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REVIEW

Nonstructural protein 3-4A: the Swiss army knife of hepatitis C virus

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SUMMARY. Hepatitis C virus (HCV) nonstructural protein 3-4A (NS3-4A) is a complex composed of NS3 and its cofactor NS4A. It harbours serine protease as well as NTPase/RNA helicase activities and is essential for viral polyprotein processing, RNA replication and virion formation. Specific inhibitors of the NS3-4A protease significantly improve sustained virological response rates in patients with chronic hepatitis C when combined with pegylated interferon-α and ribavirin. The NS3-4A protease can also target selected cellular proteins, thereby blocking innate immune pathways and modulating growth factor signalling. Hence, NS3-4A is not only an essential component of the viral replication complex and prime target for antiviral intervention but also a key player in the persistence and pathogenesis of HCV. This review provides a concise update on the biochemical and structural aspects of NS3-4A, its role in the pathogenesis of chronic hepatitis C and the clinical development of NS3-4A protease inhibitors.

Keywords: directly acting antiviral, hepatitis C virus, helicase, innate immunity, interferon, mitochondrial antiviral signalling protein, NS3-4A, replication, serine protease.

INTRODUCTION

The last years have seen great progress in deciphering the life cycle of hepatitis C virus (HCV), a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma worldwide. Progress in this area has translated into the development of novel antiviral agents, with approval of a first generation of specific inhibitors of the HCV nonstructural protein 3-4A (NS3-4A) protease expected in 2011. In addition, recent work has identified the NS3-4A protease as a key viral protein blocking innate immune pathways and thereby contributing to the pathogenesis of hepatitis C. It appears timely, therefore, to review current understanding of this multifunctional viral protein.

Abbreviations: AA, amino acid; DAA, directly acting antiviral; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; HCV, hepatitis C virus; IFN, interferon; IRES, internal ribosome entry site; IRF3, interferon regulatory factor 3; ISG, interferon-stimulated gene; Jak-STAT, Janus kinase-signal transducer and activator of transcription; MAVS, mitochondrial antiviral signalling protein; NCR, noncoding region; NS3-4A, nonstructural protein 3-4A; PAMP, pathogen-associated molecular pattern; PEG-IFN-α, pegylated interferon-α; PI3K, phosphatidylinositol 3-kinase; PRR, pattern recognition receptor; RIG-I, retinoic acid-inducible gene I; SVR, sustained virological response; TC-PTP, T-cell protein tyrosine phosphatase; TIR, toll/IL-1 receptor homology; TLR, toll-like receptor; TRIF, TIR domain-containing adaptor inducing IFN-β.

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BIOCHEMICAL AND STRUCTURAL PROPERTIES OF THE NS3-4A COMPLEX

Hepatitis C virus contains a 9.6-kb positive-strand RNA genome composed of a 5′ noncoding region (NCR), which includes an internal ribosome entry site (IRES), an open reading frame that encodes the structural and nonstructural proteins and a 3′ NCR [1]. IRES-mediated translation of the HCV open reading frame yields a polyprotein precursor that is co- and post-translationally processed by cellular and viral proteases into the mature structural and nonstructural proteins. The structural proteins, which form the viral particle, include the core protein and the envelope glycoproteins E1 and E2. The nonstructural proteins include the p7 viroporin, the NS2-3 and NS3-4A proteases, the NS3 RNA helicase, the NS4B and NS5A proteins, and the NS5B RNA-dependent RNA polymerase. The structural proteins

and the p7 polypeptide are processed by the endoplasmic reticulum (ER) signal peptidase and signal peptide peptidase. Cleavage between NS2 and NS3 occurs by the NS2 protease, while the NS3-4A protease is responsible for processing at the downstream cleavage sites. Polyprotein processing mediated by the NS3-4A protease follows a preferential order ([2] and references therein). The first cleavage occurs *in cis* at the NS3/NS4A site. Subsequent processing events can be mediated *in trans*, with rapid processing at the NS5A/NS5B site to produce an NS4A-5A intermediate. NS3-4A-mediated cleavage then occurs between NS4A and NS4B, to produce a relatively stable NS4B-5A precursor, and finally between NS4B and NS5A.

Hepatitis C virus NS3-4A is a noncovalent complex made of NS3 and the cofactor NS4A. NS3 is a 70-kDa multifunctional protein, with a serine protease domain located in the N-terminal one-third (amino acid [aa] 1–180) and an NTPase/RNA helicase domain in the C-terminal two-thirds (aa 181–631). Both enzyme activities have been well characterized, and high-resolution structures have been solved (reviewed in Ref. [3,4]). The NS3 serine protease domain adopts a chymotrypsin-like fold with two β-barrel subdomains. The structure is stabilized by a Zn^{2+} ion that is coordinated by Cys 97, Cys 99, Cys 145 and His 149. This Zn^{2+} binding site also plays an important role in the processing of the NS2/NS3 site by the NS2 protease [5]. The catalytic triad of the NS3-4A protease is formed by His 57, Asp 81 and Ser 139 (Figs 1a,b). The 54-aa NS4A polypeptide functions as a cofactor for the NS3 serine protease. The central portion of NS4A (aa 21–32) is required for proper folding of NS3 through the formation of a β-strand incorporated into the N-terminal β-barrel of NS3. The N-terminal hydrophobic portion of NS4A (aa 1–21) forms a transmembrane α-helix required for the integral membrane association of the NS3-4A complex ([6] and references therein), while the C-terminal acidic portion (aa 40–54) has been shown to interact with other replicase components to contribute to HCV RNA replication and virus particle assembly [7,8].

