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BRIEF REVIEW

# Current and future therapies for hepatitis C virus infection: from viral proteins to host targets

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**Abstract** Hepatitis C virus (HCV) infection is the most important problem across the world. It causes acute and chronic liver infection. Different approaches are in use to inhibit HCV infection, including small organic compounds, siRNA, shRNA and peptide inhibitors. This review article summarizes the current and future therapies for HCV infection. PubMed and Google Scholar were searched for articles published in English to give an insight into the current inhibitors against this life-threatening virus. HCV NS3/4A protease inhibitors and nucleoside/nucleotide inhibitors of NS5B polymerase are presently in the most progressive stage of clinical development, but they are linked with the development of resistance and viral breakthrough. Boceprevir and telaprevir are the two most important protease inhibitors that have been approved recently for the treatment of HCV infection. These two drugs are now the part of standard-of-care treatment (SOC). There are also many other drugs in phase III of clinical development. When exploring the various host-cell-targeting compounds, the most hopeful results have been demonstrated by cyclophilin inhibitors. The current SOC treatment of HCV infection is Peg-interferon, ribavirin and protease inhibitors (boceprevir or telaprevir). The future treatment of this life-threatening disease must involve combinations of therapies hitting multiple targets of HCV and host factors. It is strongly expected that the near future, treatment of HCV infection will be a combination of direct-acting agents (DAA) without the involvement of interferon to eliminate its side effects.

## Introduction

HCV is a major health burden affecting about 200 million people worldwide [1]. Chronic HCV infection is associated with a 25 % lifetime risk of developing cirrhosis and a 20 % lifetime risk of developing hepatocellular carcinoma [2]. HCV is a member of the virus family *Flaviviridae*. Its

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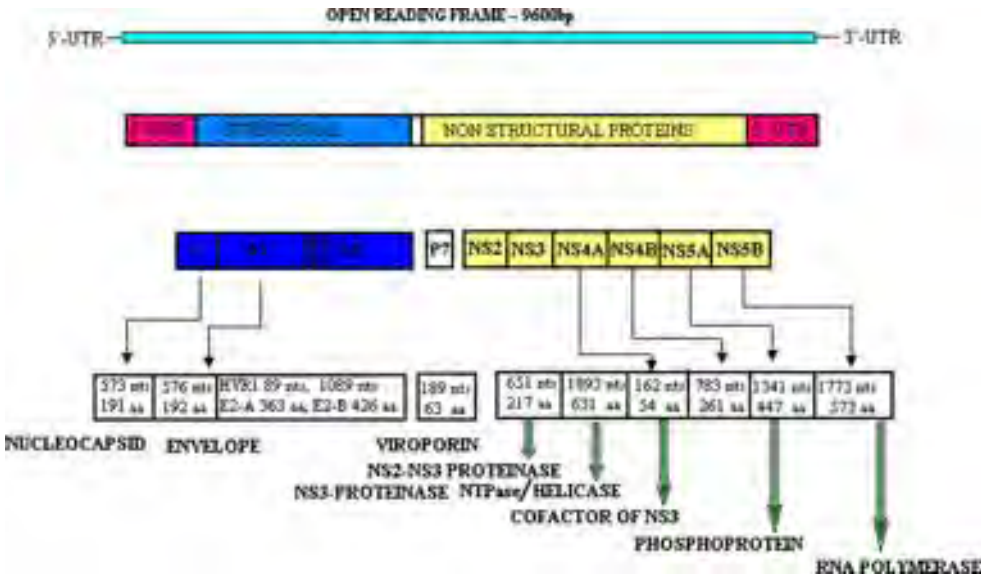


**Fig. 1** The hepatitis C virus genome, its structural and non-structural proteins, their nucleotide sequence lengths, their amino acid sequence lengths, and their functions

genome is an approximately 9.6-kb, positive-sense, single-stranded RNA [3] that encodes a single polyprotein of about 3000 amino acids. The polypeptide is cleaved by viral and host enzymes to produce at least 10 mature viral proteins, including core, E1 and E2 envelope proteins, the p7 ion channel protein, and the non-structural (NS) proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B, as shown in Fig. 1 [4]. The virus life cycle has been elucidated in multiple recent reviews [5].

The current most standard treatment for HCV infection includes a protease inhibitor (either telaprevir or boceprevir) along with the PEG-IFN/ribavirin [6]. It is chiefly effective against HCV genotype 2 or 3 [7]. The treatment duration for genotype 2 and 3 is six months, while for genotype 1 and 4 it may extend up to 12 months [8]. There are also side effects associated with therapy, which include pancytopenia, flu-like symptoms, depression, pain in the body, and elevated temperature. These symptoms are most commonly observed and may be the reason for discontinuation of treatment before elimination of the virus has been achieved. As our understanding about the HCV life cycle increases, new options become available for the treatment of HCV infection. In recent years, researchers have focused on the development of DAA agents, which specifically target HCV replication and posttranslational processing [9].

## HCV proteins and their inhibitors

### Core

The core protein comprises 191 amino acids. Three domains, D1, D2 and D3 of the core protein facilitate comprehensive understanding of the functions of this protein. The N-terminal domain D1 binds to HCV RNA and acts as a powerful chaperon. This domain is basic in nature and is involved in RNA dimerization and nucleocapsid formation [10, 11]. The other two domains, D2 and D3, interact with several glycoproteins, including the viral attachment glycoproteins E1 and E2. These two domains are hydrophobic in nature and also interact with lipid droplets and several host proteins. The mature core protein consists of 177 amino acids [12]. Its processing takes place in the endoplasmic reticulum (ER), where precursor core protein is cleaved, and a short peptide, which serves as a signal sequence for the E1 glycoprotein, remains in the ER [13, 14].

The HCV core protein is an attractive target for drug development because it is the most conserved of all HCV proteins. Its exceptional level of conservation is uniform across all the HCV genotypes, reflecting its essential role in the HCV life cycle [15]. It is most likely that an inhibitor against this target can effectively be used as a therapeutic agent across all the known HCV genotypes.

