Abstract

Hepatitis C virus (HCV) is a significant medical problem and has become one of the leading causes of chronic liver disease. HCV replicates at a high rate, and due to inherently inaccurate nucleotide incorporation and lack of proofreading and post-replication repair, mutations are inevitable. In the era of direct acting antivirals (DAAs), treatment for HCV has become highly effective, but there are still about 5–10% of treated patients who do not achieve sustained virological response (SVR). There are many factors that affect SVR rates including the absorption and metabolism of DAAs, genetic make-up, the presence or absence of cirrhosis, and severity and resistance of HCV to DAAs. An important factor influencing treatment failure is HCV resistance. The majority of treatment failures while on DAAs are not due to on-treatment failures, but due to relapses. The exact mechanism for mutation-associated relapse is unclear, but possible theories include persistent intra-hepatocytic viral replication and/or differences in the levels of host immune response.

Keywords: HCV, molecular, treatment, drug resistance, mutation

1. Introduction

Hepatitis C virus (HCV) is an important medical problem, affecting millions of people worldwide [1]. HCV is one of the leading causes of chronic liver disease with one third of those affected eventually developing liver cirrhosis or hepatocellular carcinoma [2]. Additionally, HCV infection is asymptomatic in the majority of cases, and persons often do not receive necessary medical care as they are unaware of their infection. [3] Worldwide, HCV-related complications are responsible for about 350,000 deaths annually [2, 4].

HCV is an enveloped, positive-strand RNA virus and encodes a single polyprotein. This single polyprotein is cotranslationally and post-translationally processed by host and viral proteases to create 10 viral proteins: N terminus, Core, E1, E2, p7, NS2, NS3, NS4A, NS4B,
NS5A, NS5B, C-terminus. Of these, NS3, NS4A, NS4B, NS5A, and NS5B are nonstructural proteins that are the major players in RNA viral replication (Figure 1). The life cycle and replication of HCV is similar to other positive-strand RNA viruses. First, the virus enters the hepatocyte by receptor-mediated endocytosis, and after fusion and uncoating of the virion, it is released into the cytoplasm. The viral genome is then used as mRNA for translation of the viral polyprotein. After cleavage and processing of the viral polyprotein, the nonstructural proteins involved in replication (NS3-N5B) are incorporated into a membranous web to make replication complexes. Replication occurs by the synthesis of a negative-strand RNA from the positive-strand RNA, from which multiple copies of positive-strand RNA are synthesized. Infectious viral particles are then assembled by combining the structural proteins and positive-strand viral RNA. The infectious viral particles are then able to be transported out of the cell using the host VLDL-secretory pathway [1].

The high rate of HCV replication and low fidelity of the HCV polymerase results in heterogeneous virus populations [5]. Due to these factors, mutations are inevitable and the genomic composition is constantly changing. For RNA viruses, the mutation rate is about $10^{-3}$–$10^{-5}$ per nucleotide copied. The low fidelity of HCV RNA polymerases is due to the inherent inaccuracy in nucleotide incorporation and lack of proofreading and post-replication repair [6, 7].

With the advent of direct acting antivirals (DAAs), treatment for HCV has become highly effective. However, even with these new treatments, still about 5–10% of people with HCV fail treatment [1, 3]. Treatment success is measured based on sustained virological response (SVR), which is defined as an undetectable level of HCV RNA at 12 weeks or 24 weeks after the completion of treatment. For those who do not achieve SVR, there are many types of treatment failures that are described. Null responders are persons who fail to suppress HCV RNA by at least two logs by completion of treatment, whereas partial responders refer to those who achieve a decrease in HCV RNA levels by $\leq$2 logs, but never become undetectable. There are also treatment failures whose HCV RNA becomes undetectable, but then reappears in the serum. Of these, viral breakthrough refers to HCV RNA reemerging in the serum while still on treatment reappearance of HCV RNA in the serum occurs after treatment completion is referred to as virological relapse [8].

![Hepatitis C viral genome configuration.](image)

**Figure 1.** Hepatitis C viral genome configuration. The 5'- and 3'-designations indicate nontranslational regions (NTRs), and the 5'-region contains the internal ribosome entry site (IRES). The structural proteins (C, E1, E2) along with p7 and NS2 encompass the assembly module. The remainder of the nonstructural proteins makes up the replication complex.
The ability to achieve SVR depends on a combination of viral and host genetic factors. Until recently, little evidence was available to explain host differences associated with chronic HCV infection. The discovery of a human polymorphism at the IL28B gene, a variation in a single nucleotide polymorphism (SNP) on chromosome 19 that is associated with the poor interferon response, has been crucial in distinguishing responders and nonresponders to interferon-based antiviral therapy.