The substrate-binding site can accommodate six aa residues (P4-P2′), but cleavage is most efficient when substrates include 10 aa residues (P6-P4′). His 57 is believed to deprotonate and thereby activate Ser 139 for nucleophilic attack of the carbon atom of the scissile bond, leading to the

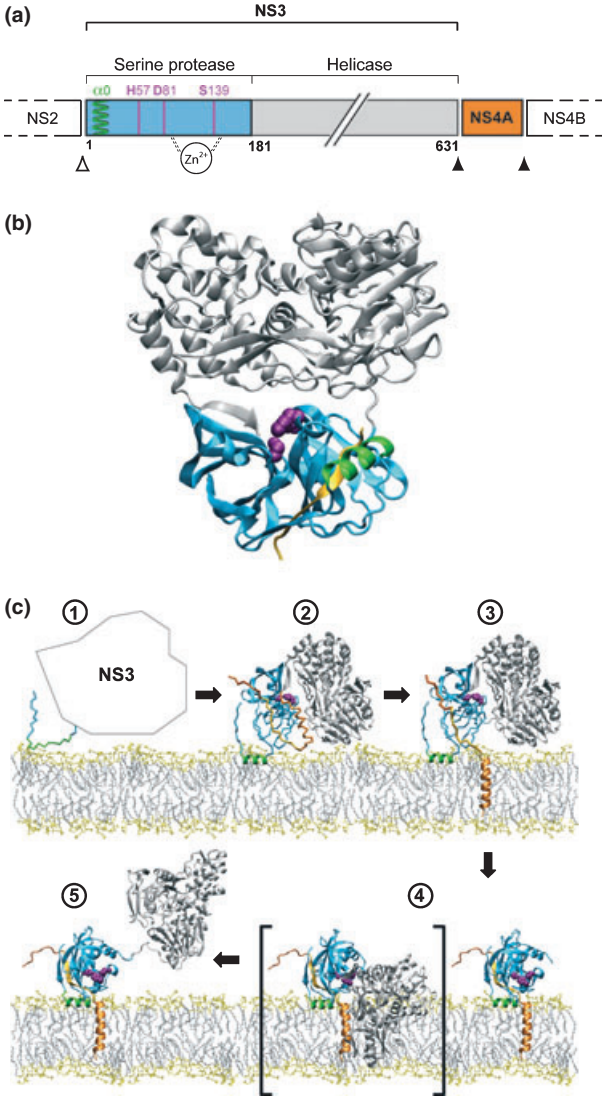

Fig. 1 Structure and membrane association of the HCV NS3-4A complex. (a) Schematic representation of the NS3-4A region of the HCV polyprotein. The NS3 serine protease and RNA helicase domains are depicted in cyan and grey, respectively. Serine protease active site residues His 57, Asp 81 and Ser 139 are highlighted in purple and N-terminal amphipathic helix α_0 in green. A Zn^{2+} atom coordinated by three cysteine residues and one histidine stabilizes the serine protease structure. NS4A is shown in orange. The white arrowhead denotes cleavage by the NS2 protease, while the black arrowheads illustrate *cis* (NS3/NS4A site) and *trans* (NS4A/NS4B site) cleavages by the NS3-4A protease. (b) Structure of the NS3-4A complex, as resolved from crystals of a single chain comprising NS3 and the central part of NS4A [68]. The same colours as in panel A are used to highlight the different elements. (c) Dynamic model for the membrane association and structural organization of NS3-4A. ① Translation of NS3 occurs at the membrane. ② Amphipathic helix α_0 folds upon interaction with the membrane interface, followed by folding of the serine protease and helicase domains). ③ The hydrophobic N-terminal segment of NS4A is inserted into the membrane after processing at the NS3/NS4A site. ④ Complete folding and membrane association lock the serine protease in a strictly defined position onto the membrane. ⑤ At the same time, the helicase domain has to move away from the serine protease domain through a rotation of the linker segment connecting the two domains. Adapted from Ref. [6] where a video illustrating the different steps can also be found.

formation of an intermediate that is stabilized by the oxyanion hole of the enzyme (aa 135–139). Collapse of this intermediate yields an acyl-enzyme intermediate and the N-terminal product peptide. Dissociation of this product peptide permits the binding of water, hydrolysis of the acyl-enzyme intermediate, and production of the C-terminal product peptide. Interestingly, N-terminal product peptides derived from the *trans*-cleavage sites were found to inhibit the enzymatic reaction [9,10]. This important observation formed the basis for the development and optimization of peptidomimetic inhibitors of the NS3-4A protease [11].

