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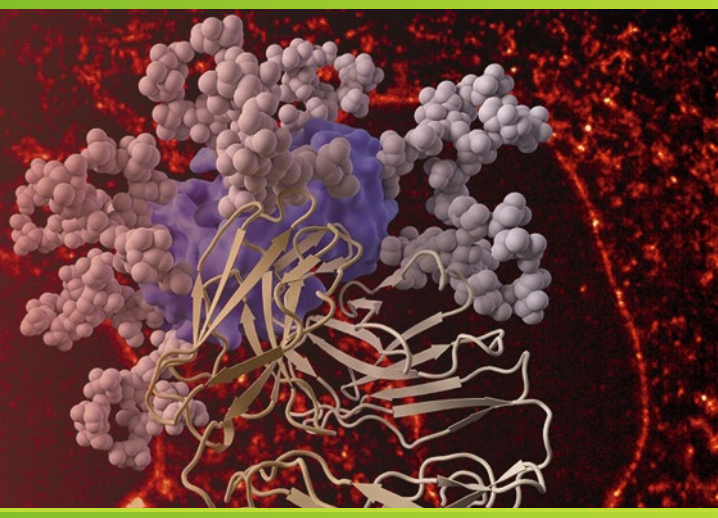

Mansun Law *Editor*

Hepatitis C Virus Protocols

Humana Press

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Hepatitis C Virus Protocols

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Cover illustration: The protein complex structure illustrates binding of broadly neutralizing antibody AR3C (ribbon) to the neutralizing face of HCV E2 core domain (blue) surrounded by N-glycans (grey). Background is a super-resolution microscopy image of HCV-infected cells labelled by an anti-E2 antibody (red). Image designed by Joe Grove, Christina Corbaci, Leopold Kong, and Mansun Law.

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Foreword

The history of hepatitis virus discovery has depended greatly on the application of emerging research technologies, which has culminated in the discovery of five major hepatitis viruses within the last 50 years. Such breakthrough methods included electron microscopy in the discovery of HAV, ouchterlony rocket immunodiffusion in the identification of HBV, cDNA expression immunoscreening in the cases of HCV and HEV, and tissue immunofluorescence staining in the discovery of HDV. Not only have emerging methods been instrumental in hepatitis virus discovery but of course they, along with the huge progress in genomics, proteomics, and structural biology, have been instrumental in gaining knowledge of how these agents work and interact in the body. As such, it is vital that new technologies and methods are continually updated and appraised constantly by the research community since such activity is critical to the innovative scientific process. This excellent volume describes in detail a comprehensive and detailed selection of methods now available in HCV research. Not only will the application of these methods teach us more about HCV pathogenesis and help us to develop preventative strategies but they will also be of great relevance to other viruses, including, crucially, ones that are sure to emerge in the future with drastic consequences for mankind. While we are now better placed than ever to combat emerging viral disease, the regularity of their occurrence and the huge challenges that still exist in developing preventative strategies make this volume of HCV methodologies most timely and highly relevant.

Edmonton, AB, Canada *Michael Houghton*

Preface

This third edition of *Hepatitis C Virus Protocols* targets readers interested in basic virology, host-microbe interactions, and antiviral drug and vaccine research. Hepatitis C virus (HCV) is a blood-borne virus responsible for approximately half a million deaths from liver cancer and end-stage liver diseases each year. Until 2011, HCV was the sole member of the *Hepacivirus* genus in the *Flaviviridae* virus family. New hepaciviruses and the genetically related pegiviruses have now been found in nonhuman primates and many other species, revealing HCV-like viruses are much more commonly circulated than previously thought. Although the impact of these findings on human and veterinary health is yet to be determined, the research strategies developed to study HCV are generally applicable to the study of these new viruses.