#### *Inhibition by peptides*

The interaction between two proteins can also be interrupted by peptides derived from either one of the two interacting proteins [16]. Keeping this technique in mind, fourteen 18-residue peptides were taken from HCV core protein [17]. It was noted that core dimerization was inhibited by three small peptides, SL173, SL-174 and SL175, by 63, 68 and 50 %, respectively. These peptides shared an amino acids sequence of 11 residues that contributed most of the free energy of dimerization. These residues were considered a hot spot for protein

dimerization. Moreover, it was also found that these peptides also have inhibitory effects on HCV production and release.

*Inhibition by small compounds*

Compounds SL-201 and SL-209, as shown in Fig. 2, were also used as small organic inhibitors against HCV core oligomerization. These compounds are basically tetracyclic in structure, having similarity to aspidosperma alkaloids, which are well known for their biological activities [18]. These compounds were studied at T1 (72 h) and T2 (144 h) for inhibiting HCV production in cell culture. It was noted that SL-201 reduced HCV replication by two logs with a 50 % effective concentration ($EC_{50}$) of 8.8 μM and 8.1 μM at T1 and T2, while SL-209 achieved the same amount of HCV reduction with an $EC_{50}$ of 2.3 μM and 3.4 μM at T1 and T2 [17].

*Inhibition by siRNAs*

The molecular approach of RNA interference has been used against HCV. It is a sequence-specific gene-silencing mechanism. Basically, there are two approaches to RNA interference. In the first approach, there is direct use of short chemically synthesized RNAs of 18-20 nucleotides, while in the second approach, small short hairpins (shRNA) of 80-100 nucleotides are used, which are then processed by the cellular machinery into small expression cassettes of active siRNA [19]. Neither of these techniques activates interferon responses, and both result in posttranscriptional gene silencing [20]. It was shown that siRNA designed against the core protein of HCV genotype-3a (HCV-3a) inhibited HCV production by 80 % when compared to mock-treated cells of the Huh-7 cell line. Permanent effects were achieved by vector-based short hairpin siRNAs [21]. Co-transfection of HEK293T cells with plasmid pEGFP-C, which contains an EGFP reporter gene and the core gene as the silencing target, and siRNA yielded similar results, indicating that siRNA is a powerful tool against HCV [22].

E1 and E2

Two envelope glycoproteins, E1 and E2, are encoded by the HCV genome. These glycoproteins play an important role in virus entry into hepatocytes, interacting with various cell-surface proteins [23]. There is a possible role of glycosaminoglycan and low-density lipoprotein receptor in attachment of virus to cells. There are four essential entry factors: scavenger receptor class B type 1, tetraspanin CD81, claudin-1 and occludin. These factors are involved successively after virus attachment. In the last step, the virus enters the cells by means of clathrin-mediated endocytosis [24].

*(−)-Epigallocatechin-3-gallate*

Green tea has been shown to have a useful role against various diseases, particularly against cancers. This effect of green tea is mostly attributed to a polyphenol flavonoid, (−)-epigallocatechin-3-gallate (EGCG) [25]. It has shown antiviral activities against various viruses such as human immunodeficiency virus (HIV) and influenza virus [26]. As this flavonoid interferes with cellular lipid metabolism, it was likely to be useful against HCV infection. It was shown to inhibit HCV infection at a very low dose and have significant specificity and tolerability. This flavonoid does not directly act on HCV particles by inhibiting their replication; it blocks infection by obstructing attachment of the virus to hepatocytes. Thus, it also blocks cell-to-cell spread of the virus. The antiviral activity of EGCG was also demonstrated using pseudoparticles (HCVpp) [27]. HCVpp are basically comprised of full-length HCV E1E2 envelope glycoproteins that are added to retroviral core particles with a retrovirus genome and a luciferase gene [28].

*Triazine*

Triazine is an HCV-specific inhibitor. Its median $EC_{50}$ against HCV genotype 1a and 1b is 0.134 and 0.027 mM, respectively. Time-of-addition experiments demonstrated that it inhibits the entry step of HCV infection. HCV enters the cell via clathrin-coated endocytosis after which a fusion step occurs in the acidic endosomal environment. The fusion step can be inhibited by treatment with bafilomycin A, which neutralizes the endosomal pH and impairs endocytosis. It has been suggested that triazine inhibits HCV entry prior to or at the same time inhibition of endosomal acidification by bafilomycin. Moreover, it has been shown that it blocks cell-to-cell transmission of the virus [29].

*Inhibition by peptides*

The interactions between proteins involved in HCV entry were also probed by short polypeptides derived from viral envelope sequences. A 16-residue polypeptide (V-S-F-A-I-K-W-E-Y-V-L-L-L-F-L-L), named peptide 75, possessing a portion of the E2 transmembrane domain blocked infection by HCV pseudoparticles. The 50 % inhibitory concentration ($IC_{50}$) was approximately 0.3 μM. Structure-activity analysis of peptide 75 demonstrated that antiviral activity was dependent on the hydrophobic character and L-configuration. Moreover, this native sequence was