Determinants of NS3-4A protease substrate specificity include an acidic aa at the P6 position, a P1 cysteine (*trans*-cleavage sites) or threonine (*cis*-cleavage site between NS3 and NS4A), and an aa with a small side chain, i.e., alanine or serine, at the P1′ position (consensus cleavage sequence D/E-X-X-X-X-C/T | S/A-X-X-X). However, the recent identification of cellular substrates of the NS3-4A protease has revealed a much more complex scenario. On the one hand, a vast number of cellular proteins display the consensus cleavage sequence and yet only very few are cleaved by NS3-4A. On the other hand, the cellular substrates identified so far have less canonical cleavage sites (Table 1). Therefore, substrate specificity appears to be conferred by additional mechanisms such as the positioning of the NS3-4A protease active site with respect to the membrane.

The NS3 helicase is a member of the superfamily 2 DExH/D-box helicases (see [12,13] and [4] for in-depth reviews). It couples ATP hydrolysis to the unwinding of double-stranded RNA or of single-stranded RNA regions with extensive secondary structure. The NS3 NTPase/RNA helicase is essential for HCV RNA replication and also plays a role in viral particle assembly (reviewed in [14]). However, its precise function(s) in the viral life cycle remain(s) elusive. The NS3 helicase unwinds RNA in an 'inchworm' or 'ratchet-like' fashion ([15] and references therein). Similar to other HCV nonstructural proteins, it has been proposed to form higher-order oligomers [16]. It is unknown why the serine protease and RNA helicase domains are physically linked, but evidence for cross-talk between these two essential enzymatic activities is emerging ([17] and references therein). Although appealing as an antiviral target, specific inhibitors of the HCV NS3 helicase have not advanced beyond preclinical development. Indeed, it may be difficult to identify molecules that specifically block the viral enzyme without affecting the many cellular helicases with similar active sites.

As all other positive-strand RNA viruses investigated thus far, HCV replicates its genome in a membrane-associated replication complex [1,18,19]. Membrane association and structural organization of the NS3-4A complex are ensured in a sequential manner by two determinants: an in-plane amphipathic α-helix at the N terminus of NS3, designated helix α_0, and the N-terminal 21 aa of NS4A, which form a transmembrane α-helix [6]. As shown in Fig. 1c, sequential membrane association by these two determinants plays an active role in the processing and structural organization of NS3-4A and positions the serine protease active site in a strictly defined topology with respect to the membrane. This mechanistic model has implications for the functional architecture of the HCV replication complex, proteolytic targeting of host factors, and possibly drug design.

Interestingly, NS3-4A is located not only on membranes of the ER and in replication complexes but also, to a minor extent, on mitochondrial or mitochondria-associated membranes [20]. These observations may explain how the NS3-4A protease can cleave and thereby inactivate a mitochondrial host protein, the caspase recruitment domain (CARD)-containing crucial adaptor molecule Cardif [21] (also known as mitochondrial antiviral signalling protein (MAVS [22]), IPS-1 (interferon-β promotor stimulator 1 [23]) and virus-induced signalling adaptor (VISA [24]) and referred to in the following as MAVS) in the retinoic acid-inducible gene I (RIG-I) viral RNA-sensing pathway.

Table 1 Cellular targets of the NS3-4A protease

Cellular target	Cleavage site	Consequence of cleavage	Validation	References
MAVS	EREVPC | HRPS	Blocked RIG-I signalling	Heterologous expression, replicon, HCVcc Liver biopsies from patients with chronic hepatitis C	[21,30] and others [33,34]
TRIF	PSSTPC | SAHL	Blocked toll-like receptor 3 signalling	Heterologous expression	[28] and others
TC-PTP	KESVKC | AQYW PAVIHC | SAGI	Enhanced EGF signalling and basal Akt activity	Heterologous expression, replicon Liver biopsies from patients with chronic hepatitis C	[29]

HCVcc, cell culture-derived hepatitis C virus; EGF, epithelial growth factor; MAVS, mitochondrial antiviral signalling protein; TC-PTP, T-cell protein tyrosine phosphatase; TRIF, TIR domain-containing adaptor inducing IFN-β. See text for the other abbreviations.

ROLES OF THE NS3-4A PROTEASE IN THE PATHOGENESIS OF HEPATITIS C

Hepatitis C virus has evolved various strategies to counteract the host immune response and to establish persistent infection [25,26]. Innate immune responses represent the first line of defence and are triggered through host recognition of pathogen-associated molecular patterns (PAMPs). RIG-I is believed to play a key role in the sensing of PAMPs within HCV RNA and the induction of type I interferons and interferon-stimulated genes (ISGs) [27]. As illustrated in a simplified manner in Fig. 2a, RIG-I signals through the adaptor molecule MAVS to activate interferon regulatory factor 3 (IRF3), which in turn induces the expression of IFN-β. Upon release from cells, IFN-β binds to the type I IFN-α/β receptor, resulting in the auto- and paracrine activation of the Jak-STAT pathway, which leads in turn to the expression of dozens or even hundreds of ISGs that establish an antiviral state in infected and neighbouring cells.

