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Chapter 10

Tissue-Based Model of HCV Replication as a Replacement for Animal Models in Drug Testing

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Additional information is available at the end of the chapter

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1. Introduction

It is estimated that hepatitis C virus (HCV) has infected at least 170 million people worldwide [1]. More than 80% of infected patients develop the chronic infection, which leads to serious liver diseases, such as liver cirrhosis and hepatocellular carcinoma [2,3]. Chronic HCV infections may proceed with asymptomatic or with non-specific symptoms (fatigue, depression) for many years. Despite increasing knowledge about the virus pathogenesis, there is still no vaccine and successful antiviral therapy. The current standard of chronic hepatitis C therapy based on pegylated IFN-α 2a (PegIFN-α) and ribavirin (RBV) has limited efficacy and undesirable side effects. There is an urgent need for more effective therapies.

HCV is classified as a member of the Flaviviridae family and serves as a sole member of genus Hepacivirus. The genome of this enveloped virus consists of a single-stranded positive-sense RNA of approximately 9.6kb, which contains an open reading frame (ORF) encoding a polyprotein precursor of around 3000 amino acids (Figure 1). This single, large ORF is flanked by well conserved 5′ and 3′ untranslated regions (UTRs) [4]. The HCV 5′ UTR is a highly structured element, which includes internal ribosome entry site (IRES), a fragment required for genome translation [5,6]. The 3′ UTR contains short variable region, poly(U/UC) tract and X-tail region. The X-tail region forms highly conserved three stable stem-loop structures, which together with the poly(U/UC) are crucial for RNA replication [7,8]. Besides viral UTRs which are significant for translation and RNA replication, rest of the genome is diverse among several HCV isolates. According to genome differences, HCV isolates are divided into six particular genotypes, that differ in their nucleotide sequences by 31-34% [9]. Infections with genotype 1 are the most prevalent and dangerous, leading to liver injury and hepatocellular carcinoma [10-12]. Patients infected with genotypes 1, 4, 5 and 6 respond to treatment less effectively than patients infected with genotypes 2 and 3 [12,13].

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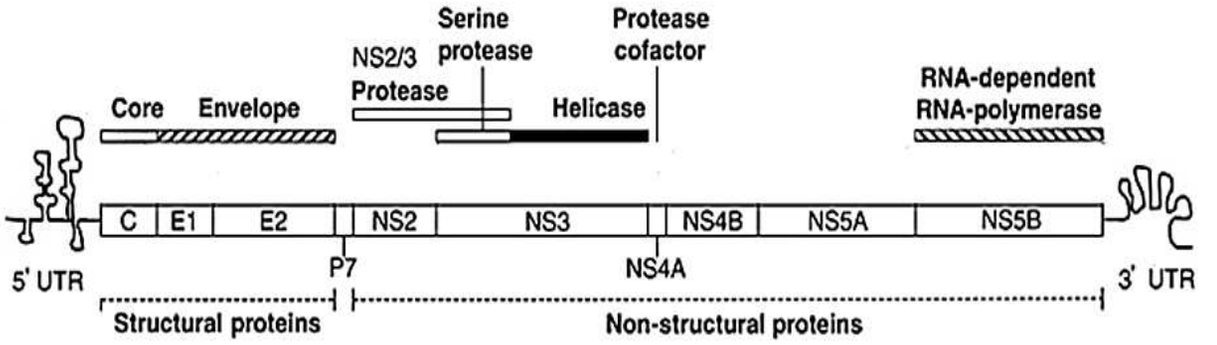

Figure 1. Genome organization of HCV

The HCV polyprotein precursor is co- and post-translationally processed into 10 proteins, that are divided into two groups, structural proteins: core protein, two glycoproteins (E1, E2), p7; and non-structural proteins: NS2, NS3, NS4A, NS4B, NS5A and NS5B. The HCV core protein forms viral nucleocapsid and also regulates cellular and viral gene expression, cell transformation and apoptosis by the interaction with cellular proteins and signaling pathways [14,15]. The two glycoproteins, E1 and E2, are essential components of the virion envelope and necessary for viral entry [16]. E2 plays a crucial role in attachment of the virus to a cell, by the interaction with host receptor – CD81 [17,18]. The p7 protein is a small polypeptide that forms an ion channel, suggesting that it belongs to the viroporin family [19,20]. The NS2 protein is a transmembrane protein, which together with amino-terminal domain of the NS3 protein create NS2-3 protease that cleaves the site between NS2 and NS3 during processing of the polyprotein [21]. The NS3 and NS4A (NS3-NS4A) built a multi-functional complex essential for viral polyprotein processing and RNA replication. The NS4A protein is a cofactor of NS3 protease activity and catalyzes polyprotein cleavage. The 442 C-terminal amino acids of the NS3 is a helicase that plays a crucial role in RNA replication [22]. The NS4B is an integral membrane protein that serves as a membrane anchor for the replication complex [23]. The NS5A protein is a membrane-anchored phosphoprotein, important in viral replication, but exact function of this protein is not known [22]. The last protein, NS5B is a viral RNA-dependent RNA polymerase which is crucial for RNA replication.

The first step of the HCV life cycle is attachment of the virus to a cell via E2 and cell receptor interaction [Figure 2]. CD81 has been the most extensively studied as a putative HCV receptor [17]. Several cell surface molecules, like: scavenger receptor B type I (SR-BI), low-density lipoprotein receptor (LDL-R) or asialoglycoprotein receptor (ASGP-R) have been also proposed to mediate HCV binding [24-27]. HCV entry into cells by pH-dependent endocytosis, but the mechanism of HCV fusion remains controversial [27,28]. Released viral RNA into the cytoplasm of infected cell serves directly as messenger RNA in an internal ribosome entry site-directed translation of the HCV polyprotein. The precursor polyprotein, which is targeted to the ER membrane is processed by cellular and viral proteases into 10 proteins. HCV replication starts with synthesis of complemantary negative-strand RNA which then serves as a template for production of numerous positive-strand genomic RNAs,

both steps are catalyzed by the NS5B RNA-dependent RNA polymerase (RdRp).These RNAs serve as mRNA in translation, as a template for synthesis more negative strands and as substrates for viral assembly. Little is known about HCV assembly and release. It is suggested that core protein is sufficient for viral assembly and its interaction with RNA may play a role in switching from RNA replication to packaging [29,30]. After viral RNA association with core, viral nucleocapsids are formed and bud into the ER. Newly produced virions may leave the cell through the constitutive secretory pathway [28].