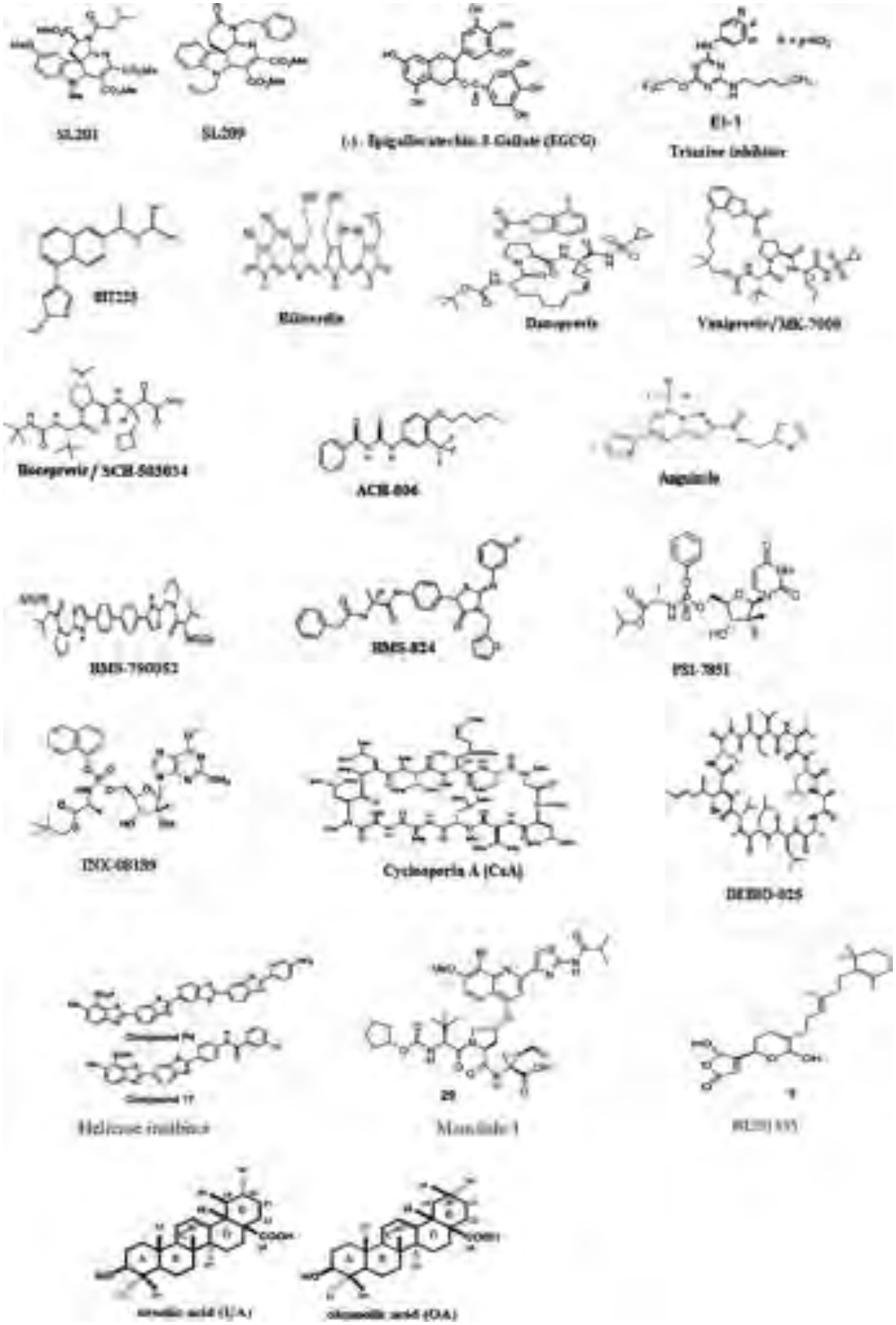


**Fig. 2** Structures of various HCV protein inhibitors

**Table 1** Drugs against Hepatitis C Virus infection

| Company | Drug | Mechanism of action | Stage of clinical development |
|---|---|---|---|
| Biotron Limited | BIT225 | Inhibits HCV P7 protein | Completed phase IIa |
| Roche | Danoprevir | Inhibits NS3 protein | Phase II |
| Merck & Co. | Vaniprevir | Inhibits NS3 protein | Phase II |
| Merck | Boceprevir | Inhibits NS3 protein | APPROVED |
| Vertex | Telaprevir | Inhibits NS3 protein | APPROVED |
| Bristol-Myers Squibb | TMC-435 | Inhibits NS3 protein | Phase III |
| BoehringerIngelheim | BI 201335 | Inhibits NS3 protein | Phase III |
| Gilead and Achillion Pharmaceuticals | ACH-806 | NS4A antagonist | Phase 1b/2 |
| EigerBioPharmaceuticals | Clemizole hydrochloride | Inhibitor of NS4B:RNA | Phase 1b |
| Med Chem express | Anguizole | Inhibitor of HCV RNA replication | Phase 1b |
| Brystol Mayer Squibb | BMS-790052 | NS5A inhibitor | Phase III |
| Brystol Mayer Squibb | BMS 824 | NS5A inhibitor | Phase II |
| Gilead Sciences Inc | GS-5885 | NS5A inhibitor | Phase II |
| GlaxoSmithKline | GSK2336805 | NS5A inhibitor | Phase II |
| Presidio Pharmaceuticals | PPI-668 | NS5A inhibitor | PhaseII |
| Pharmasset | PSI-7851 | NS5B inhibitor | Phase II |
| Abbott | ABT-072 | Non-nucleoside polymerase inhibitor | Phase II |
| Abbott | ABT-333 | Non-nucleoside polymerase inhibitor | Phase III |
| SantarisPharma | SPC3649 | Lock nucleic acid mRNA122 inhibitor | Phase 1 |
| Human Genome Sciences | Zalbin | Immunomodulator | Phase III |
| Novartis/Debiopharm | Debio 025 | Cyclophilin inhibitor | Phase III |
| SciClonePharma/Sigmatau | Zadaxin-thymalfasin | Immunomodulator | Phase III |

important for maximum blocking activity against HCV pseudoparticles [30].

P7

HCV P7 is an important protein of 63 amino acids. It is a member of an expanding family of proteins known as viroporins because it forms oligomers and ion channels in the lipid membrane. Physiological studies of the P7 protein have demonstrated that its N-terminal helix is oriented towards channel pores, and it allows the influx of cations through the membrane. In the HCV genome, the genes for these proteins are located at the junction of structural and nonstructural proteins [31, 32].

*BIT225*

Several p7 inhibitors for which antiviral activity has been established at least in cells have been reported. These include amantadine [33], hexamethylene amiloride (HMA) [34] and BIT225 (Table 1). A single-dose safety trial of BIT225 has been successfully completed in healthy volunteers. It is an orally administered drug for which a phase IIa trial has been completed to assess its safety and pharmacokinetics. BIT225 was administered to HCV-infected patients along with PEG-INF/RBV. Patients receiving 400 mg of BIT225 demonstrated a significant reduction in viral load ($\sim$ 1 log) when compared to the patients treated with PEG-INF/RBV and placebo [35]. Consequently, some of the p7 inhibitors characterized by basic science at the laboratory bench may expand therapeutic options for the treatment of hepatitis C.

*Inhibition by derivatives of iminosugars*

HCV p7 forms ion channels in the lipid membrane. These ions channels were shown to be blocked by the iminosugar derivatives deoxynojirimycin (DNJ) and deoxygalactonojirimycin (DGJ). The study was conducted by measuring *in vitro* infectivity of bovine viral diarrhea virus (BVDV). It was shown that these iminosugar derivatives inhibit ER-glucosidases I and II [36], which in turn leads to misfolding of many host and viral proteins. These long-alkyl-chain iminosugar derivatives do not interfere with viral replication or protein synthesis [37]. It was suggested that these iminosugar derivatives influence the dimerization of glycoproteins and that the membrane composition of secreted glycoproteins is altered. It was also found that these derivatives directly interact with p7 ion channels and either block the formation of these channels or block the open channels [38].