As stated earlier, the HCV NS3-4A protease cleaves and thereby inactivates MAVS [21]. It also targets TRIF (toll/IL-1 receptor homology [TIR] domain-containing adaptor inducing IFN-β), a key adaptor molecule in the toll-like receptor 3 (TLR3) double-strand RNA-sensing pathway [28]. Hence, HCV may establish persistent infection by cleaving and inactivating cellular proteins essential for the induction of the first line of immune defence. In addition, NS3-4A has been shown to cleave T-cell protein tyrosine phosphatase (TC-PTP), thereby modulating growth factor signalling [29]. Hence, the HCV NS3-4A protease is not only an essential component of the viral replication complex but also a key player in the persistence and pathogenesis of HCV.

MAVS

Mitochondrial antiviral signalling protein cleavage by NS3-4A occurs after Cys 508, adjacent to the C-terminal transmembrane domain, and results in dislocation of MAVS from the mitochondria, thereby abolishing RIG-I-mediated signal transduction [21,30] (Fig. 2b). Oligomerization of MAVS through its C-terminal transmembrane domain is required for signalling and disrupted by NS3-4A cleavage [31]. A recent study has reported the presence of functional MAVS on peroxisomes [32]. However, the exact subcellular site of NS3-4A-mediated MAVS cleavage and inactivation remains to be determined.

Mitochondrial antiviral signalling protein cleavage was also observed in liver biopsies [33]. A recent study has demonstrated MAVS cleavage in 62 of 129 (48%) liver biopsy samples from patients with chronic hepatitis C but in none of 39 control samples from patients with other forms of chronic hepatitis [34]. MAVS cleavage was more extensive in patients with a high viral load. MAVS was cleaved by all HCV genotypes, but more efficiently by genotypes 2 and 3 than by genotypes 1 and 4. Importantly, the IFN-induced Jak-STAT pathway was less frequently activated in patients with cleaved MAVS, and there was a significant inverse correlation between cleavage of MAVS and the expression level of certain ISGs. This study validated the RIG-I pathway and NS3-4A-mediated MAVS cleavage as important but not unique determinants of

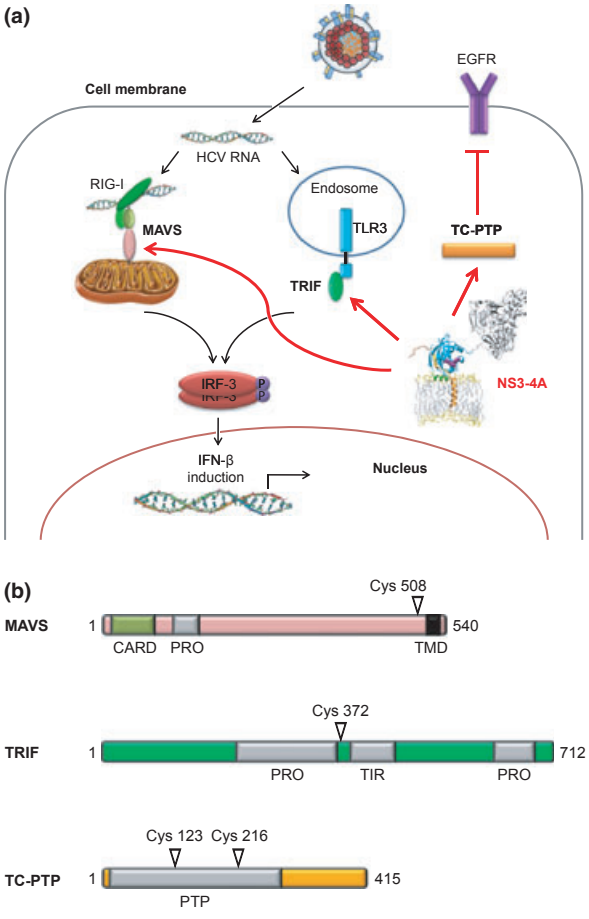

Fig. 2 Cellular targets of the hepatitis C virus (HCV) NS3-4A protease. (a) Targeting of mitochondrial antiviral signalling protein (MAVS), TIR domain-containing adaptor inducing IFN-β (TRIF) and T-cell protein tyrosine phosphatase (TC-PTP) by the NS3-4A protease. Hepatocytes are believed to sense HCV RNA through RIG-I and toll-like receptor 3 (TLR 3). Cleavage of the adaptor molecules MAVS and TRIF blocks IRF3 activation and IFN production. In addition, NS3-4A cleaves TC-PTP, thereby modulating signalling through the epidermal growth factor receptor (EGFR). (b) Schematic representation of the cellular targets of the NS3-4A protease. The caspase recruitment domain (CARD), proline-rich region (PRO) and transmembrane domain (TMD) of MAVS, the PRO and toll/IL-1 receptor homology (TIR) domains of TRIF, as well as the protein tyrosine phosphatase (PTP) domain of TC-PTP are indicated. Arrowheads denote the NS3-4A cleavage sites (see Table 1 for the corresponding sequences).

activation of the IFN system in the liver of patients with chronic hepatitis C.

Recent elegant studies have exploited MAVS cleavage by NS3-4A as a sensitive means to visualize HCV infection in live cells [35] and to explore novel cell culture systems for HCV [36].

