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# Resistance to Direct-Acting Antiviral Agents in Treatment of Hepatitis C Virus Infections

Mostafa K. El Awady and Reham M. Dawood

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## Abstract

Compounds targeting nonstructural (NS) proteins of hepatitis C virus (HCV) demonstrate clinical promise, suggesting that NS3/NS4a, NS5A, or NS5B inhibitors are potential components in direct-acting antiviral (DAA) combination therapies. In vitro studies revealed dramatic inhibition of viral replication or alteration in subcellular localization of NS proteins. DAAs bind either to catalytic sites (NS3 and NS5B) or to domain-1 of NS5A. Although >90% of the patients clear HCV RNA from their sera, a significant portion of cirrhotic patients suffer from resistance or virological relapse. Mutations in specific residues (Q80K) in NS3 (M28, A30, L31, and Y93 in genotypes 1a and 1b or L28, L30, M31, and Y93 in genotype 4) in NS5A and A282T in NS5B are associated with resistance to DAA [resistance-associated variants (RAVs)]. Current knowledge on the NS functions, mode of action of DAAs, and impacts of RAVs on treatment response are discussed. Not only mutations affecting the binding of DAAs to target proteins but also substitutions affecting the replication fitness of mutant quasispecies are major determinants of treatment failures. These resistance-associated substitutions (RASs) are now considered the major viral mutants that influence the virological outcome after DAA treatment.

**Keywords:** hepatitis C virus, direct-acting antiviral agents, resistance-associated variants, resistance-associated substitutions

## 1. Introduction

Hepatitis C virus (HCV) infection is a major etiological factor for liver cirrhosis, steatosis, and hepatocellular carcinoma and represents a primary reason for liver transplantation in patients with end-stage disease. It is estimated that around 350,000 deaths each year occur worldwide as a result of HCV-related liver diseases [1]. Chronic infection with HCV afflicts around 185 million people which represents 2.8% of the world's population [2]. Phylogenetically, HCV

exists as seven distinct genotypes each comprises several subtypes and many quasispecies. It was reported that more than 10% of the Egyptian population is infected with HCV, where genotype 4 represents >93% of the chronic infections [3]. The development of direct-acting antiviral (DAA) agents has dramatically enhanced sustained virological response (SVR) rates in genotype 1-infected patients [4]. Although the approval of IFN-free DAA combination treatments has been associated with high cure rates, the emergence of resistant HCV variants has an important role in treatment failure with DAA therapies.

## 2. Replication cycle of HCV

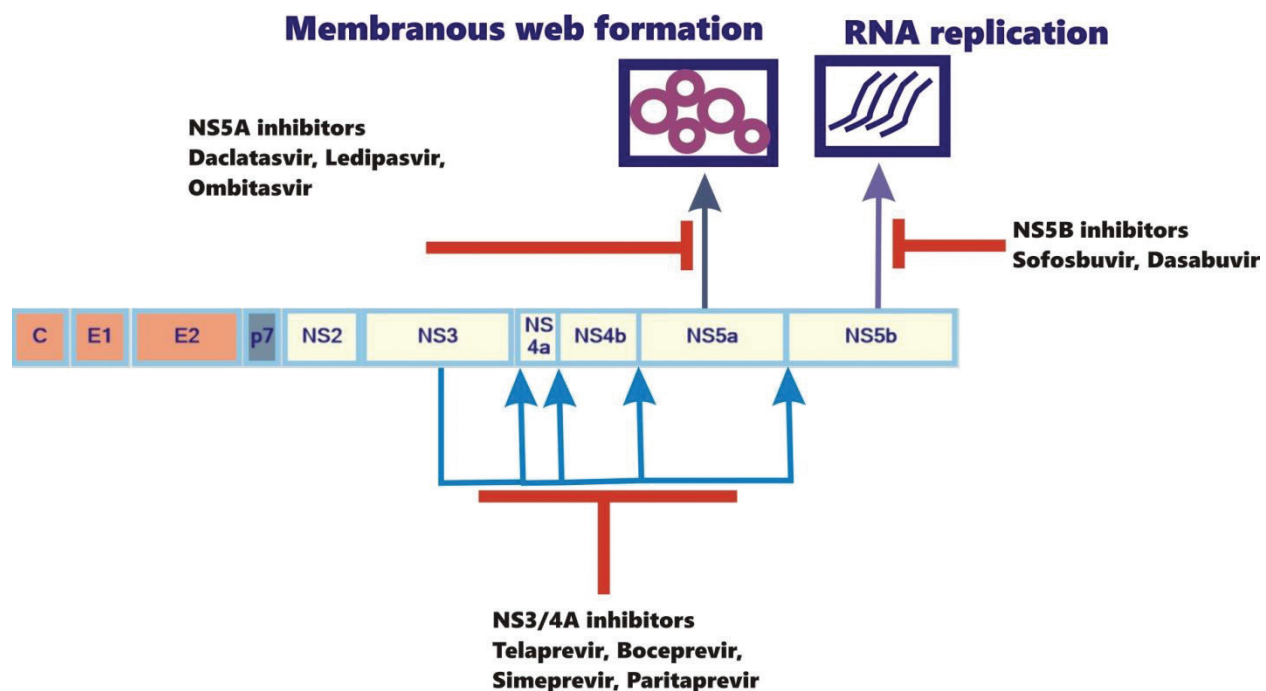
Elucidation of intracellular HCV replication has fostered the efforts toward development of DAA agents, since in the principle, each step represents a potential target for development of new DAA. Viral particles enter the host cell by endocytosis. After the release of HCV RNA from the virion, the former has two alternative pathways: (a) translated as (+) RNA strand at the rough endoplasmic reticulum into the polyprotein precursor that is cleaved by host and viral proteases into mature proteins, where viral proteins, with the help of host cell factors, stimulate the formation of a membranous web (MW). In the alternative pathway, (b) the negative-sense strand (−) RNA serves as a template for the production of extra copies of positive-sense (+) RNA strands. Since the nascent viral RNA could be a subject to excessive nucleases in the cytoplasm, the MW sequesters both viral and host factors are required for viral genomic replication process. Viral assembly occurs in the MW close to the ER and lipid, where core protein and viral RNA accumulate. During the lipoprotein synthesis in ER membrane, the latter buds to form viral envelope, and the newly formed HCV particles are released by exocytosis [5].

