, particularly when their treatment comprises NS5A inhibitors. So in the context of DAA failure, monitoring resistance for the persistence of RAVs will lead to better management of second-line therapy. Otherwise, the usefulness of performing HCV resistance testing before starting a DAA treatment scheme is still under debate. However, the elevated costs of all-oral DAA therapies may push tailoring therapy, and in some situations like in patients with advanced cirrhosis, also baseline resistance testing can be suitable in the clinical practice to decide which DAA is the best (cost effective) treatment option for a given patient.

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**References**

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Di Bisceglie AM. Natural history of hepatitis C: its impact on clinical management. Hepatology. 2000;31:1014–1018.
This review summarizes the most relevant results obtained with oral DAA combinations that have been approved and/or have completed phase III clinical trials for HCV infection.

This study comprehensively describes the natural HCV varia-
tion and polymorphism in all the three targets of approved
antiviral drugs against hepatitis C virus. Nat Rev Gastroenterol Hepatol. 2015;59:5445–5454.

Feld JJ, Kowdiey KV, Coakley E, et al. Treatment of HCV with ABT-
450/r-ombitasvir and dasabuvir. N Engl J Med. 2014;370:1594–1603.

Zeuzem S, Jacobson IM, Baykal T, et al. Treatment of HCV with ABT-
450/r-ombitasvir and dasabuvir with ribavirin. N Engl J Med. 2014;370:1604–1614.

Lawitz E, Sulkowski M, Jacobson I, et al. Characterization of vani-
previr, a hepatitis C virus NS3/4A protease inhibitor, in patients with HCV genotype 1 infection: safety, antiviral activity, resistance, and pharmacokinetics. Antiviral Res. 2013;99:214–220.

Barnard RJ, McHale CM, Newhard W, et al. Emergence of resistance-
associated variants after failed triple therapy with vaniprevir in treat-
ment-experienced non-cirrhotic patients with hepatitis C genotype 1 infection: a population and clonal analysis. Virology. 2013;443:278–284.

Soriano V, Vispo E, de Mendoza C, et al. Hepatitis C therapy with
HCV NS5B polymerase inhibitors. Expert Opin Pharmacother. 2013;14:1119–25.

Lenz O, Verbinnen T, Lin T, et al. In vitro resistance profile of the hepatitis C virus NS3/4A protease inhibitor TMC435. Antimicrob Agents Chemother. 2010;54:1878–1887.

Jacobson IM, Dore GJ, Foster GR, et al. Simeprevir with pegylated interferon alfa 2a plus ribavirin in treatment-naive patients with chronic hepatitis C virus genotype 1 infection (QUEST-1): a phase 3, randomised, double-blind, placebo-controlled trial. Lancet. 2014;384:403–413.

Manns M, Marcellin P, Poordad F, et al. Simeprevir with pegylated interferon alfa 2a or 2b plus ribavirin in treatment-naive patients with chronic hepatitis C virus genotype 1 infection (QUEST-2): a randomised, double-blind, placebo-controlled phase 3 trial. Lancet. 2014;384:414–426.

Paolucci S, Fiorina L, Piralla A, et al. Naturally occurring mutations to HCV protease inhibitors in treatment-naive patients. Virol J. 2012;9:245.