A number of mitochondrial proteins have recently been implicated in innate immune responses, either through interaction with MAVS or as components of distinct pathways. Stimulator of interferon genes (STING; also known as mediator of IRF3 activation, MITA) has been localized both to mitochondria and the ER, and may act downstream of RIG-I and MAVS in the initiation of innate immune responses [37,38]. Nucleotide-binding domain and leucine-rich-repeat-containing protein X1 (NLRX1) [39], receptor for globular head domain of complement component C1q (gC1qR) [40] and mitofusin-2 (Mfn2) [41] have been proposed as regulators of MAVS. Further work will be required to explore whether these proteins play a role in the pathogenesis of hepatitis C.

TRIF

NS3-4A has also been shown to cleave TRIF, the signalling adaptor for TLR3, to block TLR3-mediated antiviral signalling [28]. Cleavage of TRIF occurs after Cys 372, separating its TIR domain from the tumour necrosis factor receptor-associated factor 6 (TRAF-6) binding and TBK1 (TANK-binding kinase 1)-interacting N-terminal domain. However, the role of TLR3/TRIF signalling in HCV infection has not been studied in detail, and validation in liver biopsies from patients with hepatitis C is pending.

Cleavage of MAVS and TRIF appears to be a strategy adopted by several viruses. MAVS is also cleaved by the hepatitis A virus 3ABC precursor of the 3C cysteine protease [42] and the GB virus B NS3-4A protease [43]. Moreover, MAVS and TRIF are cleaved by caspases during poliovirus infection [44]. The role of MAVS and/or TRIF cleavage in the pathogenesis of these acute viral infections, as opposed to HCV that typically establishes persistent infection, remains to be further defined [45].

TC-PTP

As stated earlier, the third cellular target of HCV NS3-4A identified to date is TC-PTP [29]. The maintenance of proper protein tyrosine phosphorylation levels is critical for cellular homeostasis. Epithelial growth factor (EGF) stimulation causes TC-PTP to exit from the nucleus and to dephosphorylate the EGF receptor, leading to decreased downstream activation of the phosphatidylinositol 3-kinase (PI3K)-Akt pathway [46]. Inactivation of TC-PTP by the NS3-4A protease results in enhanced EGF-induced signalling, findings with possible implications for the development of hepatocellular carcinoma.

THE NS3-4A PROTEASE AS A DRUG TARGET

Current standard therapy for chronic hepatitis C, pegylated interferon-α (PEG-IFN-α) combined with ribavirin, results in a sustained virological response (SVR) in only about 50% of patients [47]. As a consequence, the number of patients presenting with long-term sequelae of chronic hepatitis C, including decompensated cirrhosis and hepatocellular carcinoma, is expected to increase further over the next 10 years [48]. On this background, intensive efforts have been made to develop directly acting antivirals (DAAs) against HCV [11]. Most advanced are specific inhibitors of the NS3-4A protease, which can be divided into two chemical classes, macrocyclic inhibitors [e.g., ciluprevir (BILN 2061) and vaniprevir (MK-7009)] and linear tetra-peptide α-ketoamide derivatives [e.g., telaprevir (VX-950) and boceprevir (SCH 503034)] (Fig. 3). As stated earlier, inhibitors of both classes have been developed based on the observation that the NS3-4A protease is susceptible to inhibition by the N-terminal peptide products released from the substrates upon proteolytic cleavage.

The first NS3-4A inhibitor evaluated in a clinical study was ciluprevir, an orally bioavailable macrocyclic drug binding noncovalently to the protease active site [49]. Strikingly, ciluprevir, administered twice daily for 2 days led to a rapid and pronounced decrease in HCV RNA serum levels in patients infected with HCV genotype 1 [50]. Although further clinical development of ciluprevir was halted because of cardiotoxicity in laboratory animals, these landmark studies provided the proof-of-concept for successful

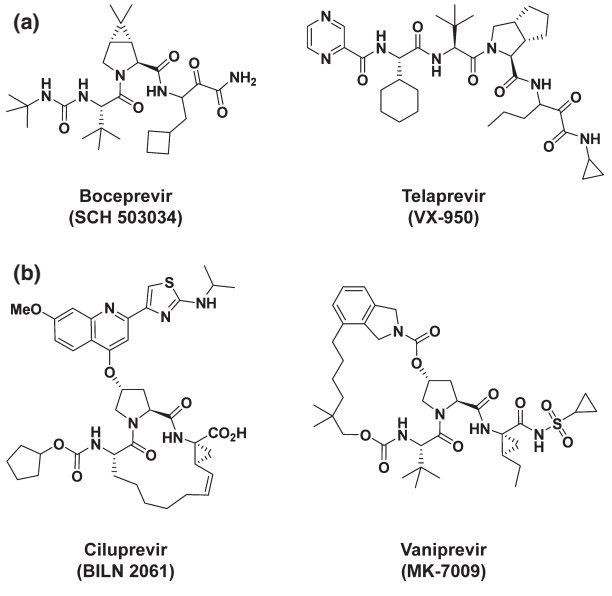

Fig. 3 Chemical structures of (a) linear and (b) macrocyclic hepatitis C virus NS3-4A protease inhibitors. Drawings were kindly provided by Dr. Steve W. Ludmerer from Merck Research Laboratories, West Point, PA.

suppression of HCV replication by NS3-4A inhibitors in patients with chronic hepatitis C.