### 2.1. Direct-acting antiviral agents

The development of DAAs has been progressed through the accumulative information on HCV life cycle, improved cell culture technology, and establishment of a robust in vitro viral propagation system.

So far, four classes of DAAs targeting three HCV proteins [NS3, NS5A, and NS5B] [nucleotide/nucleoside polymerase inhibitors (NPI) and non-nucleotide polymerase inhibitors (NNPI)] are approved for HCV treatment in several countries around the globe as shown in **Figure 1**. Multiple DAAs target specific HCV-encoded nonstructural proteins leading to arrest of viral replication [6], thus achieving higher rates of SVR even in cirrhotic and difficult-to-treat patients.

Inhibitors of HCV replication target the NS3/NS4A protease, the NS5A, or the viral polymerase (NS5B) [7]. The first generation of DAAs included NS3/NS4A protease inhibitors (PI), telaprevir (TPV), and boceprevir (BOC). These drugs when given in combination with IFN + RBV, more than 30% increase in SVR rates were achieved, as compared with IFN + RBV; however, 20–40% of the patients suffer from breakthroughs or relapses after the end of treatment. New

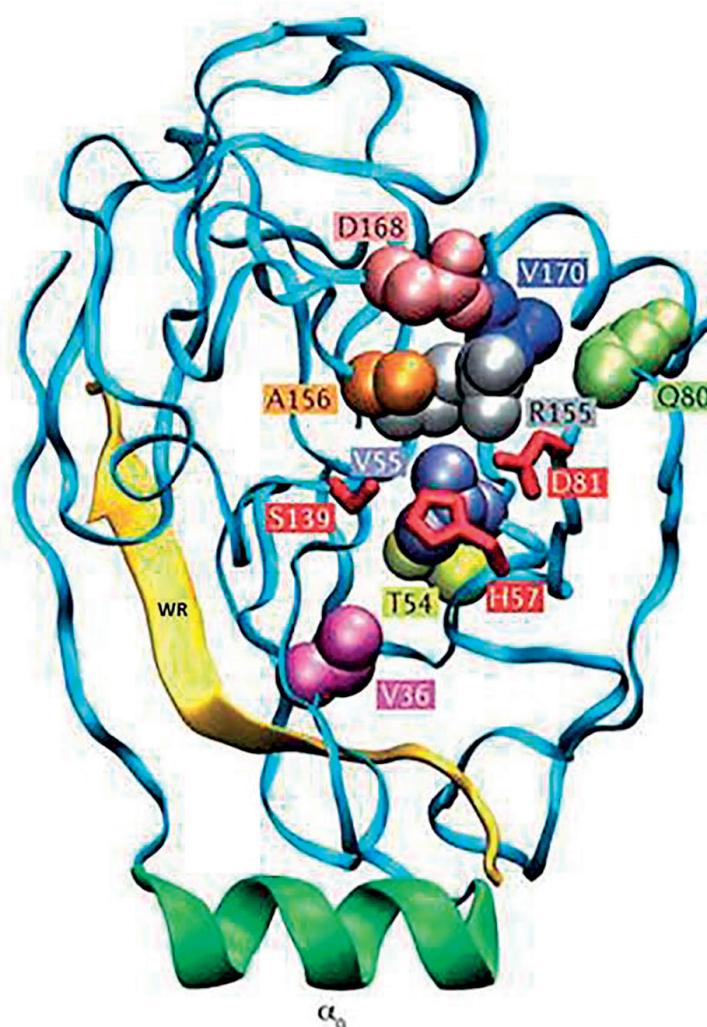


**Figure 1.** HCV genome and potential drug discovery targets shows the currently approved (particularly in Egypt) protease, polymerase, and NS5A inhibitors used as part of a multitargeted approach to HCV treatment.

