Direct-acting antiviral therapy for chronic hepatitis C

de Bruijne, J.

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General Introduction

Adapted from:

Treatment of chronic hepatitis C virus infection
Dutch national guidelines
The Netherlands Journal of Medicine 2008; 66(7): 311-22

Novel therapies in hepatitis B and C
Bart Takkenberg, Joep de Bruijne, Christine Weegink, Peter Jansen, Hendrik W. Reesink
Current Gastroenterology Reports 2008; 10(1): 81-90

New developments in the antiviral treatment of hepatitis C
Joep de Bruijne, Christine J. Weegink, Peter L. Jansen, Hendrik W. Reesink
Vox Sanguinis 2009; 97(1): 1-12

Controlled-release interferon alpha 2b, a new member of the interferon family for the treatment of chronic hepatitis C
Peter Jansen, Joep de Bruijne
Expert Opinion on Investigational Drugs 2012; 21(1): 111-8
Hepatitis C virus (HCV) is a single-stranded positive-sense RNA virus classified in the genus Hepacivirus of the Flaviviridae family. Until the first identification of HCV in the late 1980s, the clinical presentation of infection with HCV was referred to as a non-A, non-B hepatitis. HCV is a major causative agent of blood-borne infections and chronic liver disease throughout the world.

**EPIDEMIOLOGY**

Chronic hepatitis C is an important health problem in both developed and developing countries. The global epidemic of HCV infection affects approximately 200 million individuals worldwide (i.e. 3% of the world population) and is thought to be increasing with an estimated 3–4 million new infections every year. In the Netherlands the number of people with chronic HCV infection is estimated to be 15,000-60,000, corresponding to a prevalence of 0.1-0.4% in the general population. Six major HCV genotypes and over 100 subtypes have been identified. Each genotype differs by 30–35% of its nucleotide sequence. Globally, genotype 1 (subtypes 1a and 1b) is the predominant genotype, followed by genotype 2 and 3. Genotypes 4, 5 and 6 are characterized by specific geographical distribution. In the Netherlands genotypes 1, 2, 3 and 4 are the most prevalent.

The main route of transmission is parenteral via contaminated blood. The risk of perinatal transmission is low, while several reports have been published on outbreaks of acute HCV infection presumably transmitted via sexual exposure among human immunodeficiency virus (HIV)-positive men who have sex with men (MSM). The main groups at risk for HCV infection are persons who have ever used intravenous drugs, recipients of blood or blood products before 1992 and immigrants from high endemic areas. Intravenous drug use (IDU) contributed to spread of HCV infection in the 1970s and 1980s. Epidemiological studies have revealed that this mode of transmission is now declining in developed countries. Due to the shared routes of transmission of HCV and HIV, co-infection often occurs in high risk populations. Almost all HIV-infected IDUs and haemophiliacs are co-infected with HCV, whereas almost all HCV-infected MSM are co-infected with HIV. Increasing use of parenteral therapies and blood transfusions has led to an iatrogenically induced spread of HCV. This iatrogenically induced spread can be illustrated by two striking examples. First, almost all haemophiliacs who received large-pool clotting factor concentrates before 1986 became infected with HCV. Second, the highest HCV prevalence is recorded in Egypt, where approximately >10% of the general population is infected.

**NATURAL HISTORY**

Acute HCV infection is rarely observed and is usually asymptomatic. In approximately 20% of cases specific symptoms like fatigue, nausea, fever and abdominal pain occur. Fulminant hepatitis is rare. HCV RNA is first detectable seven to ten days after exposure. HCV-specific antibodies are detectable after 8-20 weeks. Failure to spontaneously eradicate HCV infection occurs in 50-90% of cases depending on the route of transmission and the age at time of infection. After spontaneous viral clearance individuals do not seem to be protected from a new HCV infection, since re-infection after clearance and super-infection have been described. However, partial immunity might occur after spontaneous clearance, as evidenced by lower peak HCV RNA titres in re-infected individuals compared to peak HCV RNA titre during primary HCV infection.