The DAAs currently target the proteins involved in HCV RNA replication, specifically NS3, NS5A, and NS5B (Table 1) [1]. Given high mutation rate, HCV is predisposed to the development of resistance to DAAs. Large numbers of genetically distinct HCV viral variants are generated daily in infected individuals. Collectively, these variants can create unique “quasi-species,” possibly resulting in reduced susceptibility to DAAs if polymorphisms are created in drug-targeted genes.

Viral resistance is an important factor associated with HCV treatment failure. Resistant variants may be selected or enriched, and drug resistance may emerge during HCV antiviral treatment. While viral resistance is a consequence of treatment failure, it is not always the cause. Resistant variants occur naturally and often exist before antiviral drug treatment [10]. The prevalence of intrinsically resistant variants is partially related to replicative fitness. In viral quasispecies, a dominant variant is usually identified along with other less fit variants, which exist at lower frequencies. These small groups of resistance-associated substitutions (RASs) apparent before the initiation of treatment can become dominant in the presence of selective treatment with DAAs. This, in turn, may affect treatment outcomes, leading to virological breakthrough or more commonly, relapse after treatment cessation.

Of note, there is a discrepancy in the term used to describe amino acid substitutions that reduce susceptibility of a virus to a drug or drug class, or the viral variants that carry the substitution resulting in reduced susceptibility. The term resistance-associated variants (RAVs) have been used previously to describe these mutants. Some investigators have stated that this term should be replaced by a different term, resistance-associated substitutions (RASs), to refer to the amino acid substitutions that confer resistance.

| NS3   | NS5A   | NS5B   |
|-------|--------|--------|
| Boceprevir | Daclatasvir | Sofosbuvir |
| Telaprevir | Ledipasvir | Dasabuvir |
| Simeprevir | Ombitasvir | Beclabuvir |
| Asunaprevir | Elbasvir | |
| Paritaprevir | Velpatasvir | |
| Grazoprevir | Pibrentasvir | |
| Glecaprevir | |

Table 1. Primary targets for DAAs for the treatment of HCV.
2. Identification of mutations

To identify barriers to resistance of experimental antiviral drugs, *in vitro* resistance selection studies are utilized. Many tests have been developed to identify HCV resistance including replicon systems in hepatoma cell lines, *in vitro* cell-free biochemical assays, and structural studies. However, these *in vitro* studies are not necessarily predictive of clinical resistance [7].

2.1. Replicon systems

Cell culture systems were developed to identify specific HCV mutations and how they affect drug resistance. The first cell culture replicon system was described in 1999 and is now available for the majority of HCV genotypes. This replicon system supports HCV replication in Huh7 hepatoma cells. Some replicons are unable to support the production of infectious virus particles, while more recent models are. The HCV pseudoparticle system, a cell culture replicon assay, was developed in 2003. This system works by creating a retrovirus coated with HCV envelope glycoproteins E1 and E2, which allows investigators to follow the steps of the specific HCV entry pathway. With this method, the entry of the virus can be monitored either visually or quantitatively by integrating reporter genes. In 2005, the first cell culture-infectious clone was introduced using the genotype 2a JFH1 isolate. With this method, the entire HCV viral life cycle is replicated in cell culture [1].

2.2. Cell-free biochemical assays

Cell-free assays are useful to examine the susceptibility of HCV to treatment with DAAs. This method can detect the effects of individual and complex substitutions on HCV enzyme activity under the influence of an investigational drug [7]. One such test is the NS3/4a enzyme assay, which uses a purified NS3 protease *in vitro*. In this assay, the NS3/4A fragment is cloned into an *Escherichia coli* expression plasmid for protein synthesis [7]. The protease activity is compared to various drug concentrations, and resistance is measured as inhibitory concentrations of either 50 and 90% (IC50 and IC90, respectively), drug concentrations that inhibit by 50 and 90%, respectively. [7]

Enzyme-based assays can be expensive and time consuming. These tests are based on coupled *in vitro* transcription/translation systems and have a turnaround time of about 10 hours. Several tools have been developed to study HCV replication, which evaluate viral enzyme efficacy and resistance to an RdRp inhibitor. The RdRp enzyme catalyzes the synthesis of both positive- and negative-strand RNAs. As for NS3/4A assays, the IC50 and IC90 can be calculated [7].