This volume collects the most updated concepts and experimental protocols developed by leading researchers in the field. The book chapters are organized into five topics:

1. Review of hepatitis C virus and bioinformatic tools
2. Methods for HCV cloning, culture, and purification
3. Methods for the study of HCV life cycle
4. Methods for the study of host immune responses
5. Small animal models

The book focuses on providing an easy guide to readers explaining the essential methods for the study of this interesting virus, and the experimental systems relevant for vaccine development. A broadly effective HCV vaccine is an unmet public health need for the eradication of this human disease. The scientific challenges in designing a vaccine against antigenically variable virus are shared by other viruses including HIV and influenza. Since the publication of the last edition, we have witnessed important conceptual and technological breakthroughs in the study of antibody and T cell responses to HCV to facilitate vaccine development. HCV will continue to serve as an important model for the study of basic virology, virus pathogenesis, human immunology, and vaccinology.

La Jolla, CA, USA ***Mansun Law***

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Part I

Review of Hepatitis C Virus and Bioinformatic Tools

Chapter 1

Overview of Direct-Acting Antiviral Drugs and Drug Resistance of Hepatitis C Virus

Darrick K. Li and Raymond T. Chung

Abstract

The advent of direct-acting antivirals (DAAs) has brought about a sudden renaissance in the treatment of chronic hepatitis C virus (HCV) infection with SVR rates now routinely >90%. However, due to the error-prone nature of the HCV RNA polymerase, resistance-associated substitutions (RASs) to DAAs may be present at baseline and can result in a significant effect on treatment outcomes and hamper the achievement of sustained virologic response. By further understanding the patterns and nature of these RASs, it is anticipated that the incidence of treatment failure will continue to decrease in frequency with the development of drug regimens with increasing potency, barrier to resistance, and genotypic efficacy. This review summarizes our current knowledge of RASs associated with HCV infection as well as the clinical effect of RASs on treatment with currently available DAA regimens.

Key words Direct-acting antiviral, Hepatitis C virus, Sustained virologic response, Resistance-associated substitution

1 HCV Virology

HCV is a member of the *Flavivirus* family (which also includes the yellow fever and dengue viruses) and is an enveloped (+)-strand RNA virus. The genome is approximately 9.6 kb in length and encodes a single large polyprotein which is ultimately cleaved to form ten proteins by cellular and viral proteases. These include three structural proteins, the nucleocapsid protein (C), and two envelope proteins (E1 and E2), as well as seven nonstructural proteins which include two proteins required for virion production (p7 and NS2) as well as five proteins that form the cytoplasmic viral replication complex (NS3, NS4A, NS4B, NS5A, and NS5B) (Fig. 1).

The following model synthesizes much of what is known to date [1]. HCV virions enter the hepatocyte via interaction with a number of co-receptors including CD81, claudin-1, occludin, and SR-B1 and are endocytosed into the cell. Following entry, the

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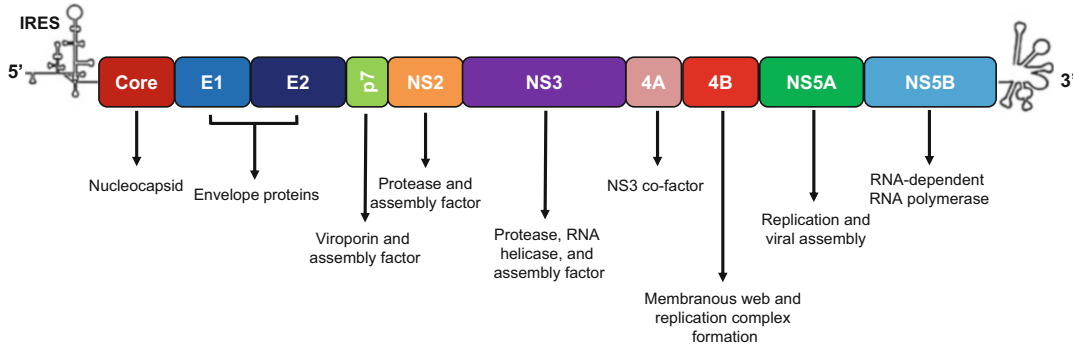

Fig. 1 Genomic organization of the hepatitis C virus. *IRES*, internal ribosome entry site, *NS* nonstructural protein

endosome becomes acidified which changes the conformation of the envelope proteins, releasing the viral (+)-strand RNA genome into the cytoplasm, which become associated with the ER. The RNA then becomes the template for the production of viral proteins. The envelope proteins are secreted into the lumen of the ER, while the core protein remains cytoplasmic. The replication complex of NS3, NS4A, NS4B, NS5A, and NS5B then forms "membranous webs" derived from the ER membrane and directs transcription of a (−)-strand genome which then becomes the template for further production of (+)-strand genomes, which are then packaged with the structural proteins to form mature virions, which are then released.