DAAAs were then approved such as simeprevir, an NS3/NS4A inhibitor; daclatasvir (DCV), an NS5A inhibitor; and sofosbuvir (SOF), an NS5B inhibitor as well as IFN-free combinations such as Harvoni [ledipasvir (LDV) + SOF] and paritaprevir/ritonavir + ombitasvir + dasabuvir (targeting NS3, NS5A, and NS5B, respectively). Rates of SVR were significantly increased (>90%) using these new combinations [8].

### 3. NS3/NS4A protease inhibitors

The viral protease NS3/NS4A is required to cleave the HCV polyprotein into individual viral proteins which are important for viral replication and assembly. It is formed by a heterodimer complex including NS3 and NS4A proteins. NS3 possesses the proteolytic site, while NS4 is a cofactor. This protease cleaves the HCV polyprotein at four sites to produce nonstructural viral proteins NS3, NS4A, NS4B, NS5A, and NS5B. The NS3 protease has a chymotrypsin-like fold consisted of two  $\beta$ -barrel subdomains separated by a groove containing the active site (comprising His57, Asp81, and Ser139) as in **Figure 2**. NS3/NS4A inhibitors block the NS3 catalytic site or inhibit NS3/NS4A interaction, thereby blocking HCV polyprotein cleavage [9]. In addition to this direct action, it is worth noting that NS3/NS4A protease has the ability to block interferon gene expression through the impairment of the retinoic acid-inducible gene I (RIG-I) and Toll-like receptor 3 (TLR3) pathways. Therefore, inhibition of NS3/NS4A protease restores the interferon expression and TLR3 production. Baseline sequencing analysis for NS3/NS4A region revealed the presence of resistance-associated variants (RAVs) in



**Figure 2.** Membrane topology of NS3-4A and positions of mutations that confer resistance to NS3-4A inhibitors. A ribbon diagram of the NS3 protease domain with the central NS4A activation domain (wide ribbon, WR). The  $\alpha_0$  helix serves as an additional membrane anchor of NS3. The catalytic triad (His57, Asp81 and Ser139) is indicated as sticks (H 57, D81 and S139). Mutation of certain residues confers resistance to NS3-4A inhibitors, and the side chains of these residues are represented as van der Waals spheres. (cited with permission from Dr. Ralf Bartenschlager University of Heidelberg Department of Infectious Diseases, Molecular Virology, INF 345, 1st. Floor D-69120 Heidelberg Germany).

patients with HCV genotype 1. Although their presence did not have an impact on treatment success [10–12], the RAVs, however, have been detected during the breakthrough in the majority of non-SVR patients. Furthermore, sequence analysis for NS3/NS4A region in relapsers who received TPV-based regimen identified the following variants as RAV hotspots, as shown in **Figure 2**: V36A/V36M, T54A/T54S, R155K/R155T, A156S/A156T, and D168N [10]. These RAVs are situated close to the catalytic site in the NS3 protease domain, consistent with the mechanism of action of a protease inhibitor. Variants conferring low-level resistance had a 3–25-fold increase in IC<sub>50</sub> from wild type, while those conferring the high level had >25-fold increase in IC<sub>50</sub>. The low genetic barrier for developing resistance in the second-generation protease inhibitors is still the major obstacle facing their activities. The sequence analysis for NS3 protease of genotype 1a in patients who received the simeprevir regimens detected