Studies have shown that some mutations reduce affinity for NS5A and decrease replication because NS5A regulates RdRp activity, although NS5A has no intrinsic enzyme activity [7].

2.3. Structural studies

Structural studies used to determine the structure of HCV proteins, and interactions with potential drugs include X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, and computational methods [7].
X-ray crystallography was used to examine the conformational flexibility and interaction of the investigational drug with conserved or mutated viral structures. By using crystallography, insight can be obtained into the cross-resistance of drugs in relation to a specific viral protein as well as the genetic barrier to resistance can be measured [7].

The NMR spectroscopy method provides data on proteins in solution without requiring protein crystallization and, therefore, allows for structural and functional studies. For unstable disordered proteins such as NS5, this is a particularly useful method [7].

Computational methods involve creating a software-based structural modeling analysis that analyze the X-ray structures of mutated NS3 or NS5B proteins. Using wild-type structures obtained from the Protein Data Bank, three-dimensional analyses of drug-binding sites and the impact of varying amino acid substitutions can be determined [7].

It is the data from structural studies that led to the modeling and understanding of structure–function relationships that ultimately led to development of highly effective DAAs with few side effects. However, the factors involved in clinical resistance could only be identified by clinical studies.

3. Clinical resistance studies

Samples from treatment failure patients have been sequenced and compared to known mutations identified from cell culture phenotypic analysis. In this way, mutations and amino acid substitutions known to impact drug susceptibility have been correlated [5, 11]. The RdRp and NS5B proteins have high barriers, whereas NS5A inhibitors and NS34A protease inhibitors have low barriers to resistance [11]. Information on the prevalence of RASs at baseline has been heterogeneous. This is not only due to differences in methods but also because studies generally select which RASs to study and which can affect their clinical significance [11]. Furthermore, most studies have been performed on HCV genotype 1 with very little data on other genotypes.

3.1. NS3/NS4A

The HCV NS3/NS4A protease cleaves four sites along the encoded protein. Rapid development of resistance due to NS3/NS4A mutations is common in patients on treatment with protease inhibitor therapy. In patients with genotype 1 infection, the most frequent substitution noted was Q80K, which was found in 13.6% of cases [11]. The R155K mutation, which is seen in genotype 1a virus, causes resistance against nearly all protease inhibitors. In genotype 1b, various resistance mutations can arise based on the protease inhibitor class to which the patient has been exposed. In response to ketoamide protease inhibitors, A156, V36, T54, and V36 + A155 mutations have been observed. When macrocyclic inhibitors were used, however, mutations in R155K and D168A were seen. Given this information, even though NS3/NS4A inhibitors have been very effective in the treatment of HCV, it is evident that drug resistance challenges the success of these agents [5].

The majority of drug-resistant mutations in the NS3/NS4A protease occur at the active site, as alterations in these areas can modify drug binding while also having minimal impact on
substrate binding and viral fitness. Danoprevir, simeprevir, and boceprevir, all project from the substrate envelope in areas known to have resistant mutations, leading to multi-drug-resistant variants. For instance, the large P2 moieties of danoprevir and simeprevir bind at the S2 subsite resulting in high interaction rates with the R155, D168, and A156 residues. It is not completely understood how these molecular alterations reduce inhibitor binding without affecting the binding of viral substrates [5] (Figure 2).

3.2. NS5A/NS5B

Substitutions in NS5B affecting efficacy of nucleoside analogs and non-nucleoside RdRp Palm-1 inhibitors are rare at baseline, while NS5A RASs are often detected in treatment naïve

Figure 2. The binding conformations of telaprevir, danoprevir, vaniprevir, and grazoprevir. Surface representations of the wild-type protease in complex with (a) telaprevir, (b) danoprevir, (c) vaniprevir, and (d) grazoprevir. The catalytic triad consists of D81, H57, and S139A. The R155, A156, and D168 side chains are also labeled for each binding conformation. (Adapted from Romano et al. [5]).
patients. By using a 15% clinically relevant cutoff in patients with genotype 1a, one or more RASs were found in 13, 14, 7, and 16% of cases in North America, Europe, Asia-Pacific, and Oceania, respectively [11].