Chronic HCV infection is defined by the presence of HCV RNA for more than 6 months after HCV exposure. Many HCV-infected individuals do not have symptoms and are unaware of their infectious status. HCV infection is therefore often diagnosed accidentally. Some extra-hepatic manifestations, such as lichen planus, Sjögren’s syndrome and vasculitis on the basis of cryoglobulinaemia are associated with chronic HCV infection. The grade of hepatitis can vary from minimal to serious inflammation with fibrosis or cirrhosis. After 10 to 30 years, 10 to 20% of the patients develop cirrhosis. Fibrosis and cirrhosis can emerge despite normal levels of ALT. Progression of the disease is slower in females and in those who are young at the time of infection, but faster in patients with non-alcoholic fatty liver disease, alcohol consumption, or co-infection with HIV or HBV. Although the effect of HCV co-infection on HIV progression remains controversial, HIV infection clearly has an impact on HCV induced disease progression. Firstly, HIV coinfection during acute HCV infection is associated with lower rates of HCV clearance. Secondly, HIV-infected individuals have higher levels of viremia. Thirdly, progression to liver fibrosis, cirrhosis and end-stage liver disease is faster in co-infected individuals.
Liver cirrhosis can decompensate with development of jaundice, ascites, coagulopathy, variceal bleeding and hepatic encephalopathy. A major threat is the development of hepatocellular carcinoma (HCC). In patients with cirrhosis due to HCV infection, the incidence of HCC is 1 to 4% per year (Figure 1).24, 25 Decompenesed liver cirrhosis and HCC as a result of chronic HCV infection are currently the major indications for liver transplantation in Western Europe and the USA.25, 26 Liver transplantation is an effective treatment for decompensated cirrhosis and for small HCCs.27 However, hepatitis C recurrence due to graft re-infection is universal after transplantation.28 As a result 10 to 41% of the patients will develop cirrhosis of the donor liver after 5 to 10 years.29 Antiviral therapy in patients awaiting transplantation prevents graft re-infection if an sustained viral response is achieved.28, 30 Although death as a result of end-stage liver disease or HCC due to chronic hepatitis C occurs in probably less than 30% of all HCV-infected patients, the worldwide epidemic leads to a mortality rate of approximately 350,000 deaths per year.24 The incidence of HCC and the mortality due to HCV infection will probably increase in the coming decades.25, 26

HCV genome organisation, protein expression and life cycle
HCV is a hepatotropic virus that is a member of the genus hepacivirus in the Flaviviridae family. The virus is ~50 nm in diameter. HCV is a positive-sense single-stranded RNA virus consisting out of 9600 nucleotides. The viral genome is encapsidated in the nucleocapsid, which is surrounded by a host cell membrane-derived lipid bilayer envelope containing the glycosylated envelope proteins (E1 and E2). After entering the cell by receptor-mediated endocytosis, HCV RNA is released into the cytoplasm of the hepatocyte. The HCV genome contains a single open-reading frame (ORF) encoding a polyprotein of approximately 3000 amino acids (Figure 2). This ORF is flanked by highly conserved untranslated regions (UTR) that contain signals for replication and translation. Cleavage of the polyprotein by host peptidases and viral proteases results in three structural proteins and seven non-structural (NS) proteins.26 Structural proteins form the nucleocapsid (core) and contribute to the virion envelop formation (E1 and E2). E1 and E2 are transmembrane glycoproteins essential for viral attachment, entry and membrane fusion. NS proteins are involved in HCV replication, polyprotein processing and assembly. NS proteins includes a viroporin (P7), proteases (NS2, NS3), helicase, nucleoside triphosphatase (NS3), a cofactor for NS3 protease activity (NS4A), an anchor for the replication complex to the endoplasmic reticulum (NS4B), the RNA-binding protein (NS5A) and RNA-dependent RNA polymerase (NS5B). While function and crystal structure of NS5A and NS5B are known, the other proteins are less well characterized.27

HCV replication is characterized by a high turnover rate with a half-life estimated to be only 2–5 hours. The production and clearance is around $10^{10}$ to $10^{12}$ virions per day in an infected patient. The HCV RNA–dependent RNA-polymerase has poor fidelity and lacks proof-reading activity. Because of this feature and the high replication activity of HCV, a large number of viral variants are produced continuously during infection with an error rate of approximately $10^{-3}$ to $10^{-5}$ mutations per nucleotide per genomic replication. The majority of variants produced result in loss of viral replication via loss of function of encoded proteins or lack of tolerance by the host’s immune system. However, a large number of variants survive; the sum of these different isolates in an infected patient is called viral quasispecies. Typically a dominant strain (wild-type) is detectable within the viral quasispecies along with strains that are present in lower frequencies.28, 29 The swarm of viral variants that develop within one host provide one of the mechanisms by which HCV evades host immune surveillance and establishes chronic infection. The enormous diversity of viral variants seems to play a pivotal role in the development of viral resistance to direct acting antiviral therapy.30, 31
were not infectious in chimpanzees, underlining the limitations of HCV replicons as model system. The second milestone was the isolation of the HCV JFH1 strain from a patient with fulminant hepatitis.42 This strain does not require adaptive mutations to replicate efficiently in hepatoma cell lines with defective IFN responses and maintains its in vivo infectivity. Several models to study HCV binding and entry were developed in parallel. Virus-like particles produced in the baculovirus system and retroviral pseudoparticles with HCV envelope glycoproteins were used as in vitro models to study viral entry, viral assembly and neutralizing antibodies. Immunodeficient mice transplanted with human hepatocytes are now available to screen antibodies and antiviral agents in vivo.43, 44