Subsequently developed NS3-4A protease inhibitors of both molecular classes have been shown to strongly inhibit HCV replication during monotherapy but to result in the rapid selection of resistant variants, which may be followed by viral breakthrough [51]. Importantly, resistance development can be prevented by the addition of PEG-IFN-α and ribavirin, and phase II and III clinical trials have shown that such triple therapies have the potential to increase SVR rates in patients with chronic hepatitis C.

The most advanced NS3-4A inhibitors in clinical development are telaprevir and boceprevir, whose approval is expected in 2011. Both of them bind to the enzyme active site covalently but reversibly. Phase I clinical evaluation demonstrated a median reduction of HCV RNA of 4.4 $\log_{10}$ after 14 days of treatment with telaprevir at a dose of 750 mg every 8 h [52]. The antiviral activity of boceprevir monotherapy was somewhat weaker, with mean reductions in HCV RNA of up to 1.6 $\log_{10}$ [53]. Importantly, rebound of wild-type virus and selected mutants was observed in all patients after treatment completion, and some patients experienced a viral breakthrough of resistant variants already during monotherapy [54]. Subsequent studies have shown that the addition of PEG-IFN-α and ribavirin to telaprevir or boceprevir leads to an even more pronounced HCV RNA decline and reduces the frequency of resistant mutants and viral breakthrough [53,55].

Based on these encouraging results, phase II and III clinical trials were conducted that showed that both telaprevir (PROVE 1 and 2 as well as ADVANCE trials [56–58]) and boceprevir (SPRINT-1 and SPRINT-2 trials [59,60]) can significantly enhance SVR rates in treatment-naïve patients with HCV genotype 1 when combined with PEG-IFN-α and ribavirin. Overall, SVR rates could be improved from 38% to 46% with PEG-IFN-α and ribavirin to 68–75% with triple therapy regimens comprising either telaprevir or boceprevir combined with PEG-IFN-α and ribavirin. The addition of telaprevir to PEG-IFN-α and ribavirin allowed to shorten the total treatment duration from the current standard of 48 weeks to 24 weeks. Importantly, studies performed in patients who had failed previous therapy with PEG-IFN-α and ribavirin also demonstrated significantly improved SVR rates upon retreatment with a triple therapy regimen (PROVE 3 and REALIZE [61,62] as well as RESPOND trials [63]). SVR rates observed in the PROVE 2 and 3 trials are shown exemplarily in Fig. 4.

This significant improvement in SVR rates comes at the cost of additional adverse effects such as rash, gastrointestinal disorders and enhanced anaemia as well as increased cost. In addition, the selection of resistant mutants in suboptimal responders is a major concern, as discussed below. Selection of the optimal candidates for treatment with this first generation of DAAs will represent an important challenge for the months to come.

Other NS3-4A protease inhibitors are currently in phase I–II development (e.g., danoprevir [RG7227/ITMN191], vaniprevir [MK-7009], MK-5172, BI201335, TMC435350, SCH900518, BMS-650032, PHX1766, ACH-1625). In general, they exhibit potent antiviral activity in HCV genotype 1 patients, comparable to telaprevir and boceprevir. Potential advantages of these second- and third-generation protease inhibitors might be improved tolerability, broader genotype coverage (e.g., MK-5172), different resistance profiles (e.g., MK-5172), and/or improved pharmacokinetics, which may allow a once daily dosing (e.g., TMC435350 and MK-5172).

Viral resistance to NS3-4A protease inhibitors

The high replication rate of HCV and the poor fidelity of the NS5B RNA-dependent RNA polymerase form the basis for the quasispecies nature of HCV and the rapid selection of resistant variants during treatment with specific antivirals. In general, NS3-4A protease inhibitors display a low genetic barrier to resistance development, which may differ significantly between HCV subtypes because of differences in the nucleotide sequence at key resistance positions [64,65]. This led to a higher incidence of viral resistance and breakthrough in patients infected with HCV genotype 1a compared to HCV genotype 1b in all clinical studies involving telaprevir.

Table 2 summarizes the resistance profiles of selected NS3-4A inhibitors. Although the resistance profiles differ significantly, R155 is an overlapping position for resistance development and different mutations at this position confer resistance to nearly all protease inhibitors that are currently in advanced clinical development [51]. Interestingly, already the first *in vitro* study investigating resistance to protease inhibitors in the replicon system identified mutations (e.g. at position Asp 168) that were later found in patients during treatment with protease inhibitors [66]. However, many resistance mutations could be detected *in vivo* only by clonal sequencing. For example, mutations at four positions conferring telaprevir resistance have been characterized so far (V36A/M/L, T54A, R155K/M/S/T and A156S/T), but only position Ala 156 was identified *in vitro* in the replicon system. These mutations, alone or as double mutations, conferred low (V36A/M, T54A, R155K/T, A156S) to high (A156T/V, V36M + R155K, V36M + 156T) levels of resistance to telaprevir (Fig. 5). It is thought that these aa changes alter the conformation of the catalytic site of the protease, which impedes binding of the protease inhibitor. However, these changes result in significant fitness costs for the virus. In general, replicative fitness of HCV variants correlated inversely with the magnitude of telaprevir resistance conferred by single-mutation variants, though compensatory mutations may be capable to restore replication fitness [54].