4. Clinical trial results

In patients treated with sofosbuvir/ledipasvir for genotype 1 infection, resistance has been examined in the ION 1–3 and ELECTRON studies. The presence of NS3-4A protease RASs at baseline did not affect the clinical response to treatment. NS5A RASs had no effect on the SVRs in naïve patients with or without cirrhosis and with or without ribavirin. They did, however, lead to a high level of resistance to ledipasvir. This resulted in a low SVR for treatment-experienced patients infected with genotype 1a. It was noted that all patients who relapsed had this RAS leading to reduced susceptibility to ledipasvir with an SVR of only 72%. Adding ribavirin improved SVR from 88 to 94% in cirrhotic patients treated for 12 weeks and from 85 to 100% for patients treated for 24 weeks. Ribavirin appears to reduce the effects of pre-existing NS5A RASs [11, 12].

A phase 2 study with combination of ombitasvir, paritaprevir, and ritonavir (ombitasvir/paritaprevir/ritonavir) plus dasabuvir showed that patients with HCV genotype 1a infection with RASs at baseline had an SVR of 86% compared to 92% to those without RASs [13]. Researchers have reported SVR in HCV genotype 1-infected patients with and without cirrhosis who had baseline RASs. Treatment consisted of combinations of ombitasvir/paritaprevir/ritonavir plus dasabuvir with or without ribavirin for 12 or 24 weeks. Patients treated with HCV genotype 1b without ribavirin had 100% SVR. However, this was a small study with only four patients. The RAS region in these patients was NS3 protease- and paritaprevir-specific, which may explain the efficacy without ribavirin. The patients without baseline RASs treated with ombitasvir/paritaprevir/ritonavir had an SVR of 97% in all treatment groups [11].

In phase 2 and 3 studies of sofosbuvir plus daclatasvir with or without ribavirin in patients infected with HCV genotype 1, both treatment naïve and experienced, an SVR of 100% was seen in patients with baseline RASs. In patients with genotype 3 infections treated with sofosbuvir plus daclatasvir without ribavirin for 12 weeks, SVR in noncirrhotics was 97% for treatment naïve patients and 94% for treatment-experienced patients, and for patients with cirrhosis, 58% for treatment-naïve patients and 69% for treatment-experienced patients. In patients with baseline NS5A RASs with cirrhosis treated for only 12 weeks, a reduced rate of SVR was seen [11, 14]. Although the sample size was small, and the treatment lacked ribavirin, this may suggest a benefit of prolonging treatment in genotype 3 patients with baseline NS5A RRASs and cirrhosis.

In patients treated with sofosbuvir plus simeprevir without ribavirin for 12 weeks, a phase 2 study showed an SVR rate of 95% for genotype 1b, 88% for genotype 1a with Q80K present, and 94% for genotype 1a without the Q80K variant [15]. In a phase 3 study, SVR rates were studied in patients who were either treatment naïve or had been treated with pegylated-INF-based regimens with or without cirrhosis. In patients without cirrhosis, SVR rates of 97% in
genotype 1b, 96% genotype 1a with Q80K, and 97% in genotype 1a without Q80K were seen. In patients with cirrhosis, the SVR was 84% in genotype 1b, 74% genotype 1a with Q80K, and 92% genotype 1a without Q80K [16]. This suggested a decreased rate of SVR in genotype 1a in cirrhotic patients who had the Q80K RAS [11].

In phase 2 and 3 trials on patients treated with grazoprevir/elbasvir, the NS3 protease RAS was found not to affect SVR. However, the presence of NS5A RASs did affect the SVR in patients with genotype 1a. Patients without the elbasvir-specific NS5A RAS had an SVR of 98% compared to 58% in those with the RAS. NS5A RASs did not affect SVR in patients with genotype 1b. This effect was not observed with the addition of ribavirin and prolonging treatment to 16–18 weeks. The SVR was 94 and 100% with and without the NS5A RAS in genotype 1b, respectively. [11]