In peripheral blood of infected patients, HCV is physically associated with VLDL, LDL, and HDL. Viral attachment, entry, and fusion involve two groups of molecules and proteins. The first group comprises the HCV structural envelope glycoproteins, E1 and E2. These are transmembrane glycoproteins at the surface of the HCV virion. Whereas E1 is thought to be responsible for mediating the intracytoplasmic virus membrane fusion, E2 is involved in the initiating process of binding to target cells.45 Entry into hepatocytes requires the tetraspan CD81, the scavenger receptor class B type I, and the tight junction proteins claudin and occludin.45-46 HCV also binds to other molecules, such as glycosaminoglycans, the LDL receptor, receptor tyrosine kinases EGFR (epidermal growth factor receptor) and EphA2 and the lectins DC-SIGN (Dendritic cell-specific ICAM-3-grabbing nonintegrin) and L-SIGN (Liver and lymphnode-specific ICAM-3-grabbing nonintegrin), but these are not essential entry factors and do not confer tissue specificity.46-48 After clathrin-mediated endocytosis and pH-dependent release from early endosomes, HCV translation and replication is started in the cytosol. Translation is initiated through an internal ribosomal entry site (IRES) in the 5′ UTR. The polyprotein precursor consisting of approximately 3,000 amino acids is cotranslationally and posttranslationally processed by both cellular and viral proteases into 10 structural and non-structural (NS) proteins at the endoplasmic reticulum (ER) membrane.49

The non-structural viral proteins are processed by two viral proteases: NS2/3 and the NS3 protease. NS2/3 protease is responsible for auto-cleavage at the NS2/3 site.50 NS3 is a dual-functioning protein incorporating a serine protease at the N-terminus and a RNA helicase at the C-terminal domain. Heterodimerization of serine protease with its cofactor NS4A significantly enhances proteolytic processing efficiency. This protease is responsible for the cleavage of the viral polyprotein at four sites (NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B).50, 51 Subsequently, these separate functional proteins are essential for viral replication. Processing of the polyprotein encoded by the HCV RNA by the various peptidases is important in the regulation of gene production and replication. Because of the critical function of these proteins in the viral life cycle, they represent attractive targets for antiviral therapy.45 The processing of the structural proteins (E1, E2, core protein, and p7) requires at least two host cellular peptidases. Most of the core protein is found in the cytoplasm, where it is bound to ER membranes or located at the surface of lipid droplets (LDs). The core protein on LDs recruits the replication complexes to the LD-associated membranes via the core-NS5A interaction. It was found by electron microscopy that expression of NS4B induces the formation of a seemingly ER-derived membranous