Lawitz E, Sulkowski MS, Ghalib R, et al. Simeprevir plus sofosbuvir, with or without ribavirin, to treat chronic infection with hepatitis C virus genotype 1 in non-responders to pegylated interferon and ribavirin and treatment-naive patients: the COSMOS randomised study. Lancet. 2014;384:1756–1765.
protein SA replication complex inhibitor BMS-790052 in humans: in vitro and in vivo correlations. Hepatology. 2011;54:1924–1935.
39. Wang C, Jia L, O’Boyle DR, et al. Comparison of daclatasvir resistance barriers on NSSA from hepatitis C virus genotypes 1 to 6: implications for cross-genotype activity. Antimicrob Agents Chemother. 2014;58:S155–S156.
40. Kumada H, Suzuki Y, Ikeda K, et al. Daclatasvir plus asunaprevir for chronic HCV genotype 1b infection. Hepatology. 2014;59:2083–2091.
41. Sulkowski MS, Gardiner DF, Rodriguez-Torres M, et al. Daclatasvir plus sofosbuvir for previously treated or untreated chronic HCV infection. N Engl J Med. 2014;370:211–221.
42. Cheng G, Peng B, Corsa A, et al. Antiviral activity and resistance profile of the novel HCV NSSA inhibitor GS-5885. J Hepatol. 2012;56 (Suppl 2):S464.
43. Wong KA, Worth A, Martin R, et al. Characterization of hepatitis C virus resistance from a multiple-dose clinical trial of the novel NSSA inhibitor GS-5885. Antimicrob Agents Chemother. 2013;57:6333–6340.
44. Lawitz E, Poroedd FF, Pang PS, et al. Sofosbuvir and ledipasvir fixed-dose combination with and without ribavirin in treatment-naive and previously treated patients with genotype 1 hepatitis C virus infection (LONESTAR): an open-label, randomised, phase 2 trial. Lancet. 2014;383:515–523.
45. Afdhal N, Zeuzem S, Kwo P, et al. Ledipasvir and sofosbuvir for untreated HCV genotype 1 infection. N Engl J Med. 2014;370:1889–1898.
46. Afdhal N, Reddy KR, Nelson DR, et al. Ledipasvir and sofosbuvir for previously treated HCV genotype 1 infection. N Engl J Med. 2014;370:1483–1493.
47. Krishnan P, Beyer J, Mistry N, et al. In vitro and in vivo antiviral activity and resistance profile of ombitasvir, an inhibitor of hepatitis C virus NSSA. Antimicrob Agents Chemother. 2015;59:979–987.
48. Zeuzem S, Ghalib R, Reddy KR, et al. Grazoprevir-elbasvir combination therapy for treatment-naïve cirrhotic and noncirrhotic patients with chronic hepatitis C virus genotype 1, 4, or 6 infection: a randomized trial. Ann Intern Med. 2015;163(1):1–13.
49. Forns X, Gordon SC, Zuckerman E, et al. Grazoprevir and elbasvir plus ribavirin for chronic HCV genotype-1 infection after failure of combination therapy containing a direct acting antiviral agent. J Hepatol. 2015;63:564–572.
50. Sulkowski M, Hezode C, Gerroft J, et al. Efficacy and safety of 8 weeks versus 12 weeks of treatment with grazoprevir (MK-5172) and elbasvir (MK-8742) with or without ribavirin in patients with hepatitis C virus genotype 1 mono-infection and HIV/hepatitis C virus coinfection (C-WORTHY): an open-label, randomised, phase 2 trial. Lancet. 2015;385:1087–1097.
51. Feld JJ, Jacobson IM, Hezode C, et al. Sofosbuvir and velpatasvir for HCV genotype 1, 2, 4, 5, and 6 infection. N Engl J Med. 2015;373:2599–2607.
52. Foster GR, Afdhal N, Roberts SK, et al. Sofosbuvir and velpatasvir for HCV genotype 2 and 3 infection. N Engl J Med. 2015;373:2608–2617.
53. Gane EJ, Schwabe C, Hyland RH, et al. Efficacy of the combination of sofosbuvir, velpatasvir, and the NS3/4A protease inhibitor GS-9857 in treatment-naïve or previously treated patients with HCV genotype 1 or 3 infections. Gastroenterology. 2016 May 27. pii: S0016-5085(16)34513-9.
54. Lawitz EJ, O’Riordan WD, Asatryan A, et al. Potent antiviral activities of the direct-acting antivirals ABT-493 and ABT-530 with three-day monotherapy for hepatitis C virus genotype 1 infection. Antimicrob Agents Chemother. 2016;60(3):1546–1555.
55. Plaza Z, Soriano V, Vispo E, et al. Prevalence of natural polymorphisms at the HCV NS5A gene associated with resistance to daclatasvir, an NSSA inhibitor. Antivir Ther. 2012;17:921–926.