Given its central role in the viral life cycle, a number of the protein components of the viral replication complex have been a target for many of the effective antivirals that have recently been developed, in particular NS3, NS5A, and NS5B, all of which will be further described later. In brief, the NS3 protein functions as the key viral protease and is responsible for a number of the polypeptide processing events including the cleavage of the NS3/NS4A, NS4A/NS4B, NS4B/NS5A, and NS5A/NS5B junctions. This activity requires NS4A as a cofactor. NS5A is a membrane-bound RNA-binding protein whose precise role remains unclear but appears to play multiple essential roles in the regulation of viral replication, assembly, and exit. NS5B is the viral RNA-dependent RNA polymerase and the catalytic core of the viral replication machinery.

The NS5B polymerase lacks proofreading capability [2], and as such, this has led to the rapid accumulation of genetic diversity and the rise of at least six separate HCV genotypes (GTs). These GTs have important consequences for treatment as there are emerging differences in the response rate of various GTs to various antiviral regimens. The GTs also have geographic variability—genotype 1 (GT1) is the most widespread and is predominantly seen in

North America and is further split into two subtypes, GT1a and GT1b. GT2 is also widespread and is found principally in Central and West Africa. GT3 is found primarily in Asia; GT4 is found in the Middle East and Northern Africa; GT5 and GT6 are rare and can be found in regions of Africa and Asia [3].

2 A Brief History of HCV Treatment

The treatment of HCV has undergone a revolution. Indeed, until recent times, interferon (IFN)-based therapy had been the backbone of HCV therapy (Fig. 2). In fact, the first evidence of therapeutic efficacy for IFN-based therapy for HCV was performed even prior to its identification in a pilot study of ten patients for what was termed "non-A, non-B hepatitis," an entity originally described in 1976 [4–6]. Concurrent with the identification of HCV in 1989 [7], the first two randomized controlled trials for the use of IFN in HCV treatment were performed [8, 9]. In these trials, recombinant IFNα was given three times a week for 24 weeks, and treatment response was measured by a sustained normalization in alanine aminotransferase (ALT) levels in the serum. Only 10–25% of patients achieved a treatment response in these trials. IFN monotherapy was the standard of care for the next decade until the late 1990s, during which combination therapy of IFN-α and ribavirin led to the next step in the treatment of HCV. In a landmark study, 912 patients were randomized to subcutaneously injected IFNα2b with daily oral administration of ribavirin achieved on SVR (as measured by undetectable HCV RNA viral loads) in 38% of treated patients undergoing 48 weeks of therapy compared with 13% with IFNα2b monotherapy [10]. The introduction of