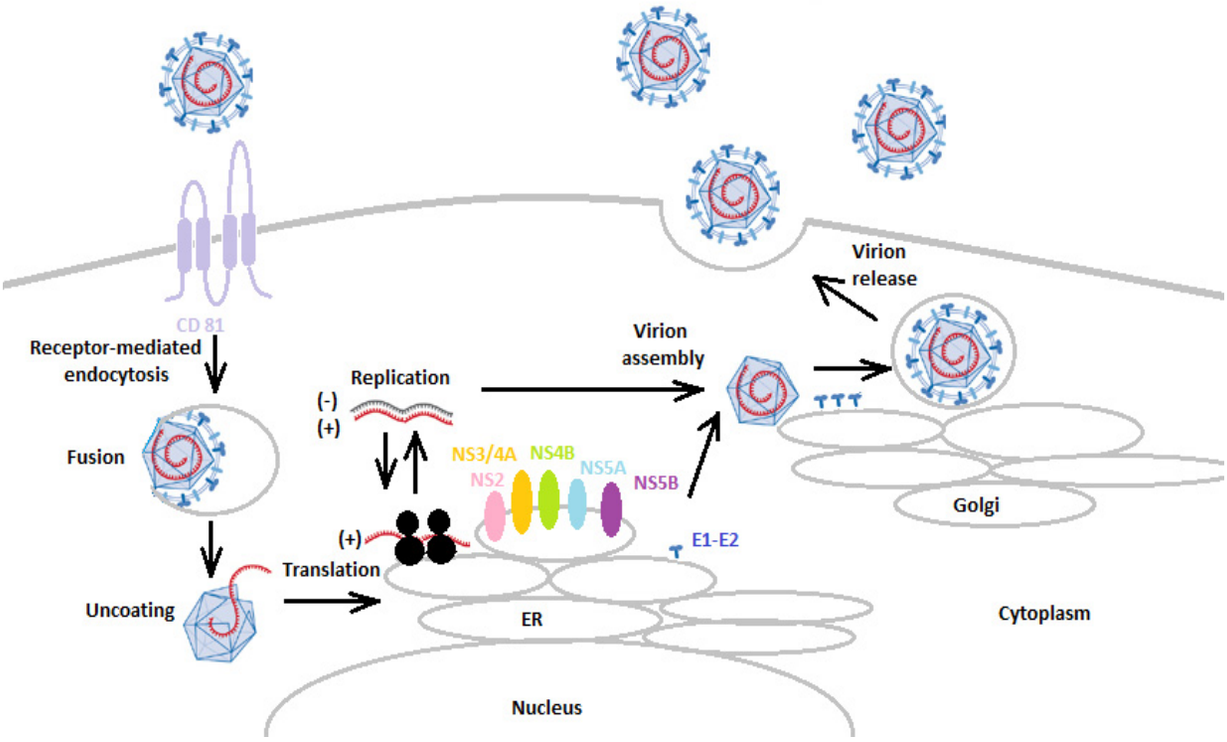

Figure 2. HCV life cycle

2. Models of HCV study

The current standard of HCV therapy that relay on combination with pegylated IFN-α 2a and ribavirin yield limited success rates and reach about 40-80%, depending on HCV genotype, viremia, age and gender of the patients [31-33]. These facts and the large number of infected individuals stress the pressing need for the development of improved antiviral strategies. Understanding of the viral life cycle is crucial to identify effective antiviral agents. The lack of small animal models for HCV hamper studies on viral replication.

2.1. Animal models

The chimpanzee is the only animal that can be infected with HCV. Unfortunately, this model is limited by restricted availability of animals, ethical dilemmas and high cost. Recently, other models have been used to study different aspects of HCV biology and novel antiviral drugs. Researchers had created severe combined immunodeficient (SCID) mice

with chimeric livers composed of human and murine hepatocytes that support robust HCV replication [34]. This model was used to demonstrate the antiviral activity of the HCV NS3/4A protease inhibitor BILN-2061. Reduction of the viral load in genotype 1b infected mice after protease inhibitor treatment was similar to results obtained in human clinical trials [35]. Unfortunately, these chimeric mice are not suitable for evaluation of immunotherapeutic agents and vaccines, because of the lack of functional immune system [34].

2.2. Tissue culture

The primary host cell supporting HCV replication is the hepatocyte. HCV replication has been detected in hepatoma, B- and T-cell lines, primary cultures of human or chimpanzee hepatocytes and peripheral blood mononuclear cells. However, the replication levels are very low and do not allow to study HCV replication in detail [36].

Since HCV discovery in 1989, intensive research to create tissue-based model of HCV replication begun. In the late 1990s few independent groups of researchers had developed the first *in vitro* hepatitis C virus replication system based on viral cDNA [37,38]. The HCV genome (called "H77") isolated from patients infected with genotype 1a was used to create plasmid containing full HCV genome transcripted to cDNA. Plasmids containing the full-length HCV cDNA were linearized with *XbaI* and then used as a template in *in vitro* transcription. Transcripted RNAs were infectious, when injected directly into chimpanzees liver [38]. These plasmids were adapted at H77 5′ terminus with the T7 promoter and at 3′ terminus with the hepatitis delta cis-acting ribozyme in continuity with T7 terminator sequences. The vectors were used in transfection of HepG2 and CV-1 cell line. The HCV genomic RNA was generated by T7 RNA polymerase that was provided by infection with recombinant vaccinia virus (vTF7-3) (Figure 3.) [39]. This system has experimental limitations, because of cytopathic and pleiotropic effects of vaccinia virus infection [40].

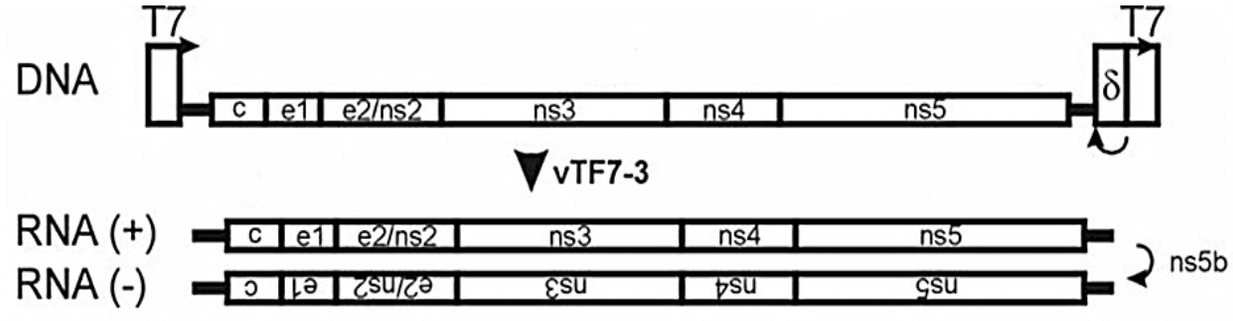

Figure 3. Construction of vectors used for the binary HCV replication system (according to [39], modified)

2.2.1. Development of HCV replicons

According to the low level of HCV replication obtained in full-length cDNA systems, researchers tried to create subgenomic replicon systems. In 1999 the first subgenomic

replicon system, which allowed HCV replication in the human hepatoma cell line Huh-7 was established. Viral RNA was isolated from the liver of the patient chronically infected with genotype 1b (Con1). These bicistronic replicons consist of 5′ HCV IRES, neomycin phosphatransferase gene (as a selectable marker), encephalomyocarditis virus (EMCV) IRES and the HCV nonstructural genes from NS2 or NS3 up to NS5B. Translation of the marker was under control of HCV IRES and translation of the HCV polyprotein was under control of EMCV IRES. As a negative control, a defective genome carrying an in-frame 10-amino acid deletion in the NS5B active site was generated (Figure 4).