NS2-3 protease

HCV NS2 is an important protein for HCV replication. It modulates the function of various host proteins for the formation of replication complex. It influences the host immune system by affecting apoptosis [39], regulating fat metabolism, altering the expression of various cytokines, arresting the cell cycle, and hampering the cAMP-dependent pathway [40–43]. Formerly, it was believed that NS2-3 is not a good target for the development of an HCV inhibitor because of a unimolecular reaction at the cleavage site of NS2-3. It was supposed that the kinetics of the reaction would be too fast for the inhibitor to act. However, later on, the discovery of the dimeric nature of NS2-3, with two active sites, opens opportunities to develop inhibitors against this newly discovered target [44].

*Inhibition by peptides*

A peptide derived from NS4A acts as an inhibitor of NS2-3 cleavage [45]. This peptide interferes with the proper placement of scissile bond at the junction between NS2 and NS3 in the active site of the protease. Thus, peptides derived from HCV polypeptide may act as an inhibitor of N2-3 cleavage and may be used as therapeutic agents against HCV [46].

NS3

The HCV NS3/4A protein is a membrane-targeted serine protease [46]. It has an important role in HCV replication because it cleaves the HCV polyprotein into NS3, NS4A, NS5A and NS5B. It has as an exposed active site, making it difficult to develop small-molecule inhibitors that tightly bind the site. The geometry of the active site also increases the capacity for cross-resistance because there are only a small number of "good" contacts that small-molecule inhibitors can make with the binding site. In spite of the challenges in the design of NS3 protease inhibitors, it was the first class of direct-acting anti-HCV drugs to be applied clinically [47]. The C-terminal portion of NS3 forms a three-domain polypeptide that possesses helicase activity, unwinding dsRNA or DNA. Like other motor proteins, NS3 helicase possesses two domains, one of which appears to rotate upon ATP binding. The two most prominent target sites on NS3 helicase are the ATP- and RNA-binding sites. Other distinctive properties of NS3 may also be utilized as targets for drug development [48]. From a biological point of view, due to the co-existence of NS3 protease and helicase activities, it may serve as a useful antiviral target against HCV [49].

Protease inhibitors (PIs) of HCV NS3/4A can be classified into two chemical classes: macrocyclic inhibitors and linear tetrapeptide derivatives. A number of PIs of these two classes are currently in clinical development. Among these inhibitors, the most important compounds are telaprevir and boceprevir, both of which are linear tetrapeptides [50]. NS3/4A protease inhibitors are the most advanced compounds in terms of clinical evaluation, producing significant reductions in HCV viral loads in patients [51].

*TMC-435*

TMC-435 is an orally administered macrocyclic inhibitor of HCV NS3/4A. It is effective against all HCV genotypes with the exception of HCV genotype 3. *In vitro* studies have shown an additive effect of this compound with INF/RBV therapy. Phase I and II clinical trials of this compound on HCV genotypes have demonstrated that it induces a significant reduction in viral load with no additional side effects [52]. An oral dose of TMC-435 given once per day was evaluated along with INF2a/RBV in HCV genotype 1 patients, and a dose-dependent antiviral activity was observed over 28 days. Its tolerability profile was also favorable. It is currently in phase III of clinical development [53].

*BI 201335 protease inhibitor*

BI 201335 protease inhibitor is also known as clinical candidate 29. It is a C8-bromoderivtive and a very potent and selective inhibitor of HCV genotype 1 NS3 [54]. Recent studies have shown that it possesses very effective absorption, distribution, metabolism and pharmacokinetics. These characteristics were studied in rats, other rodents, and monkeys [55]. The compound also showed a synergetic effect with BI 207127 (non-nucleoside inhibitor of NS5B) and ribavirin against HCV genotype 1 without any serious side effects [56].

*Biliverdin*

HCV-mediated hepatocellular damage is linked to oxidative stress [57]. Therefore, antioxidant enzymes may also serve as cytoprotective agents during HCV infection [58]. Heme oxygenase-1 (HO-1) is one of the important enzymes in this category. It causes oxidation of heme to iron, carbon monoxide, and biliverdin (BV) [59]. Following heme oxidation, there is a rapid reduction of free BV to bilirubin (BR). The reaction is catalyzed by an important enzyme, biliverdin reductase (BVR), which is abundantly present in hepatocytes. The induction or overexpression of HO-1 in replicons inhibits HCV replication [58]. Although the mechanism behind this effect is not understood, it is reasonable to postulate that one or more products of the

reaction are responsible for this effect. Supporting this concept, HCV NS5B RdRp has been shown to be inhibited by iron through divalent cation binding [60]. Recent studies have shown that BV possesses antiviral activity and has an important role in the stimulation of interferon response genes [61]. BV is also inhibitor of NS3/4As activity with an $IC_{50}$ of 9 uM. Lineweaver-Burk plots indicated mixed competitive and non-competitive inhibition of the protease by BV. In contrast, bilirubin (BR) had a much weaker effect on NS3/4A and HCV replication, and this might be due to the quick conversion of BV to BR by intracellular BVR. It has been shown that inhibition of viral replication is enhanced by BV by silencing BVR up to 80 %. Thus, BV or its derivatives may serve as a drug candidate against NS3/4A protease [62].

*Danoprevir*

Danoprevir/RG7227/ITMN-191 is an orally bioavailable macrocyclic PI possessing biochemical potency of $< 65$ pM. It acts very specifically, as it has been shown to lack the ability to inhibit other serine proteases [63]. A phase I, single-ascending-dose study of Danoprevir established its safety and tolerability in healthy adult subjects. The doses ranged from 2 to 1600 mg [64]. Phase IIb studies of Danoprevir showed that it is a potent and safe drug against HCV. An interferon-free-regimen study (INFORM-Sustained Virological Responders [SVR] study) showed that SVR12 (undetectable HCV RNA at week 12 after stopping the treatment) was achieved in 71 % of HCV genotype 1b patients. These patients were treated for just 24 weeks with ritonavir-boosted danoprevir, mericitabine and ribavirin. This compound is also under study to evaluate its efficacy when combined with the nucleoside-based polymerase inhibitor RG7128 with and without pegylated interferon and ribavirin [65].