![Figure 3. HCV lifecycle](image-url)
HCV IMMUNOLOGY

Innate immune response

Key players of the innate immune response against HCV are hepatocytes, plasmacytoid dendritic cells (pDCs) and natural killer (NK) cells. Although all nucleated mammalian cells are able to secrete type I interferon (IFN), the first response is thought to be IFN-β production by infected hepatocytes. It is initiated by two pattern-recognition receptors, toll-like receptor 3 (TLR3) and retinoic acid-inducible gene I (RIG-I). TLR3 senses dsRNA in endosomes, whereas RIG-I recognizes the polyuridine motif of the HCV 3′ UTR in the cytoplasm. Upon activation, TLR3 recruits the adapter molecule Toll–IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF), and RIG-I recruits the mitochondrial antiviral signalling protein (MAVS). Both processes result in downstream signalling, nuclear translocation of IFN regulatory factor 3 (IRF3), and synthesis of IFN-β. Binding of IFN-β to the IFN-α/β receptor activates the JAK/STAT pathway, which results in the induction of IFN-stimulated genes (ISGs) such as the OAS1/RNAse L system, which degrades viral and cellular RNA. Induction of ISGs amplifies the IFN response, because many pattern recognition and signalling molecules such as RIG-I are ISGs and because the ISG IRF7 stimulates IFN-α subtype diversification. Type I IFNs are also produced by nonparenchymal cells, especially by pDCs in inflamed tissues and draining lymphoid nodes. In HCV infection, the frequencies of pDCs in blood, and their ability to produce IFN-α upon in vitro stimulation, are reduced. NK cells are frequently found in the liver and are able to rapidly exert cytotoxicity and release cytokines during the acute phase of HCV infection. HCV attenuates the IFN response at multiple levels. A key player is the HCV NS3/4A protein, which, when over expressed in cell culture, cleaves the adapter molecules TRIF and MAVS and thereby blocks TLR3 and RIG-I signalling. Over expression of downstream signalling molecules circumvents this block and restores IFN-β production. A second key player is HCV core, which, when over expressed in cell culture, interferes with JAK/STAT signalling and ISG expression. Several additional HCV proteins interfere directly with the function of ISGs: HCV N5A inhibits 2′-5′ oligoadenylate synthetase (2′-5′ OAS) and induces IL-8. This inhibits overall ISG expression. HCV N5A forms heterodimers with protein kinase R (PKR) and thereby inhibits its function. HCV E2 acts as decoy target to PKR. These viral escape strategies, which have been identified biochemically or in transfected cell cultures, suggest that HCV has established redundant means to coexist with the host IFN response. This also raises the intriguing possibility that viral protease inhibitors may not only inhibit viral polyprotein processing, but also restore innate immune signalling.

Adaptive immune response

One of the key characteristics of HCV infection is the delayed immune response despite the early increase in HCV titre and the induction of ISGs. HCV-specific T cells are typically detectable 5–9 weeks after infection, and HCV-specific antibodies are detected 8–20 weeks after infection. HCV can be cleared without humoral immune responses in immunocompromised (e.g., hypogammaglobulinemic) patients. In immunocompetent patients, neutralizing antibodies appear late and are isolate specific. It is clear, however, that neutralizing antibodies increase in titre and breadth, typically exhibiting cross reactivity against multiple HCV genotypes once chronic HCV infection is established. Although they fail to clear the virus at this stage, they continue to exert selection pressure on viral variants and thereby contribute to the evolution of the HCV envelope sequences throughout the course of infection. The overall concentration of IgGs and the frequency of IgG-secreting B cells are also increased in chronic hepatitis C patients. In contrast to antibodies, HCV-specific T cells are critical for HCV clearance. The decrease of viral titre coincides with the appearance of HCV-specific T cells and IFN-γ expression in the liver, which suggests that viral clearance is T cell mediated. HCV-specific T cells are essential for HCV clearance. At the time of clinical presentation and ALT elevation, vigorous proliferation of HCV-specific CD4+ T cells with concomitant IL-2 and IFN-γ production is readily detectable in the blood of patients who later recover and clear the infection. In contrast, HCV-specific CD4+ T cell responses are absent or weak in those who subsequently develop chronic infection. Furthermore, loss of initially strong CD4+ T cell responses has been associated with recurrent viremia even after several months of apparent viral control. In contrast to HCV-specific CD4+ T cells, CD8+ T cells are detectable in the blood of acutely infected patients regardless of virological outcome. During acute HCV infection, CD8+ T cells appear stunned, with impaired proliferation, IFN-γ production, and cytotoxicity and increased levels of programmed death–1. Chronic HCV infection is associated with continuous activation yet impaired function and reduced breadth of HCV-specific T cells. Significant changes (>1 log) in HCV titre, ALT spikes, and spontaneous HCV clearance are exceedingly rare, which suggests coevolution of virus and host immune responses.
Signal transduction through type 1 and type 3 interferons (IFNs). The three type 3 IFNs IL28A, IL28B, and IL29 all interact with a heterodimeric class 2 cytokine receptor that consists of IL10R2 and IL28Ra (IFNkR1). Although the kinetics of signal transduction are distinct, type 1 and type 3 IFNs stimulate similar pathways, with receptor binding resulting in phosphorylation of the kinases JAK1 and Tyk2, activation of the transcription factor complex containing STAT1, STAT2, and IFN regulatory factor 9, and up-regulation of a similar set of interferon stimulated genes.