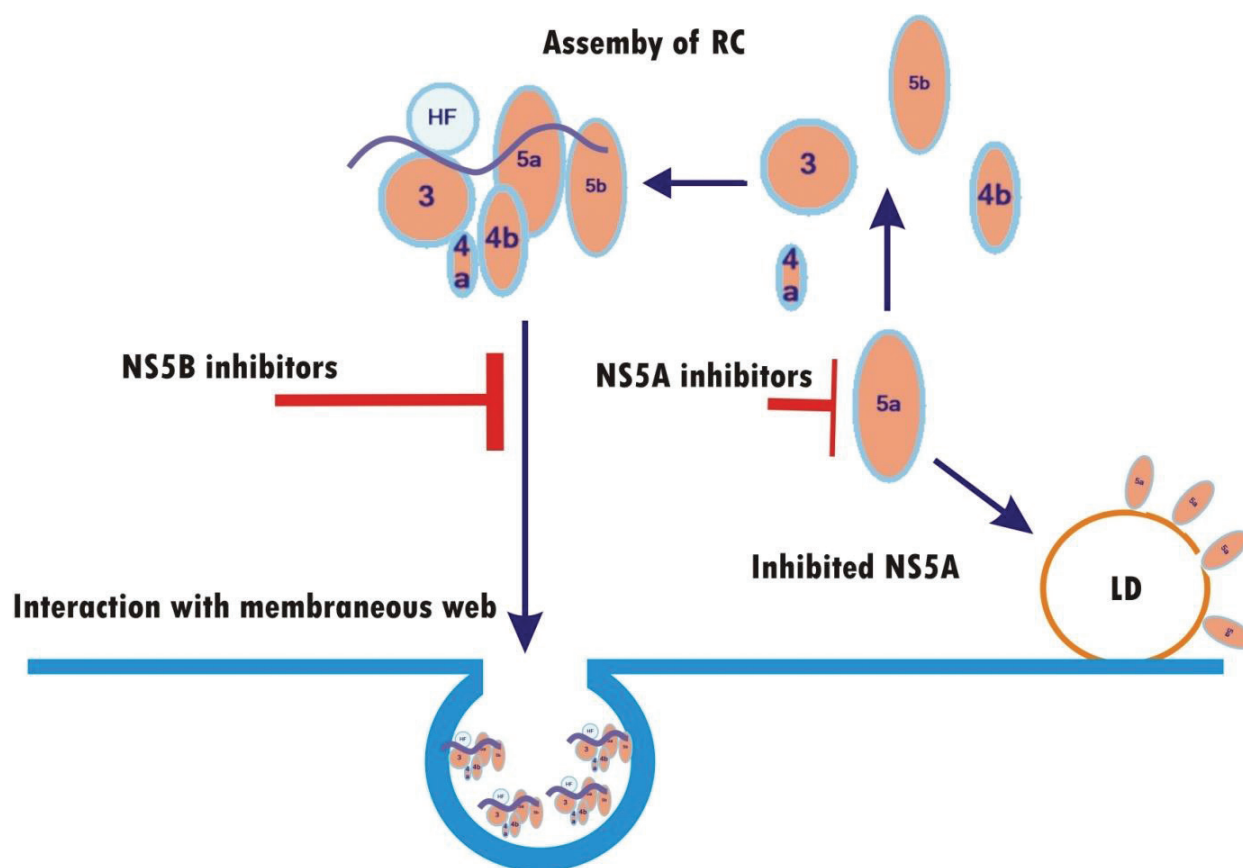
a relevant polymorphism Q80K in 19–48% [13]. In vitro studies showed that this mutation decreases viral response to simeprevir by 10folds [13]. Less profound inhibition has been observed in other NS3 inhibitors including sovalprevir and asunaprevir. The presence of the baseline mutation Q80K in HCV genotype 1a-infected patients diminishes the viral response rate to simeprevir comparable to those without this mutation (58 vs. 84%). Therefore, it was recommended to test the presence of this mutation in HCV genotype 1a-infected patients and probably other genotypes to select the best treatment protocol [14].

#### 4. NS5A inhibitors

Hepatitis C virus (NS5A) is a multifunctional phosphoprotein with an N-terminal amphipathic alpha-helix and is composed of 447 amino acids that are divided into 3 domains. It exists in two phosphorylated forms: basal (p56) and hyperphosphorylated (p58) NS5A. Phosphorylation is believed to regulate several NS5A functions, including RNA binding and self-interaction [15]. NS5A is a promiscuous protein which binds to viral (NS5B and RNA) and host factors including 13 different kinases, for example, PI4KIII $\alpha$  as well as lipid membranes (ER). NS5A is composed of NH<sub>2</sub>-terminus domain I (amino acids 1–213), domain II (amino acids 250–342), and carboxy-terminus domain III (amino acids 356–447). Domains I and II play a major role in viral replication, while domain III is essential for virion assembly. Domain I is the highly conserved region among all HCV genotypes and contains the amphipathic membrane-anchoring helix and a Zn-binding motif that renders NS5A to exhibit high affinity for HCV RNA.

Nonstructural 5A protein has no enzymatic function, and its ultimate function is not fully understood. Daclatasvir, ledipasvir, and ombitasvir are among the currently available NS5A inhibitors. Daclatasvir (DCV) is believed to target the NH<sub>2</sub>-terminal region of NS5A, and it might stop the protein function through interfering with dimer formation, downregulating the NS5A phosphorylation which leads to unusual localization, prohibiting polyprotein processing, and eventually arresting viral replication. The mode of action of DCV contributes to blocking the formation of the MW. The latter is essential for replication, which does not occur at other sites in cytoplasm where exonucleases immediately destroy RNA. A description of NS5A inhibitors' mode of action is simplified in **Figure 3**. Targett-Adams et al. [16] suggested that NS5A inhibitors can change the subcellular localization of the NS5A in the infected cells from the endoplasmic reticulum to lipid droplets. Early proof of this mechanism was provided by Francis Chisari Laboratory [17], where amphipathic alpha-helical peptide mimicking the amino acid composition of the membrane anchor domain at the amino-terminal region of HCV NS5A could change the subcellular localization of NS5A that moved from the ER membrane to the lipid droplets.