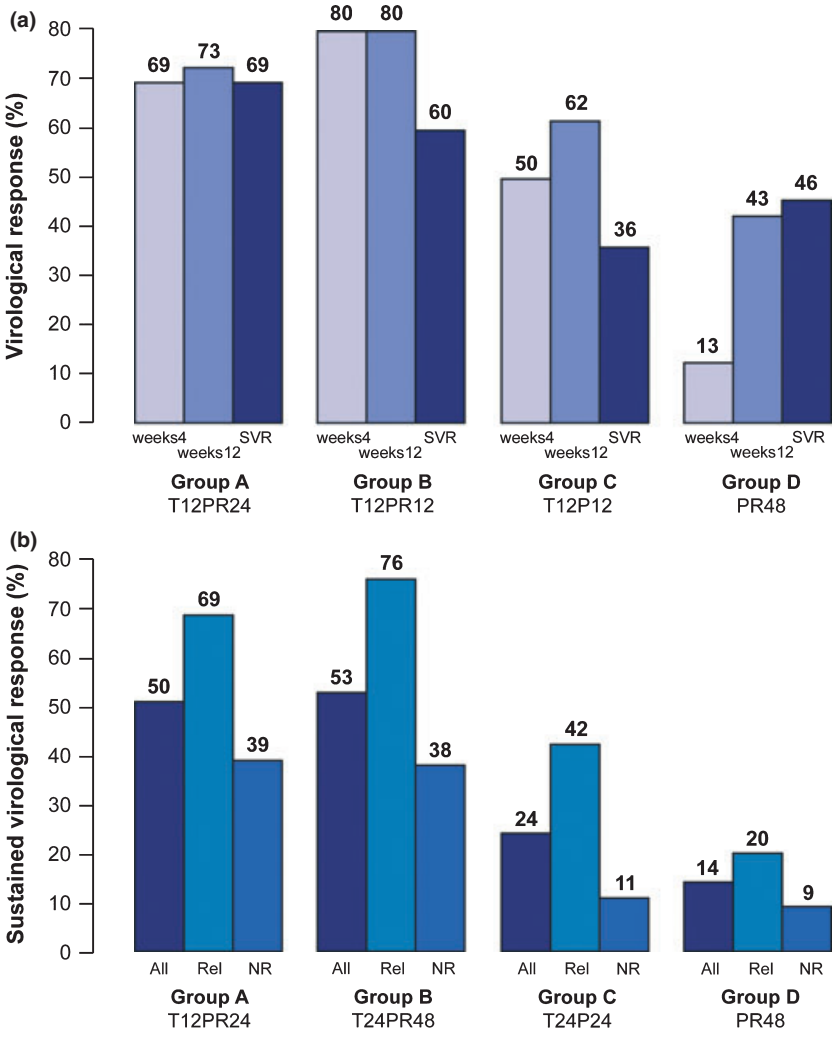

Fig. 4 Results of the PROVE 2 and 3 trials. (a) The PROVE 2 trial included 323 treatment-naïve patients with chronic hepatitis C, genotype 1 [56]. Patients were treated for 12 weeks with telaprevir plus PEG-IFN-α and ribavirin, followed by 12 weeks of PEG-IFN-α and ribavirin (Group A, T12PR24), for 12 weeks with telaprevir plus PEG-IFN-α and ribavirin (Group B, T12PR12), for 12 weeks with telaprevir plus PEG-IFN-α (Group C, T12P12), or for 48 weeks with PEG-IFN-α and ribavirin (Group D, PR48). The rate of complete virological responses at weeks 4 and 12 as well as the rates of SVRs are shown. SVR rates in groups A, B, C and D were 69, 60, 36 and 46%, respectively. (b) The PROVE 3 trial included 453 patients with chronic hepatitis C, genotype 1, that had failed previous therapy with PEG-IFN-α and ribavirin [61]. Patients were treated for 12 weeks with telaprevir plus PEG-IFN-α and ribavirin, followed by 12 weeks of PEG-IFN-α and ribavirin (Group A, T12PR24), for 24 weeks with telaprevir plus PEG-IFN-α and ribavirin, followed by 24 weeks of PEG-IFN-α and ribavirin (Group B, T24PR48), for 24 weeks with telaprevir plus PEG-IFN-α (Group C, T12P12), or for 48 weeks with PEG-IFN-α and ribavirin (Group D, PR48). SVR rates in all patients as well as in previous relapsers (Rel) *vs* nonresponders (NR) separately are shown.