56. Bartels DJ, Sullivan JC, Zhang EZ, et al. Hepatitis C virus variants with decreased sensitivity to direct-acting antivirals (DAAs) were rarely observed in DAA-naïve patients prior to treatment. J Virol. 2013;87:1544–1553.
57. Di Maio VC, Cento V, Mirabelli C, et al. Hepatitis C virus genetic variability and the presence of NS5B resistance-associated mutations as natural polymorphisms in selected genotypes could affect the response to NS5B inhibitors. Antimicrob Agents Chemother. 2014;58:2781–2797.
58. Sarrazin C, Hezode C, Zeuzem S, et al. Antiviral strategies in hepatitis C virus infection. J Hepatol. 2012;56(Suppl 1):S88–S100.
59. Pawlotsky JM. New hepatitis C virus (HCV) drugs and the hope for a cure: concepts in anti-HCV drug development. Semin Liver Dis. 2014;34:22–29.
60. Sarrazin C, Dvory-Sobol H, Svarovaška ES, et al. Baseline and post-baseline resistance analyses of phase 2/3 studies of ledipasvir/sofosbuvir plus/– RBV. Hepatology. 2014;60:1128a–1128a.
61. Finzi D, Blankson J, Siliciano JD, et al. Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. Nat Med. 1999;5:512–517.
62. Margetheron-Thermet S, Svarovaška ES, Babrzadeh F, et al. Low-level persistence of drug resistance mutations in hepatitis B virus-infected subjects with a past history of lamivudine treatment. Antimicrob Agents Chemother. 2013;57:343–349.
63. Vermehren J, Susser L, Lange CM, et al. Mutations selected in the hepatitis C virus NS3 protease domain during sequential treatment with boceprevir with and without pegylated interferon alfa-2b. J Viral Hepat. 2012;19:120–127.
64. Lenz O, de Bruijne J, Vijgen L, et al. Efficacy of re-treatment with TMC435 as combination therapy in hepatitis C virus-infected patients following TMC435 monotherapy. Gastroenterology. 2012;143:1176–1178.
65. Susser S, Flinders M, Reesink HW, et al. Evolution of hepatitis C virus quasispecies during repeated treatment with the NS3/4A protease inhibitor telaprevir. Antimicrob Agents Chemother. 2015;59:2746–2755.
66. Susser S, Vermehren J, Forester N, et al. Analysis of long-term persistence of resistance mutations within the hepatitis C virus NS3 protease after treatment with telaprevir or boceprevir. J Clin Virol. 2011;52:321–327.
67. Paolucci S, Fiorina L, Marianti B, et al. Development and persistence of DAA resistance associated mutations in patients failing HCV treatment. J Clin Virol. 2015;72:114–118.
68. Mohamed S, Bourliere M, Benali S, et al. Clinical relevance of the HCV protease inhibitor-resistant mutant viral load assessed by ultra-deep pyrosequencing in treatment failure. J Clin Virol. 2016;78:36–43.
69. McPhee F, Hernandez D, Yu F, et al. Resistance analysis of hepatitis C virus genotype 1 prior treatment null responders receiving daclatasvir and asunaprevir. Hepatology. 2013;58:902–911.
70. Dvory-Sobol H, Wyles D, Ouyang W, et al. Long-term persistence of HCV NS5A variants after treatment with NSSA inhibitor ledipasvir. J Hepatol. 2015;62:S221.
71. Krishnan P, Tripathi R, Schnell G, et al. Long-term follow-up of treatment-emergent resistance-associated variants in NS3, NSSA and NS5B with paritaprevir/r, ombitasvir and dasabuvir-based regimens. J Hepatol. 2015;62:S213–S234.
72. Sullivan JC, De Meyer S, Bartels DJ, et al. Evolution of treatment-emergent resistant variants in telaprevir phase 3 clinical trials. Clin Infect Dis. 2013;57:221–229.
73. Barnard RJ, Howe JA, Ogert RA, et al. Analysis of boceprevir resistance associated amino acid variants (RAVs) in two phase 3 boceprevir clinical studies. Virology. 2013;444:329–336.
74. Krishnan P, Tripathi R, Schnell G, et al. Pooled analysis of resistance in patients treated with ombitasvir/ABT-450/r and dasabuvir with or without ribavirin in phase 2 and phase 3 clinical trials. Hepatology. 2014;60:1134a–1135a.
75. Lenz O, Verbinnen T, Kwo P, et al. Virology analyses of HCV isolates from genotype 1-infected patients treated with simprevir plus peginterferon/ribavirin in phase IIb/III studies. J Hepatol. 2015;62:1008–1014.
This review describes all the tools available to investigate drug resistance in preclinical studies, clinical trials, and clinical practice.

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