The binding between the NS5A protein and its inhibitor changes the NS5A conformation and makes the NS5A unable to incorporate in the replication complex within the ER membrane; consequently, inhibited NS5A is no longer sequestered in the replication complex and is shuttled to the surface of the lipid droplets. It was demonstrated that the inhibited NS5A is localized at the lipid droplet surface where it remains nonfunctional and nonpermissive to



**Figure 3.** Intracellular trafficking of NS5A protein. Once cleaved out from the polyprotein precursor, NS5A mediates the formation of replication complex (RC) that contains host proteins, viral RNA, and nonstructural proteins of HCV. Host and viral components are sequestered in the membranous web to start genomic replication and viral assembly. Inhibitors of NS5A prevent it from migration to the MW, and alternatively NS5A is shuttled to the lipid droplets on the ER membrane. NS5A inhibitors therefore induce subcellular relocalization and cannot prevent replication of already formed RCs. In contrast NS5B inhibitors can arrest viral RNA replication from components within the RCs.

HCV genome synthesis (**Figure 3**) [16, 18]. An additional possible mechanism of inhibition is via inhibition of the hyperphosphorylation of the NS5A protein [19].

It was observed that the presence of single amino acid substitution in HCV genotype 1a is enough to lose response to DCV, while a couple of amino acid substitutions in HCV genotype 1b have led to minimal resistance such as Q54H-Y93H, whereas other double mutations such as L31V-Y93H in genotype 1b are associated with a high level of resistance [20]. The NS5A RAVs included M28, A30, L31, and Y93 in genotype 1a and L31 and Y93 for genotype 1b. The binding of DCV to NS5A dimer is blocked in NS5A RAVs, thus preventing the formation of MW at the ER membrane and the subsequent blocking of HCV replication. These sequels provide an explanation for the low susceptibility of HCV mutants to NS5A inhibitors *in vitro*. In phase III clinical trial, the use of combination therapy of NS5A inhibitor (DCV) with (NS3/NS4A inhibitor) asunaprevir revealed that the presence of double baseline NS5A mutations at amino acids L31 and Y93 was associated with low response to the combination therapy [21].

In HCV genotype 1a-infected patients, the baseline NS5A mutations conferred high-level resistance to NS5A inhibitors when treated for 24 weeks. These mutations included H58D, Y93H/

Y93N/Y93F, or several RAV combinations [22]. A profound impact of RAVs was observed in cirrhotic patients treated for 24 weeks with SOF and LDV. However, SVR rates have not been changed in HCV genotype 1b-infected patients with or without baseline NS5A RAVs. The same study [22] concluded that IL28B-CC genotype is significantly associated with a higher prevalence of Y93H. This kind of association is not fully understood. One of the most relevant baseline mutations that were associated with alteration in clinical outcome is Y93H which was detected in 13 out of 148 genotype 3-infected patients treated with SOF and DCV, where SVR rates were significantly lower than the rest of patients in the same cohort [23].

There is a limited information on the RAVs' influence on efficacy of NS5A inhibitors in HCV genotype 4. In a study on HCV genotype 4d-infected patients treated with DCV [24], mutations associated with resistance after breakthrough at positions L28S, M31I, and Y93H were detected. Furthermore, in genotype 4-infected cirrhotic patients from Egypt, double or multiple baseline mutations were found associated with virological relapse after 24 weeks of treatment with SOF/DCV with or without RBV: L28M-L30S and L30R-M31C-A92T-Y93P [unpublished data]. An in vitro study revealed that replicons containing multiple NS5A mutations (L28S, M31I, and Y93H) conferred high resistance to DCV [24]. Hézode et al. [25] reported treatment failure to DCV in patients infected with genotype 4a mutants harboring double substitutions at positions 28 and 30. In a parallel in vitro study, double substitutions at L28M-R30H and L28M-L30S positions conferred >10,000-fold resistance against the NS5A inhibitors DCV and LDV [25]. The influence of baseline NS5A single or multiple mutations in genotype 4-infected patients was investigated in 186 patients receiving DCV. Interestingly, wild-type genotype 4 infection represented 44.1% of the baseline structure of NS5A, while L30R mutation represents ~43% of the polymorphisms in HCV genotype 4 infections regardless of the clinical outcome [26]. In an unpublished study from our laboratory, L30R mutation was detected in relapsers to SOF/DCV ± RBV. Mutations at L30 (L30R/L30H/L30I/L30S/L30A) were the most commonly detected substitutions among relapsers to DCV-based therapy in genotype 4 infections. In vitro testing of DCV and LDV efficacies on NS5A mutants created by cloning infusion in a 2a/4 hybrid replicon revealed that EC<sub>50</sub> of both DCV and LDV was reduced >1 × 10<sup>5</sup> folds on the double mutants L30S-Y93H, L28M-L30H, L28M-L30S, and L30H-M31V as compared with wild-type hybrid. Although these polymorphisms confer high-level resistance in vitro, their presence in baseline samples prior to treatment was found not common, therefore reducing their impact on treatment response [26].

## 5. HCV population, replication fitness, and resistance to NS5A inhibitors

Hepatitis C virus exists in a mixture of related but genetically distinct viral populations known as viral quasispecies. The relative distribution of viral population depends on the replication capacity of each within a given environment. Emergence of quasispecies occurs as a result of polymorphism which either enhances or decreases the viral fitness of each quasispecies, thus leading to a change in the quasispecies distribution. Upon DAA treatment, viral polymorphisms may confer reduced susceptibility to DAAs. Such polymorphisms may be present in a fit viral population, thus leading to outgrowth of this mutant over the wild



type that clinically leads to drug resistance either during DAA (breakthrough) or posttreatment (relapse). Alternatively, substitution may occur in a less fit population which might not be detectable during or after DAA treatment. Wild-type virus does not contain amino acids conferring resistance to DAAs, while one or more amino acid substitutions are associated with resistance to DAAs. The resistant variants may contain other substitutions that provide outgrowth over other variants during DAA treatment, that is, fitness-associated substitutions that eventually lead to viral breakthrough or relapse, that is, resistant fit variants. Alternatively, resistant variants remain relatively less fit to replicate under DAA treatment with higher possibility to achieve SVR.

Baseline sequencing of quasispecies population that represent low proportions requires deep sequencing which is not available in standard virology laboratories; however, resistant variants existing in low proportion (1–15%) of the total quasispecies population do not appear to significantly influence the virological response [27]. Using a cutoff of 15%, baseline resistance-associated substitutions (RASs) were detected in 13–16% of the naïve patients infected with genotype 1a and 16–20% of the patients infected with 1b [22]. All 1a infections who experienced a relapse had baseline RASs that confer *in vitro* reduced susceptibility to LDV >1000 folds [22].

## 6. NS5B inhibitors

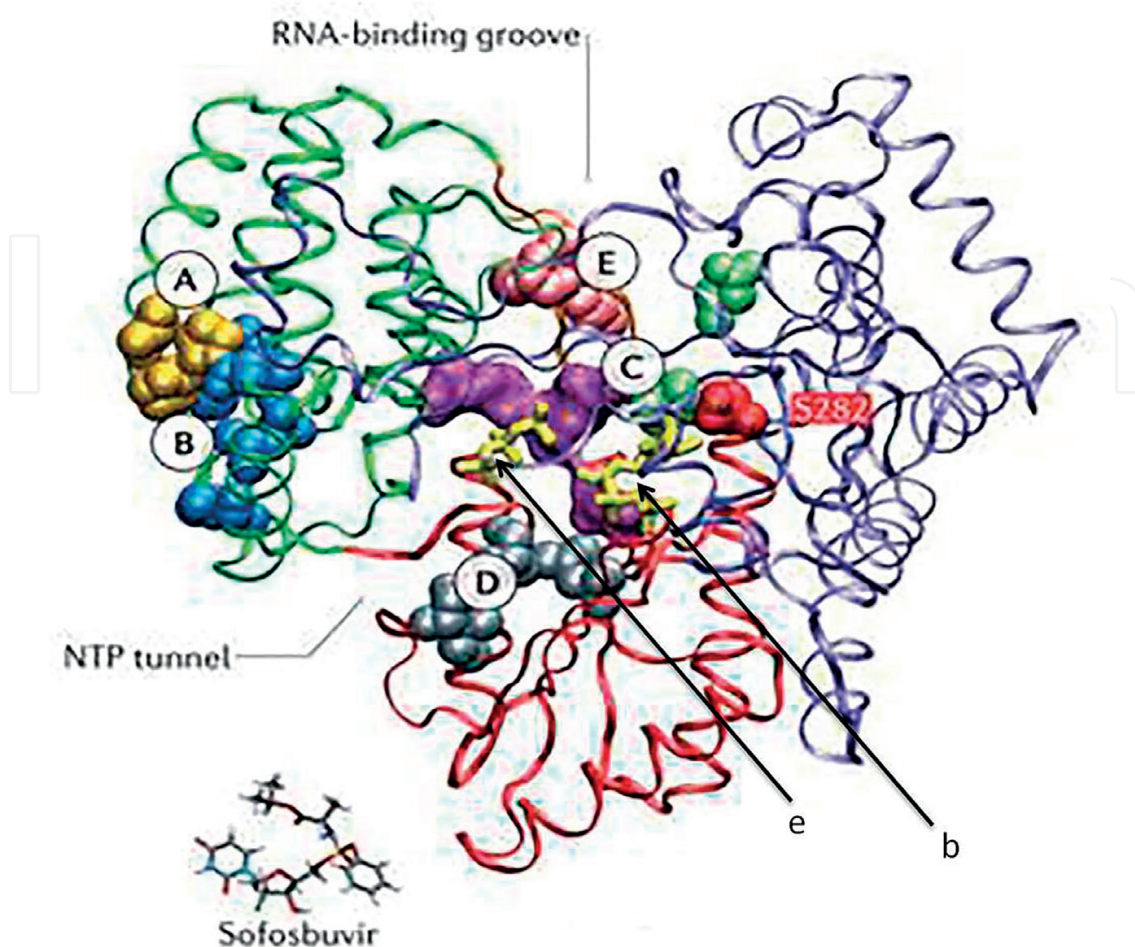
The HCV NS5B protein is the key enzyme (RNA-dependent RNA polymerase) in the HCV replication cycle. It has the ability to initiate the *de novo* synthesis of viral RNA. Structurally, the NS5B protein appears as the right-hand shape and is divided into three domains, the palm domain which has the active catalytic site and surrounded by the thumb and finger domains [28]. The thumb domain contains the allosteric site which regulates the active site (**Figure 4**).

Inhibitors of this enzyme may bind either to the catalytic site, that is, nucleoside polymerase inhibitors (NPI), or to four allosteric sites responsible for the configuration of the protein, that is, non-nucleoside polymerase inhibitors (NNPI). Since the sequence of this protein retains genetic conservation across the viral genotypes, the rates of resistance to these inhibitors seem to be relatively rare [9].

### 6.1. Nucleoside/nucleotide analogue polymerase inhibitors (NPI)

Nucleoside analogue is administered in as a prodrug to facilitate its adsorption where it is activated in the hepatocytes. Several phosphorylation steps are required to convert the nucleoside into nucleoside triphosphate, and this step is mediated by the cellular kinases [29]. The insertion of the nucleoside analogue into RNA chain terminates its elongation. It was observed that the nucleoside inhibitors showed a high genetic barrier to resistance.

Any mutation occurring in the active site of polymerase confers resistance to NPI drugs and makes the mutant virus less fit compared to the wild type which renders the virus unable to replicate. A mutation at S282T has been detected *in vitro* and rarely in treatment failures to the first developed NPI, that is, sofosbuvir (SOF). Sofosbuvir is a uridine analogue and is highly tolerable compared to other polymerase inhibitors.



**Figure 4. Ribbon diagram of full-length NS5B.** Positions of the target regions of five distinct classes of non-nucleoside NS5B inhibitors (A–E) and of some side chains of amino acid residues involved in resistance to these drugs. The position of the major resistance mutation against nucleotide and nucleoside inhibitors (at Ser282) is also indicated. The C-terminal membrane anchor and the linker have been removed for clarity. The active site is indicated by two priming nucleotides (sticks, a,b). For a better comparison with already published structures, the orientation of the finger, palm and thumb subdomains has been rotated by 180° relative to parts. (cited with permission from Dr. Ralf Bartenschlager University of Heidelberg Department of Infectious Diseases, Molecular Virology, INF 345, 1st. Floor D-69120 Heidelberg Germany.)

Three mutations were detected through in vitro exposure to SOF, with little impact in patients, namely, S282T, L159F, and E341D. In replicon assays, the mutation S282T conferred resistance to SOF by genotypes 1a and b [30]. The presence of S282T combined with other mutations such as T179A, M289L, and I293L was found to be crucial for conferring resistance to SOF in genotype 2a.

In SOF monotherapy study, the S282T polymorphism was observed in HCV genotype 2-infected patient who relapsed at week 4 posttreatment [31]. In a phase III SOF clinical trials, the substitutions L159F and V321A have been detected in several HCV genotype 3-infected patients who did not achieve SVR. Treatment failure was experienced by six genotype 1b-infected patients and relapse in a genotype 1a-infected patient. Those patients were found to carry substitutions C316N/C316H/C316F in their baseline samples [29]. In another clinical trial at phases II and III where SOF was administered, the mutation S282T was not detected at baseline. An important

phenomenon associated with the mutation S282T is that it confers low replication fitness and consequently virological failure that leads to uncommon emergence (1%).

## 6.2. Non-nucleoside polymerase inhibitors (NNPI)

The non-nucleosides target the allosteric site of the HCV polymerase. Non-nucleoside polymerase inhibitors bind to the non-catalytic site and change the conformational structure necessary for the HCV replication. The antiviral activities of these agents were determined and ranged from low to moderate, besides they have a low genetic barrier to resistance and inhibit HCV in a genotype-dependent manner. As a result of the low genetic barrier of NNPI to resistance, these agents have been studied in combination with other DAAs to target several regions of HCV genome and prevent the emergence of resistance-associated variants to an individual drug. At present, the approved NNPI is dasabuvir, which binds to palm 1 site of RNA polymerase, and beclabuvir, which binds to thumb 1 site [32].

Substitutions C316Y in genotypes 1a and b and Y448C/Y448H in genotype 1b induced resistance to dasabuvir >900 folds [33]. The 3D regimen consists of dasabuvir in combination with ombitasvir (as an NS5A inhibitor), paritaprevir (as an NS3/NS4A inhibitor), and ritonavir (as a potent inhibitor of CYP3A4). The administration of the 3D regimen with ribavirin in HCV genotype 1-infected patients achieved 95–98% SVR [34, 35]. This provides evidence that a multitargeted approach can augment the rate of response. Substitutions in NS5A, NS3, and NS5B can emerge after exposure to ombitasvir, paritaprevir, and dasabuvir, respectively. In clinical trials, the common substitutions that were detected during treatment or at treatment failure in HCV genotype 1a-infected patients were D168V in NS3, M28A/M28T/M28V and Q30E/Q30K/Q30R in NS5A, and S556G/S556R in NS5B [35–38]. Whereas the observed substitutions in HCV genotype 1b-infected patients who did not achieve SVR were Y56H and D168V in NS3, L31 M and Y93H in NS5A and S556G in NS5B at the time of treatment failure [35].

## 7. Conclusion

The extensive use of the DAAs in the near future will end with the development of viral resistance and appearance of patients who failed to achieve SVR. The majority of available data on HCV infection susceptibility to the approved treatment regimens containing combined DAAs were derived from studies on genotypes 1a and 1b.

The HCV RNA-dependent RNA polymerase (NS5B) is characterized by the absence of proof-reading activity which leads to production of a large number of viral variants. The persistence of these variants is dependent on its fitness (relative capacity of a viral variant to replicate normally). Prior to the DAA administration, most of the resistant variants are unfit to replicate, and the majority of viral variants are fit and sensitive to DAA drugs. After DAA administration, the antiviral activity of the DAAs will inhibit completely the sensitive fit wild-type variants and leads to positive selection for the resistant variants with low susceptibility to DAAs. The resistant variants may acquire substitutions rendering them fit and competently

replicating. The emergence of the fitness-associated substitutions may be either preexisted naturally or acquired by replication in the presence of the drugs allowing the virus to be actively replicating during treatment (breakthrough) or after the end treatment (relapse). Luckily, S282T mutation is very rare, and if present it afflicts the replication capacity of HCV, thus rendering NS5B inhibitors specific for catalytic site binding, for example, SOF, the most effective NS5B inhibitor. Although S282T was the first described SOF-associated RAS [30], a couple of treatment emergent substitutions were identified such as L159F and V321A [39, 40]. Indeed, several factors determine the impact of RASs on SVR including susceptibility/fitness of a given viral population, patients' genetic identity, presence of liver cirrhosis, as well as treatment regimen and duration. In patients infected with genotype 1a, the efficacy of simeprevir + SOF treatment for 8 weeks has been significantly reduced to 73% SVR in the presence of NS3 Q80K substitution compared with 84% in the absence of this substitution [41]. The RAV test is then recommended and is in fact crucial to detect the prevalence of the common NS3 Q80K RAV that affects simeprevir efficacy in the HCV genotype 1a cirrhotic patients. In Japan, the protease inhibitor asunaprevir in combination with NS5A inhibitor DCV is approved for treatment with 84% SVR. The latter is reduced to 41% in NS5A baseline substitutions at L31 and Y93 [42].

Primary substitutions in NS5A sequences of genotype 1a that are associated with resistance to LDV involves residues K24, M28, Q30, L31, P32, H58, and Y93, while genotype 1b includes mainly L31, P58, A92, and Y93 [43, 44]. In genotype 4, mutations at residues L28 and L30 are associated with relapse after DCV-based treatment (unpublished data from our laboratory). Baseline substitutions at these two residues exist in more than 40% of the genotype 4 infections [26], while in genotypes 1a and b, substitutions Y93H and L31 M exist in 15 and 6.3%, respectively [45]. Besides, other substitutions exist in 0.3–3.5% of the population. The emergence of minor less fit variants in some patients rather than reinfection is believed to be associated with treatment failure in high-risk populations [46]. Well-tolerated variants persist >6 months posttreatment such as M28T, Q30R/Q30H, L31V, and Y93R [47]. Since most formulations contain NS5A inhibitors, these mutations represent a future challenge to the next-generation regimens.

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## Author details

Mostafa K. El Awady\* and Reham M. Dawood

\*Address all correspondence to: [mkawady49@yahoo.com](mailto:mkawady49@yahoo.com)

Department of Microbial Biotechnology, National Research Center (NRC), Giza, Egypt



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