The combination of sofosbuvir/velpatasvir was studied in three phase 3 trials and the presence of NS5A RAS at baseline did not affect SVR in patients with genotypes 1a, 1b, 2, 4, 5 or 6. In patients with genotype 3 without the NS5A RAS at baseline, SVR was 97% compared to 88% in those with the RAS. Another phase 3 trial studied sofosbuvir/velpatasvir treatment in patients with decompensated cirrhosis (Child-Pugh B) and genotypes 1 to 6 HCV infections. Patients were treated for 12 weeks with ribavirin or 24 weeks without ribavirin. In patients with genotype 1 infection with and without baseline NS5A RAS, SVRs were 80% versus 96% for 12 weeks without ribavirin, 100% versus 98% for 12 weeks with ribavirin, and 90% versus 98% for 24 weeks without ribavirin. [11, 17] This suggests that adding ribavirin reduced the effect of NS5A RAS more than extending the duration of treatment [17–19]. Although asunaprevir plus daclatasvir has not been approved in the United Stated or Europe, it is used in Asia and the Middle East. Studies have suggested that patients with HCV genotype 1b with a NS5A RASs at positions 31 or 93 should not use this treatment regimen [11, 20].

In compliant patients, most treatment failures are relapses. The relapse rate has been described in several trials. One phase 3 trial studied treatment with sofosbuvir plus simeprevir in patients without cirrhosis and found a relapse rate of 17% and 3% at 8 and 12 weeks, respectively. In patients treated with grazoprevir/elbasvir, the relapse rate was 2.3% in HIV co-infected patients. A phase 3 trial studied sofosbuvir/velpatasvir and found that 20 patients with Child-Pugh B had relapse and 19 of these patients had NS5A RASs. Alternatively, 2 of 625 patients with genotype 1a, 1b, 4, 5, 6 without cirrhosis or with compensated cirrhosis experienced relapse and they both were found to have the NS5A substitution. In patients with genotype 3 and NS5A RASs, 10/277 had a relapse [11].

4.1. Retreatment studies

Retreatment strategies with DAAs in patients who have failed an interferon-free regimen can lead to SVR in the majority of patients including patients with known RASs. Studies suggested that sofosbuvir in combination with 1–3 other DAAs can be considered for retreatment. In addition, prolonging treatment to 24 weeks and/or adding ribavirin may also be considered [11]. These recommendations were based mainly on small scale studies. One study investigated 15 patients who failed a daclatasvir-based regimen. They were retreated with sofosbuvir and simeprevir without ribavirin for 12 weeks and achieved an SVR of 87%. In a study on retreatment with sofosbuvir, ombitasvir/paritaprevir/ritonavir and dasabuvir
with or without ribavirin, 92% of noncirrhotic patients with genotype 1a achieved SVR after 12 weeks with ribavirin and 100% achieved SVR after 24 weeks with ribavirin in patients with cirrhosis. In cirrhotics with genotype 1b, SVR without ribavirin achieved 100% after 12 weeks. Variants resistant to sofosbuvir were rarely selected and appeared not to affect retreatment with sofosbuvir possibly because sofosbuvir-resistant variants tend to be poorly fit and to disappear rapidly after treatment is stopped. In contrast, variants associated with NS5A RASs tend to persist which can affect re-treatment. [11].

5. Conclusions

In the era of DAAs, about 90–95% of persons treated for HCV to achieve SVR. While these new treatment regimens have significantly and dramatically improved SVR rates, about 5–10% of patients fail to achieve SVR [1]. Factors that influence SVR rates include the absorption and metabolism of the DAA, the immune response of the patient, the presence or absence of cirrhosis, and the severity and resistance of HCV to DAAs. [11] HCV resistance plays an important role in treatment failure. Most of the treatment failures on DAA treatment regimens are not due to on-treatment failures, but due to relapses. The persistence and development of resistant variants post treatment depend on the DAA class used [7].

There are several possible mechanisms of mutation-associated relapse. It seems most likely that relapse involves persistent intrahepatocytic viral replication. Treatment with DAAs is known to be biphasic with a rapid initial response followed by a slower second phase. The first phase is dependent on drug potency, exposure, and susceptibility. The second phase, which can be accelerated by ribavirin, is dependent on drug potency, host genetic features, and the severity of immune response [11]. During treatment, drug-sensitive HCV is suppressed in the blood, and the virus remains undetectable. Due to differences in host-specific hepatocyte factors or aggressiveness of the host immune system, the level of resistant variants in the hepatocytes may be higher in relapsers compared to responders.

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Abbreviations

- DAAs: direct acting antivirals
- HCV: hepatitis C virus
- SVR: sustained virological response
HIV human immunodeficiency virus
HBV hepatitis B virus
RdRp RNA-dependent RNA polymerase
SNP single nucleotide polymorphism
RASs resistance-associated substitutions
RAVs resistance-associated variants
NMR nucleic magnetic resonance

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