ANTIVIRAL THERAPY

The present

In the late 1980s, the first studies indicating that recombinant IFN-alpha based therapy might have beneficial antiviral effects in non-A non-B chronic hepatitis infection were reported. Treatment results were poor and side-effects abundant. Modifications of IFN with a polyethylene glycol (peg-IFN) molecule have improved the half-life of the drug, increased the systematic exposure and biological effect.

HCV is one of the few viruses where complete viral clearance can be obtained with antiviral therapy. This is partially due to non-integration of the virus into the cell genome. At present, the standard of care for chronic hepatitis C is a combination of peg-IFN and ribavirin, as an undetectable serum HCV RNA level 24 weeks after cessation of therapy. An SVR is durable in more than 99% of patients, even in cases with consecutive immunosuppression, and is associated with resolution of intrahepatic inflammation and regression of liver fibrosis. In patients with a suspected acute HCV infection (for example after needlestick injury with an HCV RNA-positive source) it is recommended to determine the HCV RNA load regularly and to start treatment if HCV RNA is present 12 weeks after HCV exposure. Studies have shown that the chance of achieving an SVR is 90 to 100% after treatment with peg-IFN monotherapy for 24 weeks, independent of the genotype.

In all chronically HCV-infected patients antiviral treatment should be considered. In the absence of fibrosis and inflammation in the liver biopsy, postponing treatment can be considered. Chronic HCV infection can be treated with combination therapy consisting of peg-IFN and ribavirin for 24 to 48 weeks. The treatment duration depends on the HCV genotype, the quantity of HCV RNA in plasma at the start of treatment and the viral decline during treatment. An SVR is achieved in approximately 65-82% of patients with genotype 2 or 3 HCV infection and in 40–60% of those with genotype 1 and 4 HCV infection, when given for 24 or 48 weeks, respectively. peg-IFN and ribavirin treatment duration can be tailored to the on-treatment virological response. Upon treatment, HCV RNA should be assessed at three time points, regardless of the HCV genotype: baseline, weeks 4 and 12. Treatment should be stopped at week 12 if the HCV RNA decrease is less than 2 log_{10} IU/mL, as the SVR rate in these patients is less than 2%. In patients with detectable HCV RNA (≥50 IU/mL) at week 24, treatment should also be stopped due to a minimal chance to achieve an SVR (1–3%).

The likelihood of SVR is directly proportional to the time of HCV RNA disappearance. Rapid virological response (RVR) is defined as an undetectable HCV RNA level (<50 IU/mL), at week 4 of therapy, maintained up to end of treatment. Patients infected with HCV genotype 1 who have an RVR could be treated for 24 weeks if they have a low baseline viral level (<400,000–800,000 IU/mL). In patients infected with HCV genotypes 2 and 3 with an RVR and low baseline viral load, shortening of treatment duration to 16 weeks can be considered at the expense of a slightly higher chance of post-treatment relapse. Due to insufficient evidence for equivalent efficacy in patients who have advanced fibrosis, cirrhosis or cofactors affecting response (insulin resistance, metabolic syndrome, non-viral steatosis) shortening of treatment duration should not be considered even if they have low baseline viral and RVR.

Current antiviral treatment is a long-term process and is associated with substantial side effects such as flu-like symptoms, depression, anemia, and neutropenia. Dose reduction is indicated in case of serious anaemia, thrombocytopenia or neutropenia. However, reduced administration of peg-IFN and/or ribavirin is related to an impaired treatment outcome. The strongest pre-treatment predictors of SVR are the recently identified genetic polymorphisms located in chromosome 19, close to the region coding for IL28B (or IFN l3), the HCV genotype, and the stage of fibrosis. Other predictors of treatment response include baseline HCV RNA levels, the dose and duration of therapy, body mass index, age, insulin resistance, gender or coinfection with HBV or HIV.

The likelihood of SVR is directly proportional to the time of HCV RNA disappearance.
Relapse is defined when detectable HCV RNA is present 24 weeks after stopping antiviral therapy. The relapse rate after treatment with peg-IFN and ribavirin is approximately 15-25%. Patients relapsing after treatment with standard IFN-based regimens respond to retreatment with peg-IFN and ribavirin in about 30-40% of cases (Poynard). Breakthrough is defined as detectable HCV RNA at any time during antiviral therapy after previous undetectable HCV RNA. For patients in whom a SVR is not achieved, retreatment options were limited to reexposure to the same medications, with potential modification of the dose or duration of the regimen. These retreatment strategies are associated with clinically significant morbidity and generally a limited chance of resulting in a successful outcome