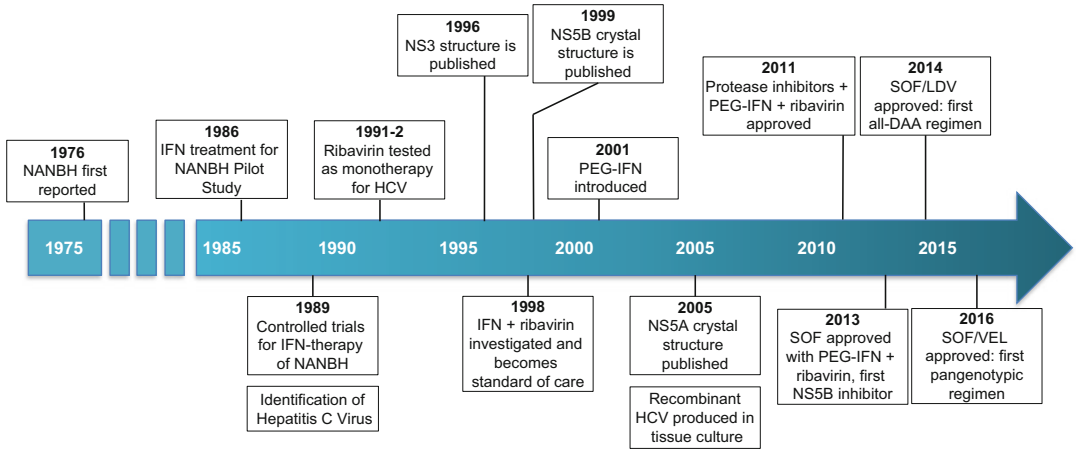

Fig. 2 Timeline of milestones in hepatitis C virus treatment. *NANBH* non-A, non-B hepatitis, *NS* nonstructural protein, *PEG-IFN* pegylated interferon; *SOF* sofosbuvir, *LDV* ledipasvir, *VEL* velpatasvir

pegylated IFN (PEG-IFN) in 2001, which had a longer half-life and more favorable pharmacokinetics in combination with ribavirin, led to additional incremental improvements in SVR rates to approximately 55% [11, 12]. For the next decade, treatment of HCV with PEG-IFN and ribavirin was the standard of care, though the treatment regimen continued to be plagued by variation in SVR rates with GT and viral load and significant side effects including asthenia, neutropenia, flu-like illness, cytopenias, and depression [13].

Significant advances in our understanding of the molecular and structural virology, life cycle, and pathogenesis of HCV as well as the ability to produce recombinant infectious HCV by tissue culture led directly to the development of the first DAAs [14]. These agents were the first-generation NS3/4A protease inhibitors, telaprevir (TVR) and boceprevir (BOC), which achieved SVR rates of 65–75% when used together with PEG-IFN and ribavirin [15, 16]. As such, they were approved by the FDA for use in "triple therapy" for HCV GT1 in 2011. Their approval was followed by a flurry of activity, notable for the development of several compounds targeting other stages of the HCV life cycle. Simeprevir (SMV), a once-daily NS3/4A protease inhibitor, was approved in 2013 to be used in combination with PEG-IFN and ribavirin for treatment of GT1, achieving comparable SVR rates as its predecessors, with better tolerability [17]. A major advance was the development of an NS5B polymerase inhibitor, sofosbuvir (SOF). SOF is a member of a family of nucleotide analogues that work by causing early chain termination after being incorporated into newly synthesized viral RNA [18]. Given its mechanism of action and the conservation of the NS5B RNA polymerase active site, it is active against all HCV GTs and has a high barrier to resistance, selecting only for viral mutants with exceedingly low replication fitness. As such, in a landmark trial enrolling individuals with predominantly HCV GT1 or GT4, SOF-anchored triple therapy was found to achieve SVR rates of 90% after 12 weeks of therapy (SVR12) [19]. Moreover, SVR12 rates of 95% and 82% were attained with SOF and ribavirin alone in treatment-naïve and treatment-experienced persons, respectively, with HCV GT2 or GT3 [19, 20]. Accordingly, in 2013, the FDA approved SOF for use as part of triple therapy with PEG-IFN for HCV GTs 1 and 4 and with ribavirin alone for GTs 2 and 3.

Of particular interest has been the development of all-oral IFN-free regimens utilizing two or more classes of DAAs to achieve the dual goal of rapid viral suppression and prevention of selection of resistant variants. This concept has been realized with the approval of SOF and ledipasvir (LDV, an NS5A inhibitor) by the FDA in October 2014 as a once-daily co-formulation for the treatment of HCV GT1. This was done on the basis of three pivotal trials that studied this combination with and without ribavirin in

both treatment-naïve and treatment-experienced patients. These clinical studies found that irrespective of ribavirin use, individuals treated with this combination achieved SVR12 rates of 94–99% [21–23]. In addition, SOF/SMV for the treatment of HCV GT1 was approved by the FDA in November 2014, based on results from the COSMOS trial, which demonstrated >90% SVR12 rates and good safety and tolerability profiles [24]. Finally, the combination regimen of ombitasvir, ritonavir-boosted paritaprevir, and dasabuvir ± ribavirin was approved in December 2014 on the basis of several trials showing SVR12 rates >90% [25, 26]. Most recently, there has been a surge of approvals for a new generation of DAA regimens with increased antiviral potency and pan-genotypic efficacy including the approval of SOF and velpatasvir (VEL, a NS5A inhibitor). A list of currently approved IFN-sparing DAA regimens and additional DAAs that are currently in development can be found in Fig. 3