CONCLUSIONS AND PERSPECTIVES

NS3-4A may be considered as Swiss army knife for HCV based on its fascinating structural plasticity, its multifunctionality and its different roles in the viral life cycle and the pathogenesis of hepatitis C. This essential viral protein has been extensively characterized at the biochemical and structural levels, and first-generation serine protease inhibitors shall be approved for clinical use shortly. However, much has to be learned with respect to the cell biology of this protein and its topology and interactions within the high-order structure of the HCV replication complex. Moreover, important questions remain to be answered concerning the role of NS3-4A in the pathogenesis of hepatitis C. For example, the cellular and viral processes regulating NS3-4A-mediated MAVS cleavage throughout the natural history of hepatitis C are largely unknown. Three cellular targets of the NS3-4A protease have been identified so far but there are

Table 2 Resistance mutations to hepatitis C virus NS3 protease inhibitors (modified from [51])

	V36A/M	T54S/A	V55A	Q80R/K	R155K/T/Q	A156S	A156T/V	D168A/V/T/H	V170A/T
Telaprevir	X	X	*		X	X	X		*
Boceprevir	X	X	X		X	X	*		X
SCH900518	X	X			X	X	X		X
BI201335					X			X	X
Ciluprevir					X		X	X	
Danoprevir					X	*	*	X	
Vaniprevir					X			X	
TMC435350				X	X			X	

*Mutations associated with resistance *in vitro* but not described in patients.
Telaprevir, boceprevir, SCH900518 and BI201335 are linear protease inhibitors, whereas ciluprevir, danoprevir, vaniprevir and TMC435350 are macrocyclic inhibitors.

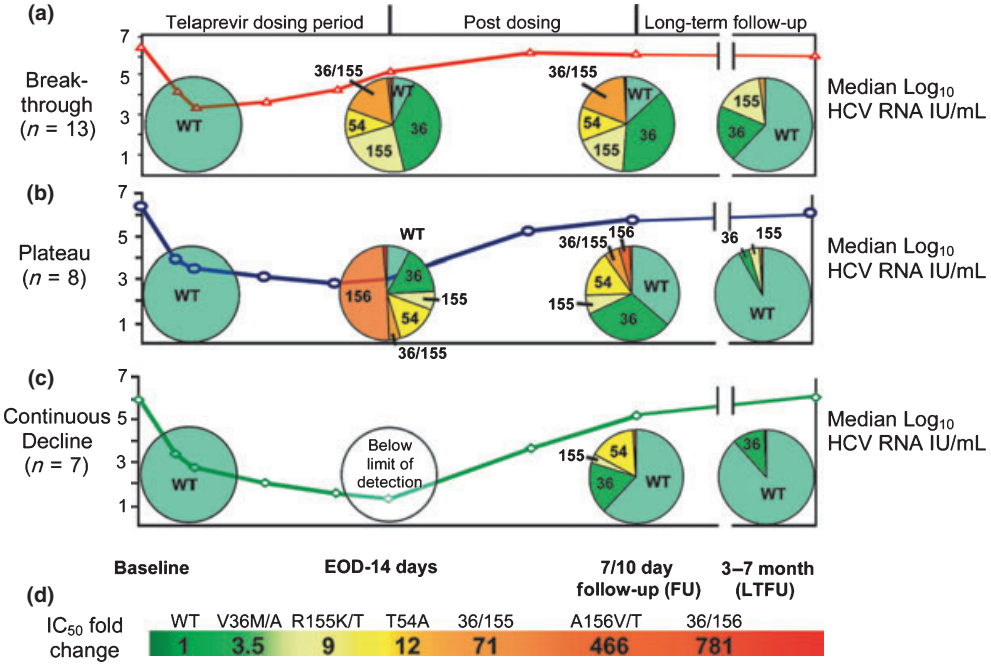

Fig. 5 Kinetics of hepatitis C virus (HCV) quasispecies during and after telaprevir monotherapy. Frequencies of resistance mutations in the NS3 protease domain are shown according to viral response in patients treated with telaprevir monotherapy for 14 days. Mean HCV RNA levels (solid lines) and the numbers of patients in each group are shown. Importantly, significant numbers of resistant variants were still detectable during long-term follow-up (3–7 months after treatment completion). (a) Patients with viral breakthrough during treatment, which was defined as plasma HCV RNA increase of >0.75 $\log_{10}$ IU/mL from nadir. (b) Patients in whom a plateau of HCV RNA levels during therapy was observed (increase of HCV RNA ≤ 0.75 $\log_{10}$ IU/mL from nadir). (c) Patients who experienced a continuous HCV RNA decline during therapy. (d) Wild type and resistant variants are listed from left to right in the order of increasing resistance, expressed as fold-changes in enzymatic IC_{50} compared to wild type (WT). From Ref. [54], with permission.

likely others. Which are they, what confers selectivity to their cleavage and what are the consequences for the infected host? The advent of specific serine protease inhibitors will significantly improve SVR rates in patients with chronic hepatitis C of genotype 1. However, new challenges and important questions will have to be addressed. Which patients should be treated with the first generation of these inhibitors, considering the requirement for an efficient PEG-IFN-α and ribavirin backbone therapy, additional adverse effects, cost, and the selection of resistant variants in patients with suboptimal response? Current efforts aimed at developing second- and third-generation protease inhibitors as well as IFN-sparing DAA combination regimens shall further improve the outlook for patients with chronic hepatitis C.

Clearly, these efforts are important and promising, given the current and expected burden of hepatitis C and the fact that HCV infection, as opposed to hepatitis B virus or HIV infection, can be cured [67].

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