*Vaniprevir*

Vaniprevir/MK-7009 demonstrated inhibitory effects against HCV NS3/4A proteases. MK-7009 possesses excellent selectivity against a series of human proteases and other enzymes. It was shown to have an inhibitory effect against genotypes 1 and 2 ($K_i = 0.07$ and 0.06 nM, respectively). It is moderately less potent against genotype 2a and 2b. Cell-based replicon assays of interferon alpha 2b and MK-7009 have revealed that these two compounds exert a synergetic effect. Moreover, the combination of MK-0608 and MK-7009 also demonstrated additive effects. MK-0608 is a nucleoside analog inhibitor of the HCV polymerase [66]. MK-7009 is presently being assessed in phase II of clinical trials. These studies showed that its administration with PEG-INF/RBV is safe and tolerable. The study was conducted on HCV genotype 1 patients ($n = 94$) and demonstrated that the patients receiving therapy along with vaniprevir showed a significant SVR with no severe side effects, although in some patients, vomiting activity was increased, but it was not severe. The resistance profile was predictable with variant R155 and D168. There was no association of this drug with IL-28B SNPs [67].

*Boceprevir*

A ketoamide inhibitor, SCH503034 (boceprevir), showed time-dependent inhibition of the NS3 protease. Boceprevir is a linear peptidomimetic NS3 inhibitor. It reversibly forms a covalent bond with NS3 ($K_i$ of 14 nM). Boceprevir monotherapy is not recommended because it leads to the emergence of viral resistance within a week. Therefore, its combimation with peginterferon and ribavirin is necessary [68, 69]. Four tablets of boceprevir are given, each as a 200-mg capsule, every 7-9 hours along with the optimal dose of peginterferon and ribavirin. It has been shown that there was a more than 4-log reduction in the replicon RNA level when replicon-bearing cell lines were exposed to six times the 90 % EC of SCH 503034 for 15 days [70]. Overall, phase III studies of boceprevir have shown that it is a very effective addition to PEG-INF/RBV, leading to its approval by the FDA (Food and Drug Administration) in 2011. According to published reports, the response rate to interferon therapy is lower in African-American patients, and therefore black and nonblack patients were analyzed as two different cohorts in the SPRINT 2 (serine protease inhibitor therapy 2) study. Non-black patients achieved 27 %-28 % higher SVR, while black patients achieved 19 %-30 % improved SVR [71].

Initially boceprevir was given only with PEG-IFN α-2b, but now it has been shown to similarly effective with PEG-IFN α-2a. Thus, both PEG-IFNs can be used along with boceprevir [72].

*Telaprevir*

Telaprevir, or VX-950, is a linear peptidomimetic NS3/4A inhibitor. It possesses an α-ketoamide group serine trap warhead. It forms a covalent but reversible complex with a steady-state inhibition constant ($K_i$) of 7 nM against NS3. Monotherapy with telaprevir is also not recommended due to the quick emergence of viral resistance. Two capsules of telaprevir were given to patients; each 375-mg capsule was given with food along with the combination of PEG-IFN/RBV every 7-9 hours. It was administered for a maximum of 12 weeks in phase III of clinical trials because longer treatment was associated with severe side effects [73]. The $K_i$ is 4- to 7-fold and 40-fold higher for genotype 2 and 3,

respectively, indicating that its potential therapeutic use would require genotype optimization. VX-950 showed antiviral activity against HCV genotype 1 at sub-micromolar concentrations. Two large phase III studies (ADVANCE and ILLUMINATE) of treatment-naïve genotype 1 patients demonstrated that the response rate to triple therapy (PEG-IFN/RBV/TLV) was higher than to double therapy (PEG-IFN/RBV). Moreover, response-guided therapy (RGT) for triple therapy was also possible [74–76]. This triple therapy for HCV infection is approved by the FDA in 2011. The relapse rate in HCV patients with liver cirrhosis was higher, and these patients also showed shorter treatment duration. Therefore, RGT triple therapy for HCV infection is approved only for patients who do not have liver cirrhosis. Moreover, measurement of HCV RNA at week 4 may also indicate whether triple therapy should be given or not [75]. Retreatment of relapse patients with PEG-IFN/RBV showed that SVR was achieved in 24 %-34 % of the patients. However, triple therapy (PEG-IFN/RBV/PI) increased SVR 69 % to 80 %. Thus, relapse patients are ideal patients for this triple therapy [77].

## NS3 Helicase inhibitors

HCV NS3 helicase inhibitors are relatively non-toxic to cells and inhibit HCV replication. Recently, it has been found that the commercial dye thioflavine S is the most important inhibitor of NS3 helicase activity. Thioflavine S is a mixture of compounds. When the dye was separated into its components, it was found that the mixture contained two major inhibitors of HCV: P4 and compound 17. Compound P4 was found to be a benzothiazole tetramer, and it inhibited more than 50 % of the helicase activity at $2 \pm 1$ μM, while compound 17 showed more than 50 % helicase inhibition activity at $2.6 \pm 1$ μM [78].

### *Manoalide (1)*

Manoalide (1) is a compound that is extracted from a marine sponge. It inhibits HCV NS3 helicase and ATPase activity with $IC_{50}$ values of 15 and 70 μM, respectively. As the apparent $K_m$ value of NS3 is not changed by this compound, so it is most likely that it acts as a noncompetitive inhibitor. It has also been demonstrated that manoalide (1) inhibits the ATPase activity of DHX36/RHAU, which is a presumed RNA helicase. Thus, manolide (1) effectively inhibits HCV NS3 ATPase, helicase and RNA-binding activities [79].

## NS4A

HCV NS4A is a protein of 54 amino acids whose main function is to act as a cofactor for NS3 protease. The N-terminal part of NS4A has also role in targeting NS3 to the ER [80]. Other important functions of NS4A involve its role in NS5A phosphorylation. Deletion analysis of NS5A revealed that amino acid residues from 2135 to 2139 were necessary for phosphorylation by NS4A [81].

### *ACH-806*

ACH-806 [1-(4-pentyloxy-3-trifluoromethylphenyl)-3-(pyridine-3-carbonyl) thiourea] is one of the most important acylthiourea compounds. It is an HCV NS4A antagonist. Its $EC_{50}$ values in a genotype 1a and genotype 1b replicon system were 30 and 14 nM, respectively. This compound selectively binds to the NS4A protein and changes the protein composition to inactivate the replication complex. In the HCV genotype 1b replicon system, it was demonstrated that this compound acts synergistically with other smaller compounds. ACH-806-resistant mutants have also been found, and these mutations were present in the region of NS3 that interacts with NS4A. It is noteworthy that *in vitro* studies of ACH-806 have shown no cross-resistance with other NS3 PIs such as VX-950 [82].

## NS4B

NS4B is a 27-kDa membrane protein. Its main function is its role in the formation of the membranous web for HCV replication, which serves as a scaffold for the assembly of the replication complex. It also possesses RNA-binding and NTPase activities in addition to anti-apoptotic properties [83].

On the basis of known functions of NS4B, its inhibitors are broadly classified into two major classes. The first class includes inhibitors that interact directly with HCV RNA, while the other class includes inhibitors that interact with membranes. Most importantly, the drugs of these two separate classes that target individual functions of NS4A are synergetic. Recently, it was discovered that NS4A also possesses the ability to hydrolyze ATP, so this function may also be utilized to develop inhibitors against this protein [83].

### *Clemizole hydrochloride*

Molecular screening for inhibitors against NS4B:RNA binding has revealed that clemizole hydrochloride is a strong inhibitor, with an $IC_{50}$ of $\sim$ 24 nM. *In vitro* studies of clemizole have shown that it inhibits HCV replication at non-cytotoxic concentrations. HCV mutants with resistance against clemizole hydrochloride have also been isolated. When the HCV genome was sequenced from these resistant cells, it was discovered that these mutations mapped to either the 3' terminus of the negative-stranded

RNA or NS4B [84]. Clemizole has also demonstrated a strong synergetic effect with other important NS3 protease inhibitors. Its degree of additive effect is higher than any other combinational therapy for HCV [85].

*Anguizole*

The anguizole is an important inhibitor of HCV RNA replication. It has low toxicity. It has been demonstrated that the second N-terminal amphipathic helix (AH2) of NS4B interacts with anguizole. This compound can disrupt the subcellular localization of membrane-associated foci (MAF). Anguizole is one of the first pharmacological compounds that affect the subcellular localization of NS4B [86].

*Inhibition by the small compounds C4 and A2*

NS4B has four transmembrane domains [87]. The N-terminal amphipathic helix 2 domain is the most important for oligomerization of NS4B and aggregation of lipid vesicles. This domain is termed NS4BAH2, consisting of 43 to 65 amino acids. This domain is amphipathic in nature, and this property is also essential for vesicle aggregation. Point mutations in this sequence lead to failure of vesicle aggregation and ultimately to the failure of viral replication. Most importantly, this domain is conserved among all reported HCV genotypes. This domain is inhibited by two compounds, C4 and A2, leading to the inhibition of virus replication. Compound C4 is effective against both genotype 1b and 2a, while A2 is effective against genotype 1b but not against 2a. This suggests a difference in their mechanism of action. It has been shown that C4 disrupts the oligomerization of NS4BAH2 peptides, while A4 prevents the interaction of oligomers with membranes [88].

NS5A

The HCV-NS5A protein is essential for viral replication, as it is the part of membrane-associated replication complex (RC). During HCV replication, a membranous web is formed by membrane vesicles that are partly derived from ER. The mechanism of assembly of the RC is not understood. It is thought that the NS5A protein plays a significant part, but its exact role in this process is still uncertain [89].

*BMS-790052*

BMS-790052 has recently been recognized as the compound that has demonstrated the most *in vitro* potency of any recognized anti-HCV compound. It exhibits significant inhibitory effects against various HCV genotypes [90]. Phase II studies of this compound have indicated that it increases the SVR24 of PEG-INF/RBV from 25 % to 83 % when used in combination with PEG-INF/RBV in HCV genotype 1 patients. Moreover, adverse effects were noted for only one patient (8.3 %) in the triple therapy group when compared to the control group [91]. This compound is presently in the phase III stage of development. The mode of action of this anti-HCV compound is obscure. Mutational analysis of NS5A has shown that the first 76 amino acids are significant in determining a replicon's susceptibility to inhibitors [92]. It exerts its antiviral effects by disturbing the efficient assembly of the replication complex. The biochemical fractionation and subcellular localization of NS5A are also altered by BMS-790052, leading to the suppression of viral replication [93].

*BMS 824393*

Compounds possessing a thiazolidinone core structure also inhibit HCV replication. BMS-824393 is one such compound, and it exhibits 50 % inhibition of HCV replicon replication at 5 nM. Its therapeutic index is $> 10{,}000$, and it displays good specificity. BMS-824393-resistant replicon cells have also been identified. Point mutations in NS5A have been found that confer resistance to BMS-824393. A leucine-to-valine substitution at residue 31 (L31V) and a, glutamine-to-leucine substitution at residue 54 (Q54L) in NS5A conferred resistance to this compound. The N-terminus of NS5A showed sensitivity to this inhibitor. As NS5A interacts with RNA, it is more likely that the BMS-824393 series may also influence NS5A binding to RNA [92].

*GS-5885*

GS-5885, also known as ledipasvir, was developed by Gilead Sciences. It is an NS5A inhibitor. Phase II studies of GS-5885 demonstrated that all 25 treatment-naïve HCV genotype 1 patients achieved SVR4. These patients were given interferon-free triple therapy with GS-5885, nucleotide sofosbuvir, and ribavirin. The drug was well tolerated and was reported to have no severe side effects in any of the patients. This drug is currently undergoing a phase III clinical trial in which 800 treatment-naïve HCV genotype 1 patients from different parts of the world will be enrolled. These patients will be given combination therapy of GS-5885 and sofosbuvir with or without ribavirin [94].

*GSK2336805*

GSK2336805 is an inhibitor of NS5A. Phase 1 single- and repeat-dose studies of this compound on chronic hepatitis C patients with genotype 1 proved its tolerability with no

serious side effects. The compound is currently undergoing phase II of clinical trials in which treatment-naïve patients with genotypes 1 and 4 are enrolled to evaluate its safety, tolerability, efficacy and pharmacokinetics at a dose of 60 mg, in combination with pegylated interferon alpha-2a (PEG-IFN α-2a) and ribavirin (RIBA) [95].

*PPI-668*

Presidio-668 is another important HCV NS5A inhibitor that has successfully completed phase 1 of clinical trials in healthy subjects and HCV patients infected with genotype 1. Recently, two companies, Presidio and Boehringer Ingelheim, have signed a collaboration agreement to combine their anti-HCV drugs for phase II studies. These combined clinical trials will be based on triple therapy with PPI-668 (NS5A inhibitor), faldaprevir, BI201335 (protease inhibitor), and BI207127 (polymerase inhibitor) with or without ribavirin [96].

NS5B

HCV NS5B is an RNA-dependent RNA polymerase (RdRP). It is also an essential part of the HCV replication complex. It initiates and catalyzes RNA synthesis [97]. The structure of NS5B resembles a right hand. For ease of understanding, its structure is divided into three domains, finger, palm and thumb. The enzyme active site is located inside the palm, which also contains the highly conserved GDD motif [98]. It also contains aspartic acid residues that are responsible for binding nucleotides during the process of oligomerization [99].

In humans, there is no homolog of NS5B, which has a very important role in viral infectivity [100] and is therefore an important candidate for the development of antiviral compounds. NS5B inhibitors can be classified into two major groups: nucleoside analogs and non-nucleoside inhibitors (NNIs). Nucleoside analogs act as substitute substrates for NS5B polymerase, while non-nucleoside inhibitors bind to the allosteric region of NS5B. The major problem associated with NNIs is their significantly different activity against various genotypes and even subtypes of HCV [9]. In contrast, the activities of nucleoside analogs are approximately the same across all HCV genotypes [101]. The major shortcoming associated with these nucleoside inhibitors is the high level of resistance [102]. Until now, only two amino acid changes have been identified in the NS5B gene that confer resistance to nucleoside inhibitors: S96Tand S282T [103]. Promising results have been documented for NS5B polymerase inhibitors in clinical trials [104], and NS5B is considered an excellent candidate for drug discovery against HCV.

*PSI-7851*

PSI-7851 is a pronucleotide inhibitor of HCV NS5B. Its structure is β-D-2′-deoxy-2′-fluoro-2′-C-methyluridine-5′-monophosphate. It is a very active nucleotide analog with a significant $EC_{50}$ value against the genotype 1b replicon. Its equivalent efficacy against other genotypes of HCV has also been established. Its inhibitory effect is also specific for HCV. There has been no mitochondrial toxicity or cytotoxicity linked with this compound. Studies of cross-resistance demonstrated that S282T replicon mutants showed resistance, while S96T/N142T replicon mutants remained susceptible to the inhibitory effect of this compound [105].

*INX-08189*

INX-08189 is a phosphoramidate of O-6-methyl-2′-C-methyl guanosine. In primary human hepatocytes, this compound efficiently produces triphosphates, which has been carried out using performed against HCV genotypes 1a, 1b, and 2a in a cell-based replicon system, with $EC_{50}$ values of 12, 10, and 0.9 nM, respectively. These values indicated that among the nucleoside-based inhibitors of NS5B, INX-08189 is the most important. Such a high level of effectiveness has been shown previously only with protease and non-nucleoside polymerase inhibitors [106]. Exposure of genotype 1b replicon-containing cells to this compound for only 24 h resulted in an $EC_{50}$ of 35 nM, suggesting rapid kinetics of antiviral activity [107].

Non-nucleoside inhibitors

*ABT-072*

ABT-072 is a non-nucleoside inhibitor of HCV NS5B. Phase II studies of this compound showed positive results. It was shown that 91 % of HCV G1 patients who received ABT-450/r and ABT-072 plus ribavirin for 12 weeks demonstrated SVR at week 24, while 82 % of patients showed SVR at week 36. There were also no severe side effects of the treatment [108].

*Oleanolic acid and ursolic acid*

HCV replication was inhibited by aqueous extract of a natural product, Fructus Ligustri Lucidi (FLL) [109]. Further studies using HPLC and inhibitory assays established that the most active component of FLL extracts were oleanolic and ursolic acids. These compounds predominately suppressed HCV replication by acting as non-competitive inhibitors of HCV RNA-dependent RNA polymerase. They at least partly suppressed its activity [110]. Thus, these two

compounds may also progress to clinical trials. There are more chances for these two compounds to be used as a therapy, because like most of the other natural products, they are likely to be well tolerated with minimal side effects.

## Targeting host factors

A large number of host factors also play an important role in the HCV life cycle. They not only have a role in species specificity but also contribute to many of the inter-individual differences that are observed in the development of HCV disease. Therefore, recent efforts have been focused on the identification of these factors. Blocking their access to HCV may provide a new tool for treatment of the life-threatening disease caused by this virus. Many new techniques, such as high-throughput assays, have been established in recent years to identify host factors that play key roles in viral diseases [111].

### Eukaryotic initiation factor 2-alpha phosphatase inhibitor

HCV causes ER stress, leading to the initiation of the unfolded protein response (UPR). The UPR in turn leads to the activation of autophagy. According to one school of thought, autophagy is important for virus replication. *In vitro* studies using the Huh-7 cell line have shown that when phenomenon of autophagy was blocked by treatment with 3-methyladenine (3-MA), an inhibitor of autophagy, HCV replication was also blocked. Some other inhibitors, such as salubrinal, 3,5-dibromosalicyladehyde and sp600125, which are inhibitors of eukaryotic initiation factor 2(eIF2)-alpha phosphatase, X-box binding protein-1 (XBP-1), and c-Jun N-terminal kinases (JNK), respectively, also inhibited HCV replication. This study was conducted in an *in vitro* model of HCV infection, and the results need to be confirmed in an *in vivo* model before going to clinical trials. It would open new doors for therapy of HCV infection [112].

### Cyclophilin A

Cyclophilin A is a chaperone that is required for HCV replication. Cyclophilins or Cyps exhibit a peptidyl-prolylcis-trans isomerase activity (PPIase). It has been reported that both CypA and NS5B interact with NS5A and a shared a common binding region on NS5A that ranges from residues Pro-306 to Glu-323 [113]. Cyclosporin A (CsA) is a compound possessing binding affinity for CypA, and its molecular target is calcineurin. *In vitro* studies have explored a number of point mutations in NS5A and NS5B that are linked to resistance to CsA [114]. Both *in vitro* and *in vivo* studies have revealed that CsA exerts anti-HCV activity [111]. Several CsA-analogues, i.e., NIM811 [115], DEBIO-025 and SCY-635 [116], are presently in preclinical and clinical stages of development. These CsA analogues preserve their affinity for binding with Cyp but do not impede calcineurin. DEBIO-025, or alisporivir, or DEB025, is an oral agent that shows potential in ongoing hepatitis clinical trials. It is currently in the phase III stage of its development [117].

### Epidermal growth factor receptor and ephrin receptor A2

The screening of a large panel of functional RNAi kinases revealed that epidermal growth factor receptor and ephrin receptor A2 are the two most important host cofactors with a role in HCV entry. Studies in cell culture and in a human liver chimeric mouse model demonstrated that HCV infection is impaired by blocking receptor kinase activity. Thus, tyrosine kinase inhibitors have significant antiviral activity and may play a role in a novel powerful approach for the treatment of HCV infection [118].

### Albinterferon alfa-2B

Albinterferon alfa-2b, also known as zalbin, is an important immunomodulator. It is the combination of interferon alpha-2b and albumin. Albumin is a blood protein that is abundant in circulating blood. It persists in blood circulation for about 19 days. Fusion of interferon alpha 2-b with albumin increases its half-life. Zalbine dosed once after two weeks has reached phase III of clinical development. The study was conducted on treatment-naïve HCV genotype 1, 2 and 3 patients, and it showed that a half dose of zalbin along with ribavirin is sufficient to achieve the same SVR rate as achieved by PEG-INF in combination with ribavirin. Moreover, there are no additional side effects associated with Zalbin as compared to peg-interferon treatment [119].

### Nitazoxanide

Nitazoxanide is another important antiviral compound. It is more commonly used as an antiprotozoal compound, and it is licensed for the treatment of *Cryptosporidium parvum* and *Giardia lamblia* infections in the United States of America. It was accidently discovered that it possesses anti-HCV activity during treatment of a patient with cryptosporidiosis, HCV and HIV. This compound activates a key antiviral enzyme, PKR (protein kinase activated by dsRNA) [120]. Clinical studies from Egypt showed that HCV RNA was undetectable in 7 out of 30 HCVG4

(genotype 4) patients [121]. Another study from the same country showed that 96 HCVG4 patients were divided into two groups. One received triple therapy (NTZ/PEG-IFN/RBV) while the other group received double therapy (PEG-IFN/RBV). The SVR rate in the triple-therapy group was 79 %, while the SVR rate in the double-therapy group was 50 %. Although the results were promising, the dataset was small, and further investigation is needed. This compound is currently in phase III of clinical trials [122].

### Zadaxin

Zadxin is an immunomodulator. It is the synthetic form of thymosin alpha. Thymosin alpha is present in blood circulation and is also produced by the thymus. Thymosin alpha-1 (Tα-1) is a peptide of 28 amino acids that plays key role in maturation of lymphocytes [123]. Zadaxin has been used against various types of cancers and also against HBV and HCV. It has no significant side effects and possesses good tolerability. Initially, SVR rates of HCV patients treated with PEG-IFN, ribavirin, and either thymosin alpha or placebo were the same, but among the patients who completed 48 weeks of therapy, the SVR rate in the thymosin alpha-1 group was 41.0 % (34/83) as compared to 26.3 % (26/99) in the placebo group ($P = 0.048$). This study indicated that thymosin alpha-1 has no role in primary therapy and it might act as an adjuvant and have a role in secondary therapy. Zadaxin is currently in phase III of clinical study [124].

### Sequestrating cellular micro RNAs

MicroRNAs are smaller RNA molecules that do not code for any protein but regulate the expression of different genes. These RNA molecules act post-transcriptionally and exert important effects on the life cycles of pathogens. The most important microRNA in the liver is microRNA 122 (MiR-122) [125]. There are three reported important binding sites in the HCV genome for miR-122: one in the 3′ untranslated region (3′ UTR) and two in the 5′ untranslated region (5′ UTR). MiR-122 binds to sequences in the 5′UTR of HCV RNA, i.e., S1 and S2, resulting in the up-regulation of viral RNA levels [126]. RNA oligonucleotides complementary to miR-122 have been demonstrated to sequester miR-122, leading to a considerable decrease in HCV RNA accumulation. This observation emphasized miR-122 as a potential therapeutic target. Locked nucleic acid (LNA) technology has been introduced in order to develop an effective approach for miR-122 targeting. LNA-modified nucleosides are basically nucleic acid analogues [127]. When incorporated into a DNA oligonucleotide, they enhance the speed and stability of duplex formation. Phosphorothioate modifications are other modifications that provide good pharmacokinetic and tissue-uptake properties. Thus, LNA-modified phosphorothioate oligonucleotide (SPC3649) complementary to the 5′ end of miR-122 provides a novel method for the treatment of HCV infection, and it was tested in chronically HCV-infected chimpanzees [128]. Treatment with high doses of SPC3649 caused a decrease of 2.3 log10 in HCV RNA levels in the liver of the animals. Moreover, no viral resistance to SPC3649 treatment has been noted. Thus, SPC3649, which blocks miR-122, offers a very potent anti-HCV treatment with no side effects or escape mutations.

## Future perspectives

Although PEG-INF/RBV/protease inhibitor is considered to be the SOC therapy for HCV infection, there is still a substantial need for alternative treatment options because the current treatment is mainly dependent on HCV genotype and also associated with a number of serious side effects. In the recent past few years, new drugs have been discovered that are used in combination with IFN/RBV. However, the addition of other treatment options to SOC therapy resulted in new side effects that were not associated with interferon therapy. The ultimate goal of antiviral therapy is to search for a cure without the involvement of interferon. Different strategies are in use to cure HCV infection, but no single therapy is perfect against HCV. It is expected that in coming few years HCV therapy will be based only on orally available DAA agents without the involvement of interferon therapy.

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