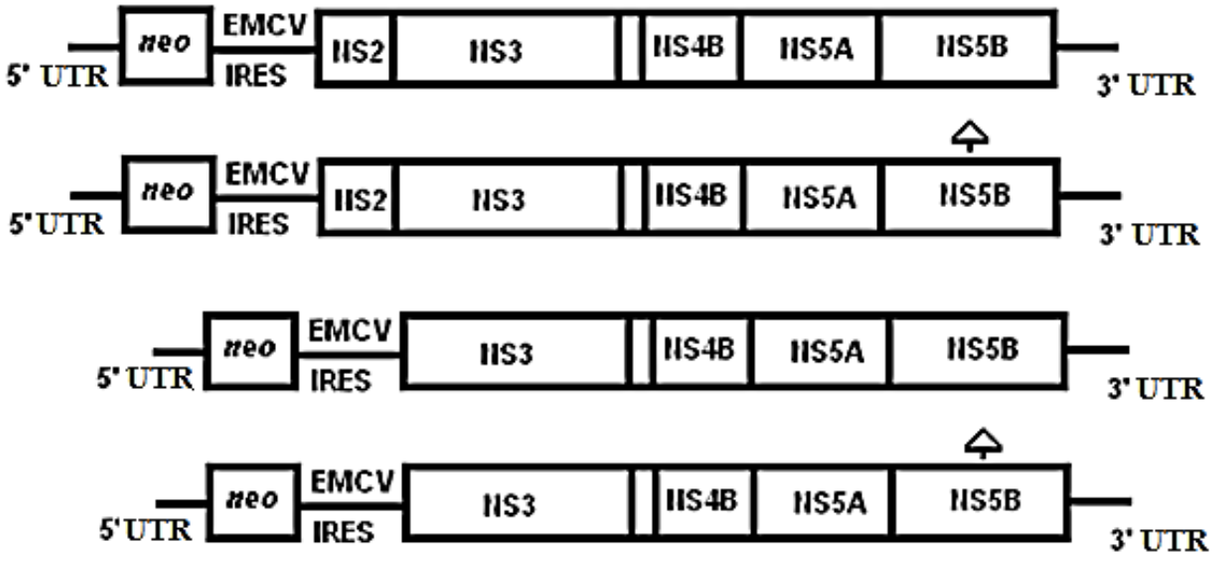

Figure 4. Structure of the HCV subgenomic replicons. The structure of the replicons composed of 5′ HCV UTR (Untranslated Region), *neo* (neomycin phosphatransferase gene), encephalomyocarditis virus (EMCV) IRES, the HCV nonstructural genes from NS2 or NS3 up to NS5B and 3′ HCV UTR. Δ indicates the position of 10-amino acid deletion in NS5B.

Although transfection of Huh-7 cells with transcripts synthetized *in vitro* and selection with neomycin resulted in a low number of surviving cell colonies, neomycin-resistant cell colonies harbored 1000-5000 copies of positive-sense HCV RNA per cell, which gave evidence of high level replication in transfected cells. To exclude the possibility that resistance was due to plasmid DNA integrated into the host cell genome, DNA of each clone was analyzed by *neo*-specific PCR [41]. Low frequency of transfected cells may indicate that replicon RNAs acquire adaptive mutations to effectively replicate in the Huh-7 cell line or only a low number of cells in the culture support efficient HCV replication. Analysis of the replicon RNAs confirmed the occurrence of cell culture-adaptive mutations that enhance RNA replication [42,43]. Mutations increasing replication were found in the non-structural coding region, especially in NS4B, NS5A and NS5B. The NS3 mutations had minimal or no impact on replication, but can enhance replication synergistically when combined with adaptive mutations in NS4B, NS5A and NS5B. In the same Huh-7 cell line up to 100-fold differences in their ability to support replicon amplification were found, which may indicate that some cellular factors also might be responsible for the different levels of permissiveness of Huh-7 cells [43].

Although HCV is divided into 6 genotypes, replicons have only been reported for genotypes 1 and 2. The establishment of efficiently replicating replicons based on genotype 1a was more challenging than generating functional genotype 1b subgenomic replicons. The first subgenomic replicons (pH77) containing sequences from genotype 1a were constructed in 2003. The replicons were created analogically to previously characterized 1b replicons, and consisted of 5′ HCV IRES, *neo*, EMCV IRES and the HCV nonstructural genes from NS2 or NS3 up to NS5B. As a positive control, constructs described in [41] were used. Unfortunately, subgenomic replicons pH77 were unable to support stable replication after RNA electroporation into Huh-7 cells with neomycin selection. Replacing first 75 residues of NS3 coding sequence from type 1a with type 1b resulted in replication. The chimeric subgenomic replicons between HCV type 1a and type 1b were able to replicate in Huh-7 cells, albeit with reduced colony formation efficiency and low viral RNA levels [44]. Transfection of highly permissive Huh-7 subline, Huh-7.5 with H77 replicons containing adaptive Ser-to-Ile substitution (S2204I) in NS5A allowed the development of the first colonies supporting H77 replication. The low frequency of cells supporting H77 replication suggested that efficient H77 replication in Huh-7.5 cells may require at least two adaptive mutations. In all cell clones analyzed, replicating RNAs had acquired a second amino acid substitution in the helicase domain of NS3. Both these mutations, when combined with NS5A S2204I, enhanced the colony-forming ability of subgenomic H77 RNA and allowed the detection of HCV RNA after RNA transfection of either subgenomic replicons [45].

The only non-genotype 1 subgenomic replicon capable of efficient replication in cell culture is the genotype 2a clone JFH1 isolated from a patient with fulminant hepatitis. Fulminant viral hepatitis is a serious form of acute hepatitis and is characterized by a broad viral replication in the host and an intensified host immune response against the virus-infected cells. The replicon was constructed according to the method used in [41], then *in vitro* transcripted and transfected into Huh-7 line. The colony-forming ability of the replicon was 60-fold higher than a Con1 (genotype 1b) subgenomic RNA harboring highly adaptive mutations. Clones of this subgenomic replicon replicated without common amino acid mutations. Furthermore, JFH1 subgenomic replicon replicated efficiently without neomycin selection in a transient replication assay. This genotype 2a subgenomic replicon is important for studying the differences in viral characteristics between genotypes 1 and 2 and to understand the mechanisms of viral replication and persistence [46]. In addition, the JFH1 subgenomic replicon produced colonies in a human hepatocyte-derived cell line, and in IMY-N9, a cell line developed by fusing human hepatocytes and HepG2 cells [47]. Replication of JFH1 subgenomic replicon was also shown in two human non-hepatocyte-derived cell lines, HeLa and 293, which provided useful information about HCV replication and cell tropisms [48].