Published data regarding real-world experience with the new DAA regimens are rapidly accumulating, and preliminary findings have been encouraging. For instance, individuals with HCV GT1 treated with SOF/SMV ± ribavirin for 12 to 16 weeks also

Class	Name	Manufacturer	Status
NS3-4A inhibitors ("**-previr**")	Telaprevir	Jannsen, Mitsubishi	Approved (2011, now discontinued)
	Boceprevir	Merck	Approved (2011, now discontinued)
	Simeprevir	Janssen	Approved (2013)
	Vaniprevir	Merck	Approved (2014, only in Japan)
	Paritaprevir	AbbVie	Approved (2015)
	Asunaprevir	Bristol-Myers Squibb	Approved (2015, only in Asia, Middle East)
	Grazoprevir	Merck	Approved (2016)
	Glecaprevir	AbbVie	Approved (2017)
	Voxilaprevir	Gilead Sciences	Approved (2017)
NS5A inhibitors ("**-asvir**")	Ledipasvir	Gilead Sciences	Approved (2014)
	Ombitasvir	AbbVie	Approved (2014)
	Daclatasvir	Bristol-Myers Squibb	Approved (2015)
	Elbasvir	Merck	Approved (2016)
	Velpatasvir	Gilead Sciences	Approved (2016)
	Pibrentasvir	AbbVie	Approved (2017)
	Odalasvir (ACH-3102)	Janssen	Phase II
	Ravidasvir (PPI-668)	Presidio	Phase II
	MK-8408	Merck	Phase II
NS5B inhibitors ("**-buvir**")			
Nucleos(t)ide inhibitors	Sofosbuvir	Gilead Sciences	Approved (2013)
	MK-3682	Merck	Phase II
	VX-135	Vertex	Phase II
	ACH-3422	Achillion/Janssen	Phase I
	ALS-335	Janssen	Phase I
Non-nucleos(t)ide inhibitors	Dasabuvir	Abbvie	Approved (2014)
	Beclabuvir (BMS-791325)	Bristol-Myers Squibb	Phase III
	ABT-072	Abbvie	Phase II
	GS-9669	Gilead Sciences	Phase II
	TMC647055	Tibotec	Phase II
	MBX-700	Microbiotix/Merck	Phase I

Fig. 3 Approved direct-acting antivirals and current pipeline agents undergoing evaluation for chronic HCV infection. Unless otherwise indicated, approved drugs have been approved in the United States and the European Union

experienced high rates of SVR that were slightly decreased (84%, 675/802 patients) from what was seen in clinical trials, but with low rates of treatment discontinuation and serious adverse effects [27]. Recently, 2099 real-world patients with GT1 infection who received treatment with SOF/LDV ± ribavirin for 8 to 24 weeks were prospectively followed and were found to have extremely high SVR rates (≥95%) regardless of treatment duration [28]. Robust SVR rates have also been seen in real-world patients treated with various SOF-based regimens with GT2 [29] or GT3 infection [30] as well as those with advanced liver disease [31].

3 Principles of HCV Resistance to DAAs

As detailed above, the evolution of IFN-sparing, DAA-based treatment regimens for HCV has progressed at an incredible pace with encouraging real-world results. However, though most treated patients are able to achieve SVR, the phenomenon of DAA resistance has become increasingly appreciated. This has led to considerable interest in identifying common resistance-associated mutations, in understanding the biochemical mechanisms underlying viral resistance, and in developing new treatment strategies to treat individuals who fail initial therapy. The next sections of this chapter will highlight and discuss this rapidly evolving field in the study of HCV.

