Review

Antiviral resistance and direct-acting antiviral agents for HCV

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Direct-acting antiviral (DAA) agents specifically target viral proteins. Two DAAs have been already been approved for the treatment of HCV infection and many more are in development. DAA treatment of HCV infection, however, leads to the selection of viral variants (produced by the error-prone HCV polymerase) that are resistant to the DAA agent in use. The selection of DAA-resistant HCV variants has been studied extensively in vitro and in vivo. Common amino acid substitution sites in each of the non-structural proteins are associated with DAA-resistance: D168, A155, A156 and V36 in NS3 protease; L31 and Y93 in NS5A; S282, S96, P495, M423, M414 and C316 in NS5B. In this review we cover the basic principles of DAA resistance, summarise the available resistance data for the various classes of DAAs and discuss the potential of DAA combination therapy for overcoming DAA-resistance, resulting in major advances in the treatment of HCV.

Introduction

Chronic HCV infection affects more than 180 million people worldwide and is now a significant cause of liver-related morbidity and mortality in the form of liver fibrosis, cirrhosis and, in some individuals, hepatocellular carcinoma. There is no vaccine and therapeutic intervention in the form of pegylated interferon-α and ribavirin combination therapy (R/I) is the common standard of care (SOC) for patients infected with HCV, though recently protease inhibitors have also been added to SOC regimes in some countries. R/I is far from ideal; only 50–60% of patients treated using R/I achieve a sustained virological response (SVR) and treatment is often associated with side effects that can make patient compliance difficult. Given the shortcomings of R/I for HCV treatment, there has been significant investigation into the development of direct-acting antiviral (DAA) agents for HCV, that is, compounds that directly interact with and inhibit the viral proteins. DAAs are being developed to target various stages of the HCV replication cycle, with specific inhibitors being developed to target the HCV non-structural proteins: NS3/4A (a serine protease), the NS5A protein and the RNA-dependent RNA-polymerase (RdRp) NS5B. Unlike current R/I therapy, in which the lack of a robust SVR is a result of complex virus–host interactions and not HCV resistance to treatment, DAAs, by virtue of their mechanism of action, will provide a selective pressure that favours dominance of HCV variants capable of replicating in the presence of the DAA (that is, drug-resistant HCV variants). Thus, implementation of a DAA into a clinical setting will require at least some understanding of HCV variants associated with resistance to the DAA. This review covers basic principles of drug resistance and summarises the available data on drug-resistant variants observed after treatment of HCV (either in vitro or in vivo) with the emerging generation of DAAs.

Factors that drive HCV resistance to DAAs

The HCV genome (Figure 1) is highly variable as a consequence of the poor fidelity of the protein responsible for replicating the genome, the RdRp NS5B. On average, $10^3$ to $10^4$ incorrect nucleoside substitutions occur per genomic replication cycle (Figure 2) [1]. This feature of NS5B RdRp, coupled with the high replication rate of HCV (estimated at around $10^{12}$ virions produced per day in an infected individual) results in the generation of a large number of viral variants [1,2]. While some of these variants have HCV genomes that are replication defective (as a result of mutations that confer loss of function to essential HCV proteins), some variants remain replication competent and constitute...
what is known as the ‘viral quasispecies’. Within an HCV-infected individual, the viral quasispecies consists of a dominant (or ‘wild-type’) HCV sequence that replicates to high efficiency, within a background of HCV variants that circulate at a lower frequency and have reduced replicative capacity compared to the dominant species. The viral quasispecies is constantly evolving and, depending on the environment in which replication occurs, the dominant HCV variant will change. For example, the presence of a DAA will favour the persistence of HCV variants within the quasispecies that are resistant to the DAA. Plasticity in the HCV genome, and the resulting quasispecies, provides a bounty of opportunity for the selection of such a DAA-resistant HCV variant. The selection of a DAA-resistant variant by DAA treatment may then be followed by selection of variants with additional amino acid substitutions that increase HCV resistance to the DAA and/or increase replication capacity of the variant. Though in fact, most DAA-resistant variants demonstrate reduced replicative capacity compared to the wild-type variant.

Resistant HCV variants may develop after DAA treatment or may already be present in the viral quasispecies [3–6]. Differentiating between pre-existing (that is, prior to DAA treatment) or developed DAA-resistant variants is challenging at present as, prior to treatment, DAA-resistant variants may exist at levels too low to detect with standard sequencing techniques. However, techniques such as Ultra-Deep sequencing, which are capable of sequencing even low-level HCV variants within the quasispecies, are shedding light on the presence of pre-existing DAA-resistant variants within HCV-infected individuals [7–9]. The existence of DAA-resistant HCV variants in untreated individuals is, of course, a concern and will need to be taken into consideration when prescribing treatment that will be affected by these substitutions [10].

The effectiveness of a DAA at targeting HCV variants within the quasispecies can be described as a ‘genetic barrier’ [11]. DAA with a high genetic barrier require multiple amino acid substitutions (compared to the dominant/wild-type HCV variant within the quasispecies) within the HCV genome to generate a variant that is resistant to the DAA. In this case, there may be one or two amino acid substitutions that generate resistance to the DAA but these substitutions come at such a cost to viral fitness that a number of subsequent amino acid substitutions are required to develop a viable DAA-resistant variant. For DAA with a low genetic barrier, a single amino acid substitution may confer resistance, and have relatively little effect on replication capacity. DAA that directly target the active site of an HCV protein, for example, the nucleoside inhibitors, generally have a higher genetic barrier to resistance than DAA that act allosterically (inhibiting function by interaction at a site on the protein that is distinct from the active site).

Though DAA half maximal effective concentration (EC50) values have been shown to vary with HCV genotype, the study of DAA resistance of genotypes (gt) other than gt1 or gt2 HCV has been somewhat limited, likely, in part, due to a relative lack of cell culture replication models for HCV genotypes 3 through 6. Some interesting work has been done with the protease inhibitors and gt2a-based chimeras that have the NS3 protease domain (NS3p) and NS4a sequences replaced with that of other genotypes [12,13]. Particularly interesting was the observation that amino acid substitutions that led to resistance in one genotype were wild-type in another (for example, D168E in gt6a

Figure 1. The HCV genome

<table>
<thead>
<tr>
<th>Structural proteins</th>
<th>Non-structural proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>NS4A</td>
</tr>
<tr>
<td>E1</td>
<td>NS3 protease cofactor</td>
</tr>
<tr>
<td>E2</td>
<td></td>
</tr>
<tr>
<td>p7</td>
<td>NS Protease/helicase</td>
</tr>
<tr>
<td>NS2</td>
<td>NS3A Replication/assembly</td>
</tr>
<tr>
<td>NS3</td>
<td>NS4B Replication/ RNA-dependent RNA-polymerase</td>
</tr>
<tr>
<td>NS4</td>
<td></td>
</tr>
<tr>
<td>NS5</td>
<td></td>
</tr>
</tbody>
</table>