The subgenomic replicon system is a powerful tool that could be used to study HCV RNA replication in tissue-based assays. It also allows to investigate functions of particular HCV proteins, but has its limitations because of no possibility to product viral particles.

2.2.2. Development of an infectious HCV cell culture system

The first robust cell culture model of HCV infection, in which infectious HCV can be produced, was reported in 2005. To create HCV constructs, HCV RNA was isolated from Japanese patient with fulminant hepatitis C (JFH1) [49]. A 32-year-old man was admitted with a 5-day history of general fatigue, fever and acute liver failure. No evidence of previous liver diseases was found. Anti-HCV antibodies were not detected at the time of admission. The titer of serum HCV RNA was 10^5 copies/ml and the genotype of the isolate was 2a. Its sequence slightly deviates from other genotype 2a strains isolated from patients with chronic hepatitis. This strain has 9,678 bp genome and contains a long open reading frame spanning nucleotide 341-9439 and coding 3033 amino acids [50]. Based on the consensus sequence of JFH1, plasmid pJHF1 containing the full-length JFH1 cDNA downstream of the T7 RNA promoter was constructed (Figure 5).

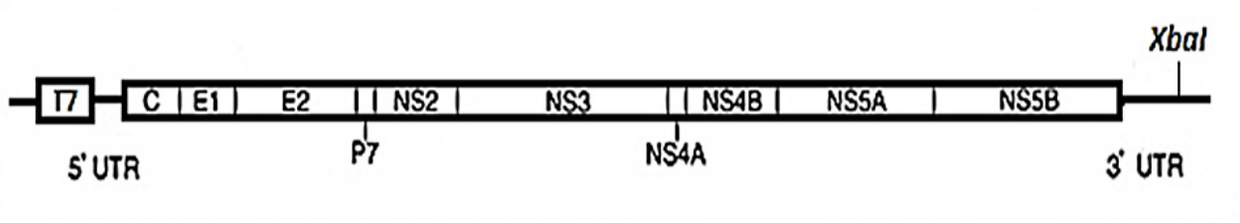

Figure 5. Organization of the full-length HCV construct pJFH1

The plasmids were linearized at the 3′ end of the HCV cDNA by *XbaI* digestion, then *in vitro* transcripted and electroporated to Huh-7. The genome-length RNA was detected in JFH1-transfected cells after 24 h and remained detectable up to 72 h. About 70-80% of the cells were positive for core and nonstructural proteins at 72 h after electroporation, indicating high levels of HCV genome replication in Huh-7 cells. In JFH1-transfected cells the viral RNA concentration in the medium increased rapidly at 5 days after transfection and remained high for the next 7 days, followed by a slow decrease. HCV particles of spherical morphology with an average diameter of about 55 nm were detected in cell medium, which confirms secretion of viral particles after JFH1 transfection into Huh-7. Secreted virus was infectious for naïve Huh-7 cells and for chimpanzee. Although JFH1 virus was infectious for the chimpanzee, infection resulted in transient viraemia and no pathological changes in the liver. Infectivity of viral particles was neutralized by CD81-specific antibodies, confirming specificity of the infection and the important role of CD81 in HCV entry [49].

The JFH1 system described above was limited by low level of infection [49]. Effective viral infection was achieved using cured cell lines such as Huh-7.5 and Huh-7.5.1 [51,52]. These cell lines were obtained by intrerferon treatment of Huh-7 supporting subgenomic replicons. Replicon-containing Huh-7 cells were cured of HCV RNA by initially passing cells twice in the absence of neomycin. Then cells were passaged four times in the presence of IFN-α creating Huh-7.5 line [51]. The Huh-7.5.1 cell line was delivered from Huh-7.5 replicon cell line by culturing 3 weeks in the presence of human IFN-γ to eradicate replicon [52]. Transfection of the JFH1 genome into Huh-7.5 and Huh-7.5.1 cells support high levels of HCV replication in more than 75% of transfected cells. Huh-7.5 line is more permissive for HCV replication than parental Huh-7 [51].

This *in vitro* HCV replication system based on genotype 2a (JFH1) is used worldwide to test the viral life cycle and new antiviral drugs. Its main advantage is markedly enhanced replication efficiency compared with other HCV clones and secretion of virus particles into the cell medium. JFH1 is the only clone without adaptive mutations that is infectious to cultured cells and chimpanzees. The limitation of this method is its restricted adaptation to genotype 2a. The most prevalent and dangerous is infection with genotype 1, which leads to liver damage and hepatocellular carcinoma. It has been difficult to disseminate non-JFH1 HCV strains in cell lines, despite establishment of chimeric viruses. Full-length chimeric genomes were constructed with the use of the core-NS2 gene regions from H77 (genotype 1a) and NS3-NS5B gene regions from JFH1 (genotype 2a). Genotype 1a/2a was able to replicate in Huh-7, but viral particles were not infectious for naïve cells [53]. The JFH1 strain is still the only HCV isolate that can be propagated in Huh-7 cells.

In vitro HCV replication system based on genotype 1a was established in 2006. *In vitro* transcribed full-length HCV RNA from clone H77 was used for transfection of immortalized human hepatocytes (IHH) by electroporation [54]. Human hepatocytes used in this experiment were immortalized by transfection of the HCV core genomic region from genotype 1a [55]. Reverse transcription-PCR of cellular RNA isolated from full-length HCV transfected cells suggested that viral RNA replication appeared. Absence of integrated H77 DNA in IHH genome confirmed HCV genomic RNA replication in the cytoplasm of IHH. The presence of HCV in IHH cell culture medium was detected. Furthermore, virus-like particles were observed in the cytoplasm. HCV infection was also observed after transferring culture media of HCV-replicating cells into naïve IHH [54]. Probably, in IHH cellular defense mechanisms against HCV infection are reduced. Further studies are necessary to test usage of this replication system in novel drug testing.

2.2.3. Cell lines permissive for HCV replication

Hepatoma cell line Huh-7 and its subline Huh-7.5 are the most permissive cell lines for *in vitro* HCV replication identified so far, indicating that a favorable cellular environment exists within these cells. Although adaptive mutations in the HCV NS proteins are required to develop HCV replication at higher frequency. The replication efficiencies of subgenomic RNAs in replication assays can vary by as much as 100-fold between different passages of Huh-7 cells. These differences suggest that effective replication depends on host cell conditions or cellular factors. Replication efficiency decreases with increasing amounts of transfected replicon RNA, indicating that viral RNA or proteins are cytopathic or that host cell factors in Huh7 cells limit RNA replication [43]. Several Huh-7 lines harboring subgenomic HCV replicons were cured of HCV RNA by prolonged treatment with IFN-α. Huh-7.5 is the most permissive cured subline identified so far. The frequency of Huh-7.5 cells able to support HCV replication is approximately three-fold higher than that of the parental Huh-7 cells. Furthermore, more than 75% of the cells that survive the transfection procedure harbor replicating HCV RNAs. The highly permissive subline (Huh-7.5) was obtained from neomycin-selected clones that harbored replicons without adaptive changes in the NS3-5B region [51].

Ihe first *in vitro* non-Huh-7 system of HCV replication was described in 2003. Subgenomic HCV RNAs (Con1) replicated in nonhepatic human epithelial cells. Subgenomic RNA isolated from Huh-7 cell lines that replicate HCV RNA were used in transfection of HeLa cells (cervix carcinoma). Neomycin-resistant cell clones were obtained. Replicons isolated from these cells carried new mutations that could be involved in the control of tropism of the virus [56].

3. Antiviral therapies for chronic hepatitis C

The current standard of treatment of chronic hepatitis C is a combination of pegylated interferon and ribavirin. This therapy leads to 40-50% sustained virological response (SVR) in patients infected with genotype 1, 93% in patients infected with genotype 2, 79% in patients infected with genotype 3 and 69% in patients infected with genotype 4 [29-31]. Among patients infected with genotype 1, only 19-24% treated with PegIFN-α and RBV can achieve rapid virological response (RVR) [57]. Pegylated interferon is associated with numerous side effects: 50% of patients experience flu-like symptoms, 25% psychiatric symptoms, 20% symptoms of fatigue and 10% symptoms of gastritis. The major side effect (36%) of ribavirin is anaemia [58].

Current standard of chronic hepatitis C therapy has limited efficacy and undesirable side effects. There is a pressing need to develop a new antiviral drugs against chronic hepatitis C.

3.1. Inhibitors of hepatitis C virus

An infectious HCV cell culture system is a powerful tool that could be used to study HCV life cycle and function of particular viral proteins. These studies allow to find viral targets for direct-acting antiviral (DDA) drugs and develop Specifically Targeted Antiviral Therapies for HCV (STAT-C). These therapies let to achieve better effectiveness of treatment, its shortening, and the diminishment and limitation of side effects. Current studies are focused on searching the new therapeutic agents for hepatitis C, which are directed against viral proteins. Several HCV inhibitors have reached clinical development, but the most advanced include inhibitors of NS3/4A protease, two of them: boceprevir and telaprevir were approved in 2011 by the Food and Drug Administration (FDA) for the treatment of chronic hepatitis C genotype 1 infection, in combination with PegIFN-α and RBV.

3.1.1. NS3/4A protease inhibitors

The NS3 protease is one of the most attractive targets for developing new therapies against HCV, because of its essential role in viral replication [59,60]. The NS3 inhibitors are divided into two groups, according to different mechanisms of action: covalent and non-covalent inhibitors. Both covalent and non-covalent NS3 protease inhibitors have been developed to bind NS3 active site.

A covalent trap called also ‘warhead’ form covalent reversible or irreversible bonds with serine hydroxyl of NS3 protease catalytic site [61]. Several classes of HCV protease inhibitors

with electrophilic functionality have been reported: aldehyde, ketone, α-ketoamide, α-ketoacid and boric acid/ester. The ketoamides have been the most successful class of covalent inhibitors. Among them, the most clinically advanced are boceprevir and telaprevir [62]. The new AASLD guidelines suggest addition of the NS3/NS4A inhibitors boceprevir or telaprevir to optimize treatment for patients infected with genotype 1.

Boceprevir (SCH 503034) (Figure 6) has been reported as safe and well tolerated in phase I and phase II clinical trials. In a phase II trial (SPRINT-1), patients treated with boceprevir for 48 weeks in combination with pegylated interferon and ribavirin demonstrated SVR rates 67-75% compared with SVR rates of 38% in the control arm [62]. Boceprevir (Victrelis) was approved by the FDA in May 2011, on the basis of the efficacy and safety results from two large phase III clinical studies that evaluated approximately 1,500 adult patients with chronic HCV genotype 1 infection. Both studies included two treatment arms with boceprevir: a response-guided therapy (RGT) arm, in which patients with undetectable virus (HCV-RNA) at week 8 of treatment were eligible for a shorter duration of therapy, as well as a 48-week treatment arm. All patients receiving boceprevir in these studies were first treated with peginterferon alfa-2b and ribavirin in a 4-week lead-in phase, followed by the addition of boceprevir after week 4. The studies also included a control arm in which patients received 48 weeks of treatment with peginterferon alfa-2b and ribavirin alone. In these studies boceprevir yielded sustained virological response rates as high as 67%.

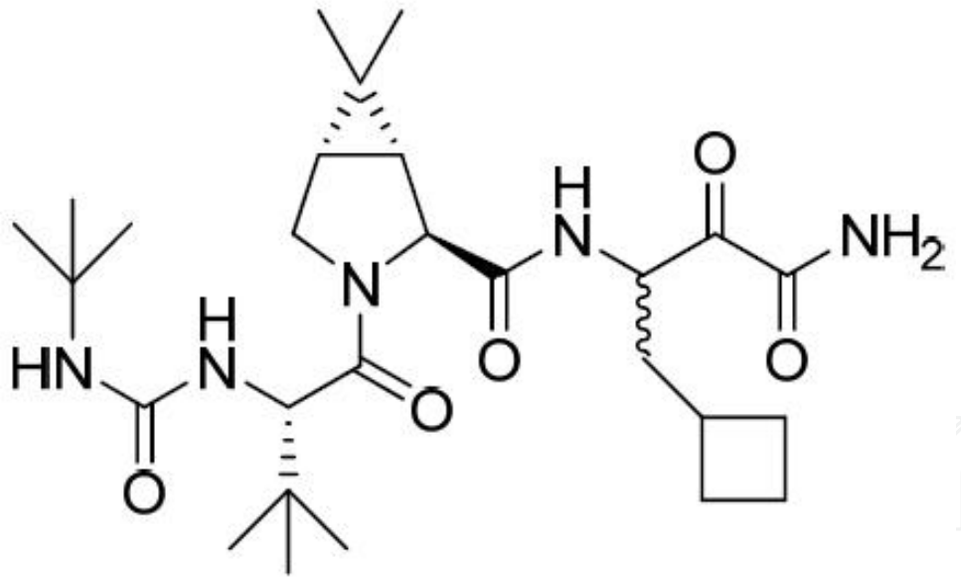

Figure 6. Chemical structure of boceprevir

Boceprevir was approved by FDA for the treatment of chronic hepatitis C genotype 1 infection, in combination with peginterferon alfa and ribavirin, in adult patients with compensated liver disease, including cirrhosis, who were previously untreated or who have failed previous interferon and ribavirin therapy.

Telaprevir (VX-950) (Figure 7) has been reported as generally safe and well tolerated. Its potent inhibition of NS3 protease activity was demonstrated in a mouse model and then

proved in a phase II clinical trial [63-65]. In a phase II clinical trial (PROVE-1) observed SVR rate was 35-67% with telaprevir compared with 41% in the control arm. In combination therapy with pegylated interferon and ribavirin (PROVE-2) resulted in 69% SVR [62].

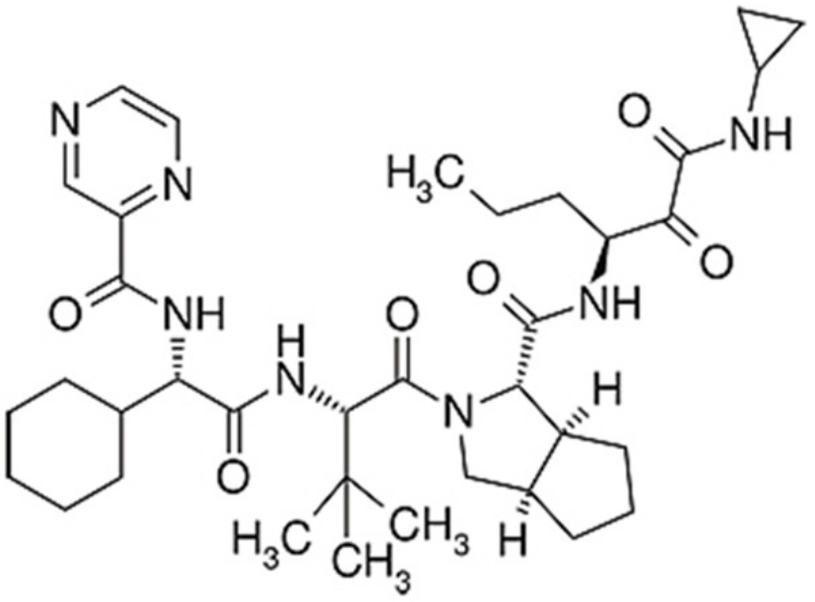

Figure 7. Chemical structure of telaprevir

FDA approved telaprevir (Incivek) in May 2011 to treat certain adults with chronic hepatitis C infection. Telaprevir is used for patients who had either not received interferon-based drug therapy for their infection or who had not responded adequately to prior therapies. Telaprevir is approved for use with combination therapy made up of peginterferon alfa and ribavirin. The safety and effectiveness of telaprevir was evaluated in III phase three clinical trials with about 2,250 adult patients who were previously untreated, or who had received prior therapy. In all studies patients also received the drug with standard of care. In previously untreated patients, 79% of those receiving telaprevir experienced a sustained virologic response (i.e. the infection was no longer detected in the blood 24 weeks after stopping treatment) compared to standard treatment alone. The sustained virologic response for patients treated with telaprevir across all studies, and across all patient groups, was between 20% and 45% higher than current standard of care. The studies indicate that treatment with telaprevir can be shortened from 48 weeks to 24 weeks in most patients. Sixty percent of previously untreated patients achieved an early response and received only 24 weeks of treatment (compared to the standard of care of 48 weeks). The SVR for these patients was 90%.

Several non-covalent inhibitors of NS3 protease have demonstrated potent reduction of HCV RNA and promising safety profiles in HCV infected patients. Non-covalent NS3 protease inhibitors rely on network of hydrogen bonding interactions and polar interactions to obtain binding energy. Ciluprevir was the first direct acting antiviral compound that demonstrated significant viral reduction in patients. The most advanced non-covalent NS3

protease inhibitors: danoprevir, vaniprevir, TMC435350, BI 201335 and BMS-650032 are currently in clinical development [66].

Ciluprevir (BILN-2061) was the first NS3 protease inhibitor tested for antiviral effect in humans. Unfortunately clinical study was terminated due to significant mitochondrial toxicity in cardiac myocytes of several preclinical species [67]. The antiviral efficacy, pharmacokinetics, and tolerability of 25, 200, and 500 mg BILN 2061 twice daily given as monotherapy for 2 days in 31 patients infected with chronic genotype 1 HCV infection and with minimal liver fibrosis were assessed in a placebo-controlled, double-blind pilot study. In 2 subsequent placebo-controlled studies of similar design, 200 mg BILN 2061 twice daily was administered for 2 days to 10 patients with advanced liver fibrosis and to 10 patients with compensated cirrhosis. Viral RNA reductions of 2–3 log10 copies/mL were achieved in most of the patients. There was a trend toward a higher number of patients receiving 500 mg BILN 2061 achieving a viral RNA reduction ≥3 log10 copies/mL as compared with patients receiving 25 mg BILN 2061 [68]. The antiviral activity of ciluprevir was also examined in patients infected with genotype 2 and 3. The antiviral efficacy of BILN-2061 was less pronounced and more variable in patients with HCV genotype 2 or 3 infection compared with previous results in patients with HCV genotype 1 [69].

Antiviral activity of danoprevir (ITMN-191/RG7227) was demonstrated in a randomized, placebo-controlled, 14-day multiple ascending dose study in patients with chronic HCV genotype 1 infection. Danoprevir displayed a slightly more than proportional increase in exposure with increasing daily dose and was rapidly eliminated from the plasma compartment. Maximal decreases in HCV RNA were: −3.9log10IU/ml and −3.2log10IU/ml [70]. Danoprevir is currently in phase IIb clinical study in combination with PegIFN/RBV. Eighty six percent of patients were HCV RNA negative by week 4 of treatment and 92% were negative by week 12 of treatment [66].

Vaniprevir (MK-7009) is another NS3 protease inhibitor, which entered phase IIb development in combination with PegIFN-α and RBV. In the early phase of clinical trial vaniprevir showed 69%-82% clearance of HCV RNA in patients after 4 weeks of treatment.

Opera-1 trial (double blind, placebo-controlled phase IIa trial) examined TMC435350 in combination with PegIFN-α and RBV in patients infected with genotype 1. TMC435350 in combination with PegIFN/RBV showed antiviral activity superior to PegIFN/RBV alone. In the 25, 75, 200 mg 4-week triple therapy arms, 6/9, 9/9 and 10/10 patients had HCV-RNA concentrations below the lower limit of detection (<25 IU/mL) and 3/9, 8/9 and 7/10 had undetectable HCV RNA (<10 IU/mL) at day 28, respectively [71]. TMC435350 is currently in phase IIb clinical trial.

Activity of BI 201335 was demonstrated in phase IIb clinical trial (SILEN-C2) that included HCV infected patients with confirmed non-response to at least 12 weeks of PegIFN/RBV treatment. After 4 weeks of treatment with BI 201335 in combination with PegIFN-α and RBV up to 69% patients had HCV RNA below the limit of detection and after 12 weeks of treatment up to 59% patients had HCV RNA below the limit of detection. SILEN-C2 confirmed robust antiviral activity with overall good tolerability and safety [72].

Asunaprevir (BMS-650032), a novel HCV NS3 protease inhibitor in clinical development, was evaluated for safety, antiviral activity, and resistance in four double-blind, placebo-controlled, sequential-panel, single- and multiple-ascending-dose (SAD and MAD) studies in healthy subjects or patients with chronic HCV genotype 1 infection. Asunaprevir at doses of 200 to 600 mg resulted in rapid HCV RNA decrease from the baseline; maximal mean changes in HCV RNA over time were 2.7 and 3.5 log(10) IU/ml in the SAD and MAD studies, respectively [73]. Currently, asunaprevir is being examined in an innovative programme in combination with NS5A inhibitor BMS-790052 in prior non-responders to PegIFN/RBV therapy [66].

3.1.2. NS3 helicase inhibitors

NS3 helicase is needed for HCV replication and is a potent STAT-C target. Small peptide and tropolones have been reported to inhibit NS3. Peptide inhibitors are quite attractive candidates for antiviral agents. It is relatively easy to design a peptide that fits a studied protein, regardless of the size and chemical properties of the target site. The first experiments performed with a radioactive helicase assay revealed the inhibitory activity of these peptides (of various lengths and composition) and pointed at a peptide composed of 14 amino acids (p14, RRGRTGRGRRGIYR) as the best helicase inhibitor. The first helicase inhibitor corresponded to a highly conserved arginine-rich sequence of domain 2 of the helicase. The 50% inhibitory activity (IC50) value was 725 ± 109 nM, indicating that the peptide is a very efficient NS3 helicase inhibitor. The antiviral activity of p14 was tested in a subgenomic HCV replicon assay that showed that the peptide at micromolar concentrations can reduce HCV RNA replication [74].

Tropolones possess multiple biological activities: antiviral, antimicrobial, and cytotoxic effects on various human tumour cell lines [75-77] . They may also exert an insecticidal as well as a metalloprotease inhibitory effects [78]. The antiviral activity of hydroxylated tropolone derivatives was demonstrated for human influenza virus and human immunodeficiency virus-type 1. Dibromo-morpholinometyltropolone (DBMTr, Figure 8A) is one of the potent NS3 helisace inhibitor among tropolones, which exert anti-helicase activity with an IC50 of 17.56 μM [79].

Other tropolones, like 3,5,7-tri[(4′-methylpiperazin-1′-yl)methyl]tropolone, 3,5,7-tri[(4′-methylpiperidin-1′-yl)methyl]tropolone and 3,5,7-tri[(3′-methylpiperidin-1′-yl)methyl] tropolone demonstrate NS3 inhibition (Figure 8B). Among them, the most active anti-helicase compound 3,5,7-tri[(4′-methylpiperazin-1′-yl)methyl]tropolone (IC50 = 3.4 μM), inhibited RNA replication by 50% at 46.9 μM (EC50) and exhibited the lowest cytotoxicity (CC50) >1 mM resulting in a selectivity index (SI = CC50/EC50) >21. The most efficient replication inhibitor, 3,5,7-tri[(4′-methylpiperidin-1′-yl)methyl]tropolone, inhibited RNA replication with an EC50 of 32.0 μM and a SI value of 17.4, whereas 3,5,7-tri[(3′-methylpiperidin-1′-yl)methyl]tropolone exhibited a slightly lower activity with an EC50 of 35.6 μM and a SI of 9.8. Moreover, these three tropolone derivatives inhibit replication of the HCV subgenomic replicon in cell cultures [80]. Despite the intensive studies, no inhibitors of HS3 helicase are in clinical use.

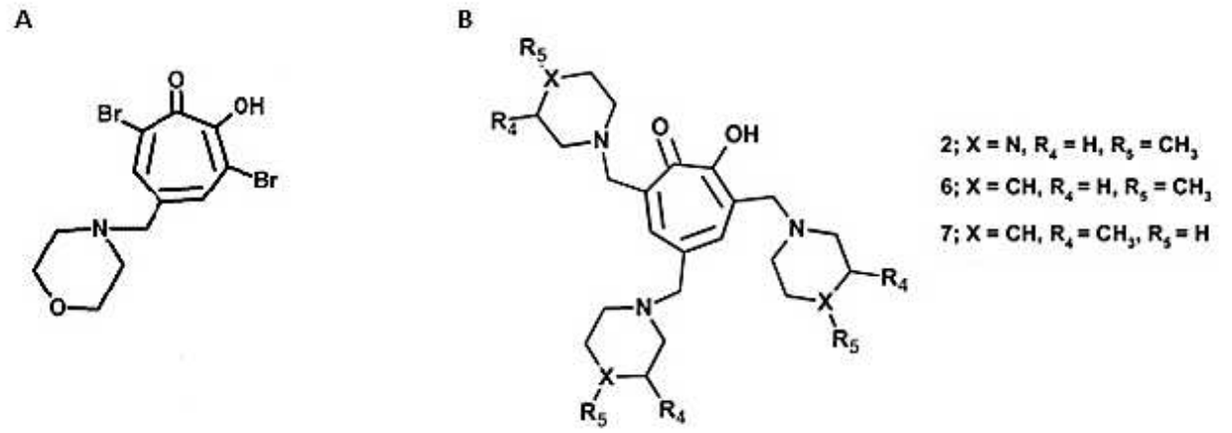

Figure 8. Chemical structure of tropolones: (A): dibromo-morpholinometyltropolone, (B): 3,5,7-tri[(4′-methylpiperazin-1′-yl)methyl]tropolone (2), 3,5,7-tri[(4′-methylpiperidin-1′-yl)methyl]tropolone (6) and 3,5,7-tri[(3′-methylpiperidin-1′-yl)methyl]tropolone (7)

3.1.3. NS5A inhibitors

The HCV NS5A as a part of replication complex is essential for RNA replication. It also has been associated with subverting host intracellular signaling pathways. The potent antiviral activity of different NS5A inhibitors has already been demonstrated in early clinical trial phase. Among them, BMS-790052 entered phase III of clinical trial. Extended Rapid Virologic Response (eRVR) was achieved up to 83% of HCV genotype 1 patients under BMS-790052 treatment in combination with peg-IFN and ribavirin. BMS-790052 was well tolerated with a safety profile comparable in the placebo group [81]. BMS-790052 has been shown *in vitro* to have a low genetic barrier of resistance [82]. Another potent NS5A inhibitor, PPI-461, with a preclinical profile similar to BMS-790052 entered clinical trial [83]. Given the successful nature of clinical trial NS5A inhibitors can now be viewed as a potential cornerstone of HCV combination therapy.