A key concept that underlies our emerging understanding of DAA resistance is the concept of a "quasispecies." The low fidelity of the HCV RNA polymerase combined with the high replication rate results in an extremely high number of different but closely related circulating HCV variants that can be observed in the plasma or liver at any time [32, 33]. The in vivo mixture of different but closely related variants is termed a "quasispecies." Each viral population that emerges from the process of random mutagenesis is then subject to selection based on the effect of the mutation(s) on overall viral fitness. Quasispecies theory stipulates that at any given timepoint, the exact distribution of viral populations within the quasispecies reflects an equilibrium between the replicative fitness of each variant, the continued generation of new variants, and the positive selective pressure applied by the environment [34]. Moreover, the quasispecies structure allows for a considerable evolutionary advantage by allowing the virus to rapidly adapt to a constantly changing milieu of stressors.

A virus's ability to evolve is thought to be determined by at least five interconnected parameters [35]. First, the development and emergence of RASs depend on the average mutation rate during viral genome replication. The HCV NS5B RNA polymerase is remarkably error-prone and does not have proofreading capacity, leading to an estimated 10^{-4} substitutions per site and round of

replication [36]. In contrast, high replicative fidelity (e.g., with DNA polymerases with an estimated 10^{-8} to 10^{-11} substitutions per site) would result in a far more homogenous and genetically static viral population and would not allow the virus to quickly explore the sequence space. The development of resistant variants is also determined by the replication rate of the virus which, in the case of HCV, is estimated at 10^{12} virions per day [37]. The extremely high rate of replication combined with the high mutation rate allows the HCV to explore the sequence space available to it at a faster rate. A third factor that contributes to the development of resistant variants is the genetic barrier to drug resistance, which involves the number and type of mutations that are needed for the emergence of a RAS. Fourth, the fitness of the resistant variant populations is critical as it determines the likelihood that any resistant variant persist within the larger viral population. Finally, the emergence of viral resistance is determined by the level of drug exposure. Indeed, exposure to suboptimal concentrations of antiviral agents will result in the selection of RASs by allowing for the maintenance of a viral load in the presence of a mild selective pressure.

As has been discussed recently, the language to describe these mutations and viral variants should be standardized [38]. Most recently, it has been proposed that the amino acid substitutions that confer resistance will be called *resistance-associated substitutions* (*RASs*) and the viral populations that carry these RASs will be called *resistant variants* (*RAVs*). These resistant variants can also acquire additional mutations, termed compensatory or fitness-associated substitutions, which may increase their fitness. This can lead to their rapid outgrowth during the course of treatment, which is termed a *breakthrough*, or after treatment, which is termed a *relapse*.

3.1 Identification of Resistance-Associated Variants

With our developing understanding of DAA resistance, there has been increasing interest in identifying pre-existing RASs that exist within HCV quasispecies. To do so, several methods have been used to perform sequencing of varying depth to identify populations of viral variants within the larger quasispecies cloud [39, 40]. In most studies, the identification of pre-existing RASs is performed using population sequencing via the traditional Sanger method. While an excellent strategy to identify major sequences present within the quasispecies, its primary weakness is its lack of sensitivity, as it is generally unable to detect viral populations that are present at proportions lower than 10–25% of the total population [41]. However, in recent years, there has been incredible advancement in the development of high-throughput, next-generation sequencing technologies (e.g., Illumina, 454, Ion Torrent, PacBio, etc.), which has rapidly improved our ability to detect viral subpopulations that are present in ever smaller proportions within the quasispecies, even those comprising just ~0.1–1% [42, 43]. The highest sensitivities for the detection of minor viral population in

NGS studies typically occur when the analysis focuses on a specific gene or short region of a gene, though the benefits of high sensitivity must be weighed against the possibility of detecting false positives as a result of the amplification and sequencing steps. Therefore, minor variants that are present as less than 0.5% of the viral population are typically excluded in these studies [42]. In addition to focused sequencing techniques, increasingly sensitive methods for whole-genome sequencing have also been devised recently that allow for the detection of minor population of RASs and mixed GT/subtype infections that may be relevant for treatment response [44].

In general, it has been found that RASs that are present in low proportions (<15%) do not significantly affect treatment outcomes, whereas RASs existing as a greater than 15% proportion of the overall population are more associated with treatment failure. As such, there has been agreement that a 15% cutoff should be used in all clinical trials and studies of real-world patients in the reporting of RASs by population and next-generation sequencing [38].