The genome of HCV has 5’ and 3’ non-translated regions (NTRs), the 5’NTR contains an internal ribosome entry site (IRES) that directs expression of the genome as a single polyprotein. HCV is composed of ten proteins, classified as structural or non-structural (NS) proteins. The structural proteins comprise core (C) and envelope (E1 and E2) and are followed by the small hydrophobic protein, p7. Core encapsidates the viral genome, E1 and E2 are involved in HCV entry into the cell, the exact functions of p7 remain to be determined though it is thought to have viroporin activity. NS2 forms an autoprotease complex with NS3, though it likely has other functions that are yet to be determined. NS4B is a transmembrane protein thought to be involved in the alteration of host membranes for initiation of the replication complex. As they are not commonly targeted by DAA, these proteins will not be discussed further. NS3 has an N-terminal protease domain (referred to here as NS3p) and a C-terminal ATP-dependent RNA helicase that unwinds RNA duplexes during genome replication. NS4A forms a complex with NS3, this complex is responsible for the cleavage of NS proteins from the polyprotein. NS5A is a membrane-bound phosphoprotein and is involved in virus particle assembly and replication. NS5B is an RNA-dependent RNA-polymerase that replicates the HCV genome.
NS3p decreases sensitivity to BILN2061, but E168 is the wild-type amino acid for the gt2a protease) [12]. A clinical trial using RG7128 for treatment of gt1a, gt1b and gt3a HCV-infected patients resulted in substitutions in NS5B that varied between patients, though assigning these substitutions as genotype specific is difficult given the low patient number [14]. Furthermore, although some of these substitutions (S282R/G) were associated with resistance in vitro, no phenotypic resistance was observed in patients after cessation of treatment. A better characterised example of DAA resistance varying with genotype is the R155K substitution in NS3p that is associated with resistance to protease inhibitors. The R155K substitution requires two nucleotide changes in gt1b but only one in gt1a HCV [15,16]. The effect of this was observed in clinical trials with telaprevir as substitutions at R155 were observed only in gt1a HCV-infected patients, and not in gt1b HCV-infected individuals [15,17]. The same phenomenon was observed in a danoprevir (ITMN-191, RG-7277) monotherapy trial, in which only 2 of 23 gt1b HCV-infected individuals demonstrated viral rebound post-treatment as compared to 8 of 14 gt1a HCV-infected patients [18]. In both cases, rebound was predominantly associated with the R155K substitution. The unlikelihood of R155K substitution in gt1b HCV can also explain the observation that substitution at R155 in gt1a HCV results in resistance to both macrocyclic and ketoamide protease inhibitors (Table 1). However, for the gt1b replicon resistance to macrocyclic inhibitors is predominantly associated with substitution at D168 and resistance to ketoamide inhibitors is predominantly associated with substitution at A156 and V36.

Although extrahepatic reservoirs of HCV is a controversial subject, such reservoirs could influence DAA resistance and the development of resistance mutations.
resistance in HCV-infected individuals. A number of studies suggest that reservoirs of HCV may exist in extrahepatic sites, including blood mononuclear cells (PBMCs), perihepatic lymph nodes and bone marrow [19,20]. Given the different immune-based selective pressures that would exist at these extrahepatic sites, it could be envisioned that the dominant HCV variant within the quasispecies would differ between sites. Indeed, HCV variants isolated from liver, PBMCs or perihepatic lymph nodes have been shown to be divergent [19,21,22]. It is, therefore, possible that extrahepatic HCV reservoirs may harbour DAA-resistant variants either pre- or post-DAA treatment. Thoroughly investigating the nature of HCV variants (including DAA-resistant variants) within a given anatomical location remains a goal of future research.

Other limitations to the identification of DAA-resistant HCV variants include: the degree of correlation between DAA-resistant variants identified in vitro versus in vivo (though, generally in vitro identified resistant variants are an excellent predictor of those observed in vivo) and determining substitutions that occur in HCV proteins other than those being targeted by the DAA. The majority of studies to date, both in vitro and in vivo, sequence only the gene of the protein targeted by the DAA and it remains to be seen if substitutions in other areas of the HCV genome may be associated with DAA resistance.

**DAAs and the resistant HCV variants associated with them**

Two DAAs, the NS3 protease inhibitors boceprevir (SCH-503034) and telaprevir, are currently in the early stages of clinical use post approval programmes and are prescribed in combination with R/I. Encouragingly, there are many other DAAs in early clinical trials and a new era in HCV treatment is certainly on the horizon. In addition to NS3 protease inhibitors, DAAs are being developed that target NS5A (an enigmatic protein that has not demonstrated enzymatic activity but is involved in several steps in the HCV replication cycle) or NS5B. Available data on resistant variants for each of these DAA classes will now be discussed. A summary of the DAA-resistant HCV variants discussed is given in Table 1.

**NS3/4A protease inhibitors**

The HCV genome is expressed as a single polyprotein (Figure 1) that requires both cis and trans-cleavage to generate the functional protein components of the virus. The NS3 protein contains an N-terminal serine protease domain and a C-terminal NTPase/helicase domain. The serine protease activity of NS3 is dependent on the non-covalent linkage of NS3 to its polyprotein neighbour, NS4A. The serine protease activity of the NS3/4A complex is responsible for the cis-cleavage of NS3/4A from the polyprotein. Subsequently, NS3/4A cleaves the NS4B/NS5A and NS5A/NS5B junctions, liberating the HCV non-structural proteins to carry out their functions in the HCV replication cycle (Morikawa et al. [23]; Figure 2). In addition to its function in the HCV replication cycle, NS3/4A also plays a role in HCV evasion of the innate immune response to viral infection. The proteolytic activity of NS3/4A cleaves two host proteins (IPS-1 and TRIF) involved in the recognition pathway of double-stranded RNA leading to an antiviral type-I interferon response [24]. Thus, in addition, to their role in blocking the action of NS3/4A at the level of HCV polyprotein processing, the protease inhibitors may also act to restore innate immune function in HCV-infected hepatocytes. The essential role of HCV NS3/4A in the HCV replication cycle, as well as its enzymatic activity on cellular proteins, make it a prime target for the development of DAAs.

NS3/4A inhibitors can be divided into two chemical classes: the macrocyclic inhibitors and the linear alpha-ketoamide inhibitors. Both classes bind within the active site of the NS3/4A protease (Figure 3) and thus inhibit polyprotein processing and HCV replication. However, the amino acids in NS3/4A with which the compounds interact are slightly different for the two classes and, as a result, amino acid substitutions associated with resistance are somewhat different for each class.

**Macrocyclic NS3/4A inhibitors**

Ciluprevir (BILN-2061) was the first NS3/4A inhibitor developed and showed significant promise in early clinical trials, with twice-daily administration leading to a rapid decrease in HCV RNA in the serum of patients infected with gt1, gt2 and gt3 HCV [25–27]. Treatment of a gt1b (Con1) replicon with ciluprevir selected for HCV variants with substitutions at D168 (D168V/A) [28]. Unfortunately, cardiotoxicity in laboratory animals halted further development of the compound. Still, ciluprevir provided proof-of-concept that targeting the NS3/4A protease could lead to a successful therapeutic strategy.

In silico studies involving drug-docking in the NS3p crystal structure led to the identification of vaniprevir (MK-7009), another macrocyclic NS3/4a protease inhibitor [29]. In clinical trials, 84% of gt1 HCV-infected patients who received 800 mg vaniprevir daily, for 4 weeks followed by 44 weeks of R/I, achieved SVR, compared to 63% of patients who received only R/I [30]. Viral breakthrough was associated with substitutions at amino acids 155 and 168 of NS3p [30].

Danoprevir (ITMN-191/R7227) is a ‘second-generation’ protease inhibitor that has shown a potent
Table 1. Summary of substitutions associated with DAA resistance

<table>
<thead>
<tr>
<th>DAA class</th>
<th>Substitutions</th>
<th>In vitro or in vivo</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NS3 protease inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ciluprevir/BILN2061 (macrocyclic)</td>
<td>D168(V/A)</td>
<td>In vitro; gt1b replicon</td>
<td>[28]</td>
</tr>
<tr>
<td>Vaniprevir/MK7009 (macrocyclic)</td>
<td>D168V, R155K</td>
<td>In vivo; gt1a HCV</td>
<td>[30]</td>
</tr>
<tr>
<td></td>
<td>R155K, D168(T/I/A/V)</td>
<td>In vivo; gt1b HCV</td>
<td>[30]</td>
</tr>
<tr>
<td>Danoprevir/ITMN-191, RG-7227</td>
<td>R155K</td>
<td>In vivo, confirmed in vitro; gt1b HCV/replicon</td>
<td>[106]</td>
</tr>
<tr>
<td>(macrocyclic)</td>
<td>R155K</td>
<td>In vivo, confirmed in vitro; gt1a HCV/replicon</td>
<td>[106]</td>
</tr>
<tr>
<td>BI201335 (macrocyclic)</td>
<td>R155K, D168V</td>
<td>In vivo; gt1a HCV</td>
<td>[33]</td>
</tr>
<tr>
<td></td>
<td>R155K, D168V, R155K</td>
<td>In vivo; gt1b HCV</td>
<td>[33]</td>
</tr>
<tr>
<td></td>
<td>A156V, R155(K/Q), D168(V/A/G)</td>
<td>In vitro; gt1a replicon</td>
<td>[32]</td>
</tr>
<tr>
<td>IDX-320 (macrocyclic)</td>
<td>D168V</td>
<td>In vitro; gt1b replicon</td>
<td>[34]</td>
</tr>
<tr>
<td>IDX-184</td>
<td>D168V</td>
<td>In vitro; gt1b replicon</td>
<td>[76]</td>
</tr>
<tr>
<td>Telaprevir/VX950 (linear, ketoamide)</td>
<td>V36(A/M), T54A, R155(K/T), A156S</td>
<td>In vivo, confirmed in vitro; gt1b replicon</td>
<td>[15]</td>
</tr>
<tr>
<td></td>
<td>A156S</td>
<td>In vitro; gt1b replicon</td>
<td>[28]</td>
</tr>
<tr>
<td></td>
<td>R155K, D168V, R155K</td>
<td>In vivo; confirmed in vitro; gt1b replicon</td>
<td>[15]</td>
</tr>
<tr>
<td></td>
<td>&gt;25-fold increase in EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A156(V/T), R155(K/Q), A156S</td>
<td>In vivo, confirmed in vitro; gt1b replicon</td>
<td>[15]</td>
</tr>
<tr>
<td></td>
<td>&gt;60-fold increase in EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boceprevir (linear, ketoamide)</td>
<td>A156(S/T), T44A, Q41R, F43S</td>
<td>In vitro; gt1b replicon</td>
<td>[38]</td>
</tr>
<tr>
<td></td>
<td>V36(M/A), T44(A/S), R155(K/T), A156S, V55A</td>
<td>In vivo; gt1a HCV</td>
<td>[39]</td>
</tr>
<tr>
<td></td>
<td>T54(A/S), A156S, V170(A/L), V55A</td>
<td>In vivo; gt1a HCV</td>
<td>[39]</td>
</tr>
<tr>
<td>Narlaprevir/SCH900516 (linear, ketoamide)</td>
<td>V36(M), R155(K/T), 156S</td>
<td>In vitro; gt1a HCV</td>
<td>[41]</td>
</tr>
<tr>
<td>GS-9256 (linear, ketoamide)</td>
<td>A156T, D168(A/G/E/N/V)</td>
<td>In vitro; gt1b replicon</td>
<td>[42]</td>
</tr>
<tr>
<td></td>
<td>R155K, D168(E/V)</td>
<td>In vivo; gt1a HCV</td>
<td>[42]</td>
</tr>
<tr>
<td></td>
<td>D168(E/V), A156V</td>
<td>In vivo; gt1b HCV</td>
<td>[42]</td>
</tr>
<tr>
<td>AVL-192</td>
<td>A156T&lt;sup&gt;a&lt;/sup&gt;, D168A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>In vivo; gt1b replicon</td>
<td>[43]</td>
</tr>
<tr>
<td>AVL-181</td>
<td>D168A&lt;sup&gt;a&lt;/sup&gt;, R155K&lt;sup&gt;a&lt;/sup&gt;</td>
<td>In vivo; gt1b replicon</td>
<td>[44]</td>
</tr>
<tr>
<td><strong>NS5A inhibitors</strong></td>
<td>Positions primarily associated with resistance: L31, Y93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daclatasvir/BMS-858 based</td>
<td>L31(M/C/V), Y93C, M28T, Q30(H/R)</td>
<td>In vitro; gt1a replicon</td>
<td>[52]</td>
</tr>
<tr>
<td>(iminothiazolidine)</td>
<td>L31(V), Y93H</td>
<td>In vitro; gt1b replicon</td>
<td>[52]</td>
</tr>
<tr>
<td></td>
<td>F28I(S/V), L31(M/V), C92R, Y93H</td>
<td>In vitro; gt2a replicon</td>
<td>[52]</td>
</tr>
<tr>
<td></td>
<td>M28T, Q30(H/R), L31M, Y93H</td>
<td>In vivo; gt1a HCV</td>
<td>[52,53]</td>
</tr>
<tr>
<td></td>
<td>Y93H</td>
<td>In vivo; gt1b HCV</td>
<td>[52,53]</td>
</tr>
<tr>
<td>Alisoporivir/DEBIO-025 (cyclosporine A)</td>
<td>D320E</td>
<td>In vitro; gt1b replicon</td>
<td>[56]</td>
</tr>
<tr>
<td><strong>NS5B inhibitors: nucleoside/nucleotide</strong></td>
<td>Positions primarily associated with resistance: S282, S96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mercitabine</td>
<td>S282T</td>
<td>In vitro; gt1b replicon</td>
<td>[65]</td>
</tr>
<tr>
<td>RG-7128 (prodrug of PSI-6130)</td>
<td>S282T+Y586C&lt;sup&gt;c&lt;/sup&gt;; A396G&lt;sup&gt;c&lt;/sup&gt;; I239(V/L)&lt;sup&gt;c&lt;/sup&gt;; K72M&lt;sup&gt;c&lt;/sup&gt;</td>
<td>In vitro; gt1b replicon</td>
<td>[64]</td>
</tr>
<tr>
<td>Balapiravir (prodrug of R1479)</td>
<td>S96T, S96T+N142T&lt;sup&gt;c&lt;/sup&gt;</td>
<td>In vitro; gt1b replicon</td>
<td>[67]</td>
</tr>
<tr>
<td>PSI-7851 (mixed isomer of PSI-7976 and PSI-7977, now GS-7977)</td>
<td>S282T</td>
<td>In vitro; gt1b replicon</td>
<td>[69,70]</td>
</tr>
<tr>
<td><strong>NS5B inhibitors: non-nucleoside inhibitors</strong></td>
<td>Tegobuvir/GS-9190</td>
<td>In vitro; gt1b replicon</td>
<td>[97,98]</td>
</tr>
<tr>
<td>Thumb I</td>
<td>Y452H, C455F, R465G, W571R, C316Y</td>
<td>In vitro; gt1b replicon</td>
<td>[97,98]</td>
</tr>
<tr>
<td></td>
<td>Position P495 primarily associated with resistance: S282, S96</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Benzimidazole-based</td>
<td>In vitro; gt1b replicon</td>
<td>[77]</td>
</tr>
</tbody>
</table>

*Variants were not selected for by drug treatment but rather drug potency was assessed on variant replicons. **Substitution alone was not associated with resistance but addition of substitution to S282T resulted in increased fitness. DAA, direct-acting antiviral; gt, genotype.*
antiviral effect in clinical trials [31]. Viral rebound following 14 days of monotherapy was associated with R155K substitution in 10 of 10 post-treatment rebound gt1 HCV-infected patients [18]. One of the 10 patients had an additional D168E substitution. Similarly, BI-201335 resistance is predominantly associated with R155K and/or D168E substitution in gt1a or gt1b in vitro and in vivo [32,33]. Treatment of a gt1b replicon with IDX-320 resulted in a D168V variant with increased resistance to IDX-320, but a replication capacity that was 19% of wild type [34]. Other variants associated with protease inhibitor resistance, such as R155K, R155Q and A156S, remained relatively susceptible (<10-fold increase in EC₅₀) to IDX-320 [35].

Linear NS3/4A inhibitors
Like ciluprevir, telaprevir was the result of structure-based drug design [28,36]. Preliminary in vitro studies using telaprevir treatment of the gt1b (Con1) replicon identified a single variant, A156S, with a telaprevir EC₅₀ of 4.65 μM, compared to 0.4 μM for the wild-type replicon [28]. Thorough sequencing analysis during the trial highlighted the HCV ‘selection’ process that occurs during DAA treatment. Being a highly conserved amino acid across all six major HCV genotypes, it was not unexpected that substitution at A156 resulted in reduced replicative fitness [15,28]. However as treatment progressed, variants with two substitutions emerged: V36(A/M) and R155(K/T); or V36M and A56T; and these variants demonstrated both increased resistance and increased fitness (though not 100% of wild type) compared to the variants with single substitutions [15]. At 3–7 months after treatment conclusion, the highly resistant variants were not detected (having been replaced by a predominantly wild-type HCV population). Importantly, in a follow-up study, it was observed that patients treated with pegylated interferon-α (for 1, 12 or 24 weeks) after 14 days of treatment with telaprevir, had undetectable HCV RNA levels 24 weeks after the conclusion of telaprevir treatment, despite the presence of telaprevir-resistant variants during the initial 14 days of the trial [17,37].

The common NS3p binding site of telaprevir and boceprevir means both drugs select for similar, or identical, NS3p variants – with amino acid substitution occurring in the active site of NS3p (Figure 3). Like telaprevir, boceprevir selects for substitution at A156; A156S and A156T; and these variants demonstrated both increased resistance and increased fitness (though not 100% of wild type) compared to the variants with single substitutions [15]. At 3–7 months after treatment conclusion, the highly resistant variants were not detected (having been replaced by a predominantly wild-type HCV population). Importantly, in a follow-up study, it was observed that patients treated with pegylated interferon-α (for 1, 12 or 24 weeks) after 14 days of treatment with telaprevir, had undetectable HCV RNA levels 24 weeks after the conclusion of telaprevir treatment, despite the presence of telaprevir-resistant variants during the initial 14 days of the trial [17,37].

Table 1. Continued

<table>
<thead>
<tr>
<th>DAA class</th>
<th>Substitutions</th>
<th>In vitro or in vivo</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thumb II</td>
<td>Position M423 primarily associated with resistance</td>
<td>In vitro confirmed in vivo; gt1a or gt1b replicon</td>
<td>[80]</td>
</tr>
<tr>
<td>VCH-759</td>
<td>M423(T/I/V), L419(V/M), I482(L/V/I), V494(A/I)</td>
<td>In vitro; gt1b replicon</td>
<td>[83,84]</td>
</tr>
<tr>
<td>Filibuvir (PF-00868554)</td>
<td>M423(T/I/V), M426T, I428T</td>
<td>In vivo; gt1a/b HCV</td>
<td>[85]</td>
</tr>
<tr>
<td>Palm I</td>
<td>Position M414 primarily associated with resistance</td>
<td>In vitro; gt1b replicon</td>
<td>[88]</td>
</tr>
<tr>
<td>Benzothiadiazine based</td>
<td>M414T, C451R, G558R, H95R</td>
<td>In vitro; gt1b replicon</td>
<td>[89]</td>
</tr>
<tr>
<td>ANAS98 (benzothiadiazine)</td>
<td>M414L, G554D, M414T+G554D</td>
<td>In vitro; gt1b replicon</td>
<td>[92]</td>
</tr>
<tr>
<td>GS–625433 (acylpyrrolidin)</td>
<td>M414T, I447F</td>
<td>In vitro; gt1a, gt1b replicon</td>
<td>[95]</td>
</tr>
<tr>
<td>Palm II</td>
<td>Position C316 primarily associated with resistance</td>
<td>In vitro; gt1b replicon</td>
<td>[94]</td>
</tr>
<tr>
<td>HCV-796</td>
<td>L314F, C316(f/Y/N/S), I363V, S365(A/T/I), M414(T/I/V)</td>
<td>In vitro; gt1b replicon</td>
<td>[96]</td>
</tr>
<tr>
<td>ABT-333</td>
<td>C316Y, M414T, Y448(H/C), S556C</td>
<td>In vitro; gt1a, gt1b replicon</td>
<td>[97]</td>
</tr>
</tbody>
</table>
HCV resistance to DAAs

Studies with a gt1b replicon and a more potent form of boceprevir, SCH567312, identified Q41R and F43S as additional sites associated with resistance to boceprevir, but these variants retained near wild-type sensitivity to telaprevir [38]. Boceprevir EC50 for the Q41R variant was threefold that of wild type, while the telaprevir EC50 for this variant was the same as for wild type. In contrast, the F43S variant generated a fivefold increase in boceprevir EC50 but only a twofold increase in telaprevir EC50. Several HCV variants, V36M/A, T54A/S, R155K/T, A156S, V170A and V55A, were detected in patients treated twice or three times daily with 400 mg boceprevir for a total of 14 days [39]. Resistant variants were observed more frequently in patients with a greater than 2 log10 decrease in viral RNA concentration and the A156S variant was only observed in patients that showed this level of viral RNA decrease. Although in the replicon system the T54A variant showed similar resistance to both telaprevir and boceprevir, the variant was detected more frequently in boceprevir-treated patients, as was also the case for the V170A variant. Importantly, the frequency of most variants (exceptions were V36 and V170) within an infected individual had declined by more than 50% two weeks after the end of treatment [39]. A very recent study observed Q41R and F43S/L variants in patients treated with 400 mg boceprevir three times a day, previously these variants had only been observed in vitro [40].

Narlaprevir (SCH-900518) and GS-9256 are examples of second-generation protease inhibitors that bind covalently and reversibly to the NS3p active site [41]. In clinical trials with narlaprevir, resistant variants were selected for in 5 of 40 patients (4 patients demonstrated viral breakthrough and 1 was a non-responder) [41]. Like first-generation ketoamide-based protease inhibitors, narlaprevir resistance was associated with V36M, R155K and A156(S/T) substitutions. GS-9256 has an EC50 of 15–20 nM against a gt1b replicon and resistance in the replicon is primarily associated with A156T and D168(A/G/E/N/V) substitutions [42]. Similarly, GS-9256 treatment of gt1a or gt1b HCV-infected patients selected for R155K (gt1a only), D168(E/V) and A156V (gt1b only) substitutions [42].

The protease inhibitors AVL-181 and AVL-192 bind irreversibly to the HCV protease and have shown highly potent (~3 nM) EC50 values against both wild-type gt1b replicon and replicons containing relevant protease substitutions [43,44]. AVL-192 retained an EC50 value of less than 10 nM for replicons containing either A156S, R155K or C159S [43]. Potency was, however, reduced ~15-fold for replicons with D168A or A156V substitutions. Similarly, AVL-181 potency was reduced when a gt1b replicon contained D168A or R155K substitutions [44].

**NS5A inhibitors**

The HCV NS5A protein has no known enzymatic activity, yet plays essential roles in both viral genome replication and virus particle assembly, and may be a mediator between the two events [45–48]. Indeed, studies with inhibitors of HCV replication, such as daclatasvir (BMS-790052) and cyclosporine A, have shed light on the function of NS5A and its roles in HCV replication [49,50]. While a number of compounds that target NS5A are listed on the Hepatitis C New Drug Pipeline website [51], clinical and resistance data is not reported for all available compounds as yet.

Daclatasvir was developed from refinement of the iminothiazolidinone BMS-858 (which itself was identified as an anti-HCV compound by screening more than one million compounds) [52,53]. In Phase I clinical trials HCV-infected patients treated with daclatasvir demonstrated a 3.3 log10 reduction in viral load 24 h after treatment [52]. Daclatasvir is a highly potent inhibitor of HCV replication, with picomolar EC50 values for gt1a, gt1b and gt2a replicons [52]. Encouragingly, daclatasvir also demonstrated picomolar EC50 values for chimeric replicons, in which the entire NS5A coding region of a gt1a or gt1b replicon was replaced with the corresponding sequence from gt3a, gt4a or gt5a HCV. Substitutions associated with daclatasvir resistance in gt1, gt1b and gt2a replicons are summarised in Table 1 [52]. Common to all
genotypes was substitution at L31 and Y93, while only gt1a and gt2a daclatasvir-resistant replicons showed substitutions at M28/F28. In fact, for both the gt2a replicon and the virus, the F28S variant was the most resistant to daclatasvir (EC\textsubscript{50} ~379 nM for the replicon and ~137 nM for the virus), yet it retained a replication capacity of at least 50% of wild type [52]. Amino acid substitutions M28T, Q30H/R and L31M were also observed \textit{in vivo} in gt1a HCV-infected patients treated with daclatasvir, and the Y93H variant was observed in gt1b HCV-infected patients.

Cyclosporine A-based NS5A inhibitors

The identification of cyclosporine A (CsA) as an inhibitor of HCV replication led to the identification of cyclophilin A (CyPA) as an essential cellular cofactor required for HCV infection [54,55]. CsA analogues bind to CypA, preventing its interaction with NS5A, and thus inhibiting HCV replication. Although CsA and its derivatives are not DAAs, D316E and Y317N substitutions in NS5A are associated with an NS5A structure that is less reliant on CypA interaction and thus is more resistant to CsA [50]. A D320E substitution has also been associated with resistance to the CsA derivative alisporivir (DEBIO-025) [56].

NS5B inhibitors

During HCV infection, the NS5B RdRp acts in a complex with other viral and cellular factors (including NS5A) to replicate the HCV genome [57,58]. Like other viral polymerases, the crystal structure of NS5B (which to date has only been crystallised in a form lacking the membrane anchoring domain of the C terminus) resembles a right hand, with three domains described as ‘thumb’, ‘palm’ and ‘finger’ domains (Figure 4) [59–61]. The active site of NS5B is located within the palm domain, contains the GDD motif common to other viral polymerases, and is encircled by the finger and thumb domains giving a ‘doughnut-like’ structure. HCV genome replication begins with synthesis of a complementary negative-strand copy of the genome, from which positive-strand genomes are produced for packaging into newly forming virions. RNA replication is associated with large conformational changes in NS5B, including a concerted movement of the finger and thumb domain to translocate the RNA [60,62].

To date, there are at least 26 compounds, in various stages of development, that target the RNA polymerase activity of NS5B. These compounds can be divided into three groups that inhibit RNA synthesis either by: incorporation into the growing RNA chain and subsequent termination of chain elongation – the nucleoside (or nucleotide) inhibitors, by binding directly to NS5B – the non-nucleoside inhibitors or by binding to metal ions at the active site of NS5B – the pyrophosphate analogues. Both clinical and \textit{in vitro} studies have suggested that non-nucleoside inhibitors demonstrate a lower genetic barrier to resistance than do nucleoside inhibitors [63].

Nucleoside and nucleotide inhibitors of NS5B

Nucleoside inhibitors act within the palm domain, at the active site of NS5B. One of the early nucleoside inhibitors in development was mericitabine (RG7128, the prodrug of PSI-6130), which demonstrated efficacy in Phase Ib clinical trials in 2008 and has entered Phase II trials [63,64]. Preliminary \textit{in vitro} studies, using a gt1b replicon treated with PSI-6130, demonstrated that a S282T substitution in the NS5B active site led to a sixfold increase in EC\textsubscript{50} for the drug [65]. However, this mutation came at cost to viral fitness, with the S282T variant having 15% replication capacity compared to the wild-type replicon [63,64]. Promisingly, short-term (less than 4 weeks) PSI-6130 treatment of a gt1b replicon, even at the EC\textsubscript{50} (~0.61 μM) concentration, failed to generate resistant variants [63,64]. Treatment of a gt1b replicon with greater than or equal to five times the EC\textsubscript{50} concentration resulted in complete clearance of the replicon. When gt1b replicon cells were cultured over several months with gradual increases in PSI-6130 concentration, a variety of substitutions in addition to S282T were observed to appear in NS5B, including Y586C,

![Figure 4. RNA-dependent RNA-polymerase NS5B](image-url)
A396G, I239 (V/L) and K72M [64]. Variants with single substitutions at these positions did not demonstrate decreased sensitivity to PSI-6130, but variants with one or more of these substitutions in combination with S282T demonstrated an increased replication capacity compared to S282T alone (though none of the variants had replication capacities similar to the PSI-6130 sensitive wild-type HCV). Most importantly, however, no viral resistance was observed in patients treated for 2 weeks with mercicitabine monotherapy or 4 weeks with mercicitabine in combination with the R/L, in the Phase Ib clinical trial [14]. In combination with R/I, treatment with mercicitabine for 24 weeks was more effective than R/I alone and resulted in very high rates of virological suppression [66].

Treatment of a gt1b (Con1) replicon with the nucleoside R1479 (of which balapiravir is the prodrug) generated S96T and S96T+N142T variants with reduced susceptibility to R1479 [67]. The S96T substitution was responsible for R1479 resistance and this variant had a fourfold reduction in sensitivity to the drug. However, the S96T variant had a replication capacity 4–5% of the wild-type replicon. Addition of the N142T substitution, reduced R1479 sensitivity by fivefold but the N142T substitution alone had no effect on sensitivity. Unfortunately, clinical trials with balapiravir have now been halted due to patient safety concerns [68].

Preliminary studies with the nucleotide inhibitor PSI-7851 demonstrated a 16-fold increase in EC₉₀ concentration for a gt1b replicon with an S282T substitution in NS5B [69]. PSI-7851 is a mixture of two diastereoisomers, PSI-7976 and PSI-7977, with PSI-7977 the Sp isomer, being the more active inhibitor of HCV RNA replication [70,71]. Subsequent clinical studies with PSI-7977 demonstrated that it is a potent, specific HCV nucleotide analogue, which was safe and well tolerated at a dose of 400 mg once a day for 7 days and resulted in a 4.7 log₁₀ IU/ml drop in viral load [72]. A Phase II study comparing a 12-week course of PSI-7977 plus ribavirin to PSI-7977 plus R/I revealed an astonishing 100% SVR at 12 weeks (all PSI-7977 plus ribavirin-treated patients were negative for HCV RNA at week 4) for gt1, 2 or 3 HCV-infected patients ([73] and E Gane, University of Auckland, personal communication). No resistance to PSI-7977 was observed in any of the treated patients, confirming a high barrier to resistance. A subsequent study attempted to treat gt1 null-responders with PSI-7977 (now GS-7977) [74]. After 4 weeks of treatment, HCV RNA was undetectable in patient serum, however all but one of the patients experienced viral relapse after cessation of the therapy.

Resistance studies with INX-189 (the prodrug of 2-C’methyl guanosine monophosphate) and gt1a or gt1b replicons resulted in S282T and I585T substitutions in the gt1b NS5B and an A540T substitution in gt1a NS5B. However, only the S282T substitution was associated with an increase (12.3-fold) in INX-189 EC₅₀ compared to a 6 nM EC₅₀ for wild-type gt1b replicon [75]. Similarly, S282T was the only observed substitution associated with resistance to IDX-184 following treatment of a replicon but replication capacity was reduced to 5% of wild type [76]. The substitution was not observed during treatment of HCV-infected non-human primates [76].

Non-nucleoside inhibitors of NS5B

While nucleoside inhibitors directly inhibit RNA replication, non-nucleoside inhibitors act allosterically by binding to sites other than the active site on NS5B, presumably inducing a conformational change in the protein and preventing its effective function. The allosteric mechanism of non-nucleoside inhibitors helps to explain the lower genetic barrier to these drugs, as amino acid substitutions can occur in regions that may not be crucial to NS5B function. At least four allosteric sites have been identified as being the target for the currently reported non-nucleoside inhibitors. There are two allosteric sites within the thumb domain of NS5B, and two within the palm domain. Accordingly, amino acid substitutions associated with resistance to non-nucleoside inhibitors are located throughout these four sites.

Thumb I in NS5B

Treatment of a gt1b (Con1) replicon with benzimidazole-based inhibitors (of which BILB-1941 and BI-207127 are members) induced substitutions (Table 1) at the thumb I site of NS5B, which is located at the junction of the thumb domain and the N-terminal finger loop of the protein [77]. Substitutions at P495 of NS5B (in a gt1b replicon) increased EC₉₀ values for this class of drugs by up to 78-fold. Substitutions at P496 and a V499A substitution also reduced potency of these compounds in a replicon system, but to a lesser extent than the P495 substitutions. While BILB-1941 proved potent in vitro, Phase I clinical trials were terminated due to gastrointestinal intolerance [78].

Thumb II in NS5B

Several classes of non-nucleoside compounds interact around a pocket, thumb II, at the base of the NS5B thumb domain [79]. Within this pocket is the residue M423, which, with surrounding residues, is involved in interactions with finger residues during RNA replication [60,62]. The thiophene-2-carboxylic acid derivative, VCH-759, interacts at this site, as does VCH-916 and the dihydroxypyrone derivative filibuvir (PF-00868554) [80–85]. In Phase I monotherapy clinical trials with VCH-759, M423T was the predominant

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HCV resistance to DAAs
NS5B substitution, with M423(V/I) and mixed substitutions at positions L419(V/M), I482(L/V/T) and V494(A/I) also being observed [80]. In vitro analysis of substitutions at these positions in a gt1a or gt1b replicon demonstrated up to a 54-fold increase in VCH-759 EC_{50} value for single substitutions, and up to 456-fold increase for a replicon with both M423I and I482L substitutions.

Filibuvir, which targets the same M423-containing pocket of NS5B, is currently in Phase II clinical trials. Filibuvir-resistant gt1b (Con1) replicons had a substitution in at least one of M423, M426 (M426T) and/or I482 (I482T), all of which form part of the thumb II site of NS5B [83,84]. The most predominant substitution (in 22 of 30 resistant replicons) occurred at position M423 with an M423T substitution resulting in a 715 to 2,202-fold increase in filibuvir EC_{50}. Replication fitness for the M423T variant was reduced to 50% of wild type. A follow-up study demonstrated that M423I, M423T and M423V substitutions in a gt1b (Con1) replicon increased filibuvir EC_{50} values by more than 560-fold compared to the wild-type replicon [85]. Though replicative capacity compared to the wild-type replicon was greatly reduced in each case, with M423I having 23% capacity, M23T 24% and M423V 11%. These substitutions at M423 (M423I/T/V) were the predominant (76% of patients treated with ≥300 mg filibuvir monotherapy, twice daily) substitution in gt1a or gt1b HCV-infected patients treated for 3–10 days [85]. However, 28 days after cessation of an 8–10 day treatment with filibuvir, 33% of patients had a complete reversion to the M423 (wild type) HCV sequence. Interestingly, a single patient with a pre-existing substitution in NS5B, R422K, did not show a reduction in viral load during filibuvir treatment. This is a rare example in which a DAA-resistant variant was first observed in vivo. In the context of a gt1b (Con1) replicon, the R422K substitution generated a replicon with 340-fold decrease in susceptibility to filibuvir, but with a severely attenuated replication capacity (2.6% of wild-type replicon).

VX-222 is a potent HCV inhibitor targeting the thumb II site, with an EC_{50} of 5 nM against the gt1b (Con1) replicon [86]. Patients treated with VX-222 demonstrated a 3 log_{10} decrease in serum HCV RNA levels after 3–4 days of treatment [87]. Although resistance data for VX-222 has not been published to date, L419M, I482L and M423T substitutions in NS5B have been associated with increases in VX-222 EC_{50} values using a recombinant polymerase activity assay [86].

Palm I in NS5B
A third group of non-nucleoside inhibitors, the benzothiadiazines and acylpyrrolidines, act at a site located within the palm and inner thumb domains of NS5B, palm I. In vitro studies with preliminary forms of benzothiadiazine-based inhibitors showed a single M414T, C451R, G558R or H95R substitution in NS5B was associated with resistance to this class of compounds [88]. Replicons with H95R or M414T substitutions showed replication capacity comparable to the wild type. Surprisingly, replicons with a C451R or G558R substitution appeared to require the benzothiadiazine compounds to generate an active polymerase conformation – as demonstrated by a dose-dependent increase in replication efficiency for these variants, compared to the wild-type replicon [88].

In vitro studies with the benzothiadiazine, ANA598, have shown the compound to have EC_{50} values of 3 nM for gt1b and 50 nM for gt1a replicons [89]. ANA598 has been used in monotherapy and combination (with R/I) therapy in clinical trials and in both treatment regimes, viral RNA levels were significantly reduced [90,91]. Additionally, for the combination therapy, viral levels were undetectable earlier than for R/I treatment only. Consistent with in vitro studies using early benzothiadiazine compounds, in vitro studies with ANA598 produced M414L, G554D and M414T+G554D variants [89]. The G554D+M414T variant demonstrated the greatest ANA598 resistance, having an ANA598 EC_{50} value of 20 μM. Importantly, however, ANA598-resistant replicon variants remained sensitive to R/I treatment.

The acylpyrrolidine GSK625433 also targets the palm I site in NS5B and has an EC_{50} of 3 nM or 290 nM for gt1b or gt1a replicons, respectively [92]. Like ANA598, resistance to GSK625433 is associated with M414T substitution, but treatment of gt1a/b replicons with GSK625433 generated an additional I447F variant with reduced susceptibility to GSK625433.

Palm II in NS5B
A hydrophobic pocket in the palm of NS5B is the target for several compounds including HCV-796 and ABT-333 [93,94]. HCV-796 demonstrated an EC_{50} of 9 nM when used on a gt1b replicon [93]. Resistance to the compound was associated with substitutions at L314, C316, I363, S365 and M414 (Table 1) [95]. Replication efficiency of all variants was reduced compared to the wild-type gt1b replicon, with substitutions at S365 having the greatest effect, reducing colony formation efficiency by 1,000-fold. In early clinical trials with HCV-796, viral rebound was associated with a C316Y substitution. Unfortunately, HCV-796 Phase II clinical trials using combination therapy with R/I were halted due to hepatotoxicity.

ABT-333 treatment of gt1a or 1b replicons most commonly induced C316Y, M414T, Y448H/C or S556G substitutions in NS5B [94]. The M414T variant...
suggests the binding site of ABT-333 overlaps with the NS5B palm I site. ABT-333 EC_{50} values for the variants were 10- to 1,000-fold increased compared to wild type, though variants also demonstrated reduced replicative capacity. In clinical trials, 2 days of ABT-333 monotherapy followed by 26 days of combination therapy with R/I resulted in 10 of 24 patients having undetectable viral RNA levels at the conclusion of the study [96]. Similar to in vitro studies, ABT-333 treatment in patients resulted in C316Y, M414 (TV), Y448 (H/C), S556G and D559G substitutions in HCV NS5B. The C316Y substitution generated the most resistant variant, but was only observed in 2 of 24 patients. Despite the presence of these HCV variants, viral response to the combination therapy was not affected, suggesting that variants remained susceptible to R/I.

**Multiple binding sites in NS5B**

The substituted imidasopyridine, tegobuvir (GS-9190), appears to target a site on NS5B that overlaps with, but is in fact, distinct from, the HCV-796 binding site [97,98]. Like BILN-2061 and HCV-796, tegobuvir was more potent against gt1 than gt2 replicons, effective concentration for the gt2a replicon was increased 20,000-fold compared to gt1b [98]. Utilizing this property, studies with tegobuvir and gt1b(gt2a) chimeric replicons demonstrated the active site of tegobuvir to be at least partially located within the β hairpin (amino acids 435 to 455) of the thumb domain of NS5B. Treatment of gt1b replicon cells with tegobuvir resulted in Y452H and C455F variants, as well as R465G, W571R and a variant common to palm II site inhibitors, C316Y. Indeed, the C316Y was the most resistant variant, with an 8.8-fold decrease in susceptibility to tegobuvir. Treatment of gt1b replicon cells with a tegobuvir analogue generated a Y448H variant, which proved to also be highly resistant to tegobuvir (36-fold decrease in sensitivity compared to wild-type replicon). Docking of tegobuvir into available NS5B crystal structures, further defined the tegobuvir binding site as being partially in the palm II site but extending across to palm site I. This predicted binding site incorporates the β-hairpin loop and the amino acids observed in resistant variants. In preliminary clinical trials, a single dose of tegobuvir produced median viral declines ranged from 0.46 to 1.49 log_{10} IU/ml [99]. In Phase II trials, a median maximal reduction of -5.7 log_{10} IU/ml was observed in patients treated with tegobuvir in combination with R/I [100]. The Y448H substitution was the most common NS5B substitution observed in patients that had viral rebound following cessation of treatment.

**Pyrophosphate analogues**

The pyrophosphate analogues are the least developed of the NS5B inhibitors. Like nucleoside inhibitors, pyrophosphate analogues (such as the dihydroxyppyrimidine carboxylates) act at the active site of NS5B, and can coordinate the two metal ions within the site, thus inhibiting RNA replication [101,102]. Studies with a prototype pyrimidine demonstrated that the compound was able to both inhibit nucleotide incorporation and pyrophosphate-mediated excision of nucleotide analogues, suggesting a role for pyrophosphate analogues in combination therapy with nucleotide analogues [103]. P156L and G152E substitutions decrease NS5B susceptibility to members of this class of compounds [103,104].

**Interferon-free, combination DAA therapies**

The large number and diversity of DAAs entering the market should allow for interferon-treatment therapies that use multiple DAAs in order to combat the distinct resistant variants that develop for a single DAA. In vitro data generally confirms that replicons with resistance substitutions to one DAA remain fully susceptible to a DAA with a different target site. However, in in vivo studies using interferon-free combination DAA therapies, viral breakthrough has been observed and associated with resistance substitutions to both DAAs.

In addressing the aim of interferon-free treatment, in vitro studies with DAAs now tend to include an assessment of the effectiveness of a DAA at inhibiting replication of a replicon/HCV that has known resistance substitutions in proteins other than that targeted by the DAA of interest. For example, in vitro studies with gt1b replicons containing substitutions associated with resistance to inhibitors targeting NS5A and NS5B were used to demonstrate the potential for the use ofIDX-320 (an NS3p inhibitor) in combination therapies [35]. IDX-320 retained the same potency for wild-type replicons as those containing L31(F/M/V), Y93(C/H) substitutions in NS5A, or S282T, C316Y, M423T, C445F, Y448H substitutions in NS5B.

Similarly, introduction of Y448H, or C316Y +C445F+Y452H into a gt1b replicon results in a replicon with 38- or 343-fold, respectively, reduced susceptibility to the NS5B inhibitor tegobuvir. However, the EC_{50} of GS-9256, an NS3p inhibitor, is the same (~1–2 nM) for both replicons [105]. Conversely, GS-9256 potency is severely (120–3,136-fold higher EC_{50}) reduced against replicons with R155K, A156T, D168E or D168V substitutions but the potency of tegobuvir is retained. Long-term treatment of the gt1b replicon with a combination of the two compounds resulted in significantly greater (~1.5 log_{10}) reduction in replicon RNA compared to treatment with either compound alone and suggested that combination therapy with tegobuvir and GS-9256 may be an effective approach. A Phase II clinical trial (gt1 HCV)
with this combination resulted in a median maximal reduction of \(-4.1 \log_{10}\) IU/ml, compared to a \(-5.1\) and \(-5.7 \log_{10}\) reduction for patients treated with tegobuvir +GS-9256+ ribavirin or tegobuvir +GS-9256+R/I, respectively [100]. However, viral breakthrough was most common in patients treated with tegobuvir and GS-9256. A total of, 14 of the 15 patients that experienced viral rebound had substitutions in both NS3p (most commonly R155K in gt1a HCV and D168E/V in gt1b HCV) and NS5B (most commonly Y448H in both gt1a and gt1b HCV). Thus, disappointingly, addition of R/I to the tegobuvir +GS9190 combination therapy was required for complete viral suppression.

A short (13 days) combination therapy with mericitabine (NS5B inhibitor) and danoprevir (NS3p inhibitor) resulted in a median \(-5.1 \log_{10}\) IU/ml reduction in HCV RNA concentration without viral rebound [106]. A subsequent clinical trial resulted in 41% of patients achieving SVR for 12 weeks (SVR12), 71% of these patients had gt1b HCV and the remaining had gt1a HCV [107]. However, the percentage of patients who experienced viral breakthrough was lower when mericitabine and danoprevir was combined with ribavirin. HCV isolated from the 26 patients who had viral rebound, showed an R155K substitution in 16 patients (14 gt1a HCV, 2 gt1b HCV), R155K+V36(M/A) in 5 (gt1a HCV) patients, D168T (gt1b HCV) in 1 patient, D168E+V36(V/G) in 1 (gt1b) patient. All of which are associated with resistance to danoprevir. HCV from just two patients had substitutions in NS5B: S282T (in combination with R155K, gt1a HCV) and R155(K/Q) in a gt1b HCV patient.

Of 34 patients involved in a Phase I clinical trial with BI-207127 (NS5B inhibitor) +BI-201335 (NS3p inhibitor) + ribavirin, only one patient demonstrated viral breakthrough [108]. Gt1a HCV isolated from this patient had the resistance-associated substitutions R155K in NS3p and P495L in NS5B. Phase II trials are under way [109].

A Phase IIa clinical trial used daclatasvir in combination with an NS5p inhibitor (asunaprevir, BMS-650032), either with or without additional R/I therapy [53,110,111]. In total, four of eleven (two of nine gt1a and two of two gt1b) patients treated with daclatasvir and asunaprevir, and ten of ten (nine gt1a and one gt1b) HCV-infected patients treated with daclatasvir and asunaprevir in combination with the R/I, had SVR at 12 weeks after treatment. Six gt1a HCV-infected patients treated with daclatasvir and asunaprevir demonstrated viral breakthrough during treatment. HCV variants isolated from these patients harboured Q30R, L31M/V and Y93C/N substitutions in NS5A, as well as R155K and D168A/E/T/V/Y in NS3p (selected for by asunaprevir treatment); all of which were previously observed in \textit{in vitro} studies.

Combination therapy with ABT-333 (NS5B inhibitor), and ABT-450 (NS3p inhibitor) resulted in SVR12 for 95% of gt1 HCV infected patients and 47% of previous non-responder patients, after a 12-week combination treatment regime [112]. DAA-resistant HCV variants were sequenced from the non-responder patients who also developed viral breakthrough during the trial. In gt1a HCV-infected patients, viral breakthrough was associated with R155K, D168(A/V/E/Y) substitutions in NS3p and G554S, M414T, C316Y, D559G, G554S and S556G substitutions in NS5B. D168K and C316Y were observed in the 1 gt1b HCV-infected patient who demonstrated viral breakthrough.

These clinical investigations with interferon-free DAA treatment all point toward a requirement for more than two DAAs to be used in combination therapy.

**Conclusion**

From the preceding discussion it can be seen that, like HBV and HIV, selection of resistant variants to the DAAs does appear to follow particular ‘pathways of resistance’ and these are summarized in Table 1. A knowledge of these pathways will aid the physician in choosing the best therapy for patients chronically infected with HCV where the treatment goal is cure.

The many DAAs in development for the treatment of HCV is reminiscent of the development of antiretroviral therapy for HIV. For HIV, the outcome of decades of intense research into antiretrovirals has resulted in effective treatment regimes and, for those patients with access to treatment, life expectancy of HIV-infected individuals is approaching that of uninfected individuals [113,114].

Two common themes emerge from the study of DAA-resistant HCV variants: firstly, DAA-resistant variants generally demonstrate reduced replicative capacity compared to ‘wild-type’ (or DAA-susceptible) variants but, over multiple rounds of replication, can acquire additional substitutions to increase viral fitness. In \textit{vivo}, removal of the DAA generally results in restoration of the susceptible/more fit variants as the dominant species within the quasispecies; and secondly, HCV variants resistant to a DAA targeting one viral protein remain susceptible to DAAs targeting another viral protein, as well as to R/I. However, combination therapy remains susceptible to failure due to selection of HCV with resistance substitutions in both targets.

With regard to the first point, changes in the dominant HCV variant within an infected individual will have consequences for determining effective long-term treatment regimes. If a DAA-resistant variant remains dominant within the quasispecies after cessation of DAA treatment, successive treatments will require use of an alternative DAA. Advances in, and increased access to, sensitive sequencing technologies...
will provide greater insight into the ever-changing HCV variants present during chronic infection. As previously mentioned, application of such technologies will also address questions related to the occurrence of pre-existing DAA-resistant variants within an infected patient. An important point regarding changes in the HCV quasispecies is the lack of a DNA intermediate in the HCV replication cycle. Unlike, for example, the HIV replication cycle which involves integration of a DNA copy of the viral genome into the infected cell genome and thus the potential for genetically storing antiretroviral resistant variants, HCV variants are more transient. Taking these factors into consideration, as well as the potential for multi-DAA therapy, the clinical relevance of HCV DAA-resistant variants may become questionable, as was recently discussed by Halfon and Sarrazin [115].

The second point confirms that, as for HIV, combination therapy using compounds that target multiple (more than two) HCV proteins/aspects of the HCV replication cycle will be the key for successful HCV treatment. Given the large number of DAAAs in development, such effective treatment strategies are indeed on the horizon and should herald a new era in the treatment of HCV.

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