3.1.4. NS5B inhibitors

The HCV NS5B RNA-dependent RNA polymerase as a catalytic component of HCV replication complex plays a crucial role in viral replication cycle. Because NS5B is structurally distinct from mammalian DNA and RNA polymerase enzymes, it is a potent target for the development of new anti-HCV agents. The inhibitors of NS5B are divided into two classes: nucleoside inhibitors (NIs) and non-nucleoside inhibitors (NNIs). The nucleoside inhibitors bind to the active site of polymerase, resulting in chain termination and premature termination of HCV RNA synthesis. Optimization of nucleoside analogues is complicated by NIs interaction with cellular polymerases and signaling pathways. The most advanced candidate for drug is RG7128, which is currently in phase II clinical trial and has shown highly promising safety, tolerability and efficacy profile. In a 14-day monotherapy RG7128 showed high antiviral efficacy, achieving viral load decreases up to 2.7 log IU/ml at the end of dosing period. In phase IIa study 88% of infected patients with genotype 1 and

90% of patients infected with genotype 2 or 3 achieved undetectable viral load after 4 weeks of combination treatment with RG7128, PEG and RBV. Moreover, there were no evidence of resistance selection during two weeks of monotherapy. These results suggest a high antiviral activity in HCV infected patients and a high barrier to resistance of nucleoside analogues as inhibitors of HCV replication [84].

The HCV non-nucleoside inhibitors bind to one of the four allosteric binding sites within the HCV polymerase: site I (Thumb I) for JTK-109, site II (Thumb II) for PF-868554, VCH-759, VCH-916 and VCH-222, site III (Palm I) for ANA-598, A-848837 and ABT-333, and site IV (Palm II) for HCV-796, resulting in conformational changes of the protein and inhibition of catalytic activity of polymerase. Currently, a number of NNIs have entered the clinical trial (Table 1).

Inhibitors	Binding site	Study phase
PF-868554	Site II	Phase II
VCH-759	Site II	Phase I
VCH-916	Site II	Phase I
VCH-222	Site II	Phase I-II
ANA-598	Site III	Phase II
ABT-333	Site III	Phase II
HCV-796	Site IV	Withdrawn

Table 1. HCV NS5B non-nucleoside inhibitors under clinical investigation

HCV-796 was the first inhibitor that showed an antiviral effect in HCV-infected patients, but due to hepatic toxicity HCV-796 was withdrawn from clinical trial. PF-868554, VCH-759, VCH-916, VCH-222, ANA-598 and ABT-333 proved antiviral activity in early clinical trials and some have been advanced to phase II.

PF-868554 was tested in monotherapy over 8 days in genotype 1 patients with chronic infection. All patients demonstrated antiviral response after 48 hours of treatment. In phase IIa clinical trials patients with genotype 1 were treated with PF-868554 in combination with Peg-IFN/RBV. By week 4 HCV RNA plasma levels dropped up to 4.43 $\log_{10}$ compared with placebo and Peg-IFN/RBV group. After 12 weeks of treatment up to 88% had undetectable HCV RNA [85].

VCH-222 antiviral activity in HCV replicons resulted in EC_{50} values of 22.3nM for genotype 1a, 11.2nM for genotype 1b and 4.6μM for genotype 2a. Patients with genotype 1 were treated with VCH-222 for 3 days in phase Ib clinical study. All patients demonstrated rapid and significant antiviral response with 3 $\log_{10}$ in plasma HCV RNA [85].

ANA598 activity against genotype 1 was shown in *in vitro* HCV replicon system [86]. In phase II clinical study genotype 1 patients received ANA598 in combination with Peg-IFN/RBV for 12 weeks. Early virological response was achieved in 73% of HCV infected patients [85].

ABT-333 entered clinical trial after showing in replicon assays its potent inhibition of genotype 1a and 1b polymerases. Antiviral activity of ABT-333 was evaluated in combination with Peg-IFN/RBV. At the last day of therapy (Day 28) 41.7% of patients had undetectable HCV RNA [85].

Non-nucleoside polymerase inhibitors selected different NS5B mutations which exhibited resistance profiles. Given the distinct binding sites and resistance profiles among different NNIs, it is likely that two to three NNIs could be used in combination [85]. Antiviral activity and tolerability of the HCV NNIs is promising, but the use of NS5B inhibitors in treatment of HCV infection need more studies in future clinical trials.

4. Conclusions

The current standard of care (SOC) of chronic hepatitis C infection based on pegylated-IFN and ribavirin has limited efficacy and undesirable side effects. Efficient therapies must be developed to eliminate HCV infections, which pose a serious worldwide health problem. More than 80% of HCV infected patients develop the persistent infection, which leads to serious liver diseases, like liver cirrhosis or hepatocellular carcinoma. The lack of small animal models for HCV hamper studies on viral replication and search for potent antiviral targets. Development of HCV replicons able to replicate in cell line Huh-7 and *in vitro* HCV infection system using the JFH-1 clone provide a good method for screening the new antiviral drugs. An infectious HCV cell culture system is a powerful tool that could be used to study HCV life cycle and function of particular viral proteins. These studies allow to find viral targets for direct-acting antiviral (DAA) drugs and develop Specifically Targeted Antiviral Therapies for HCV (STAT-C). STAT-C let to achieve better effectiveness of treatment, its shortening, and the diminishment and limitation of side effects. Current studies are focused on searching for the new therapeutic agents for hepatitis C, which are directed against viral enzymes. Several HCV pro-drugs selected in *in vitro* HCV replicon system as potent HCV inhibitors, have reached clinical development, but the most advanced include inhibitors of NS3/4A protease. Two of them: boceprevir and telaprevir were approved in 2011 by the Food and Drug Administration (FDA) for the treatment of chronic hepatitis C genotype 1 infection, in combination with PegIFN-α and RBV.

The most intensive studies are focused on searching for novel DAAs against HCV genotype 1. Infections with genotype 1 are the most prevalent and dangerous, leading to liver injury and hepatocellular carcinoma. Moreover, patients infected with genotypes 1 respond to SOC less effectively than patients infected with genotypes 2 and 3. Because an infectious HCV cell culture system is restrictive adapt to genotype 2a, subgenomic HCV replicon systems are used worldwide for screening new antivirals against genotype 1.

In conclusion, *in vitro* tissue-based model of HCV replication allowed to determine the viral life cycle and function of particular viral proteins, which is crucial for the development of novel more efficient antivirals.

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