4 Drug Resistance to DAAs

4.1 NS3/4A Protease Inhibitors

The NS3/4A viral protease is a heterodimer complex in which the NS3 protein contains the proteolytic cleavage site and NS4A functions as a cofactor. NS3/4A inhibitors block the NS3 catalytic site or inhibit NS3/4A interaction, thereby preventing the cleavage of the HCV polyprotein. The catalytic site of the NS3 protease is located within a shallow groove which has made the design of small inhibitor molecules challenging. Moreover, given the paucity of binding sites available for small molecules to the catalytic site, there is a relatively low barrier to genetic resistance to NS3/4A inhibitors [45].

The first-generation protease inhibitors (PIs) telaprevir (TVR) and boceprevir (BOC) were the first two DAAs approved for the treatment of HCV GT1 in conjunction with interferon and ribavirin [46, 47] and were part of a structural class known as linear ketoamides [45, 48]. The second wave of first-generation PIs are characterized by their increased potency and include the macrocyclic compounds: simeprevir (SMV), asunaprevir (ASV), and vaniprevir (VAN). SMV and ASV are currently approved for HCV treatment in the United States, while VAN is only approved in Japan. Given the development of these increasingly potent protease inhibitors, the production of TVR and BOC was terminated in 2014. Most recently, the second-generation protease inhibitors have been developed with a particular focus on not only continuing to increase potency and reduce susceptibility to resistance mutations but also optimizing broad genotypic activity [49]. Approved members of this class include paritaprevir (PTV), grazoprevir (GZR), glecaprevir (GLE), and voxilaprevir (VOX).

NS3 RASs can be detected at low levels in infected individuals even prior to DAA treatment. Early studies that relied on population sequencing of individuals who did not achieve SVR after treatment with a TVR-based regimen in phase II and III trials found that a number of RASs were preferentially enriched, including V36A/M, T54A/S, R155K/T, A156S/T, and D168N [50]. A subsequent study evaluating the presence of baseline RASs in individuals who did not achieve SVR after treatment with a SMV-based regimen in phase IIb and III trials found some similar mutations to that seen with the first-generation PI-based regimens, including those at positions 155, 156, and 168, along with a number of unique RASs including Q80K and S122R [49, 51]. RASs at positions 155, 156, and 168 have also been identified in individuals who were treated with PTV- or GZR-containing regimens [52–54]. However, in comparison to second-wave, first-generation PIs, in vitro replicon studies demonstrate that these mutations are associated with significantly less resistance toward second-generation PIs [52, 53]. These resistance-associated positions are all located around the catalytic site of the NS3 protease domain, and mutations at these sites lead to reduction in the ability of the inhibitor molecule to bind effectively to the active site. A complete list of known NS3 RASs to date that result in >twofold increase in resistance can be found in Fig. 4.

NS3 RASs are generally found at low levels (0.1–3.1%) at baseline because many of them incur a significant replicative cost [64]. The one exception to this is Q80K, which does not significantly impair replicative fitness. In one study of patients with GT1 infection, the Q80K RAS was identified in 13.6% of cases, with nearly all the cases being present in patients with GT1a infection [49].

After withdrawal of treatment, NS3 RASs gradually disappear with time as the environmental pressure that selected for these subpopulations is removed. This has been observed in patients treated with all generations of NS3/4A inhibitors. In one study that investigated the evolutionary dynamics of treatment-emergent RASs in 1797 patients treated with TVR, PEG-IFN, and ribavirin, 77% of those who did not achieve SVR harbored RASs at time of treatment failure, but these were lost over time after treatment cessation [65]. The median time to reversal to wild-type viruses predominating was 10.6 months for GT1a and 0.9 months for GT1b, but all patients had lost detectable RASs by 17 months and 13 months, respectively [65]. Another study of 197 patients treated with SMV, PEG-IFN, and ribavirin had similar results. 91% of those who did not achieve SVR had the presence of RASs at the time of treatment failure, and the median time until the return to dominance of the wild-type virus was 9 months for individuals with GT1a infection and 6 months for those with GT1b infection [49]. More recently, a study of patients treated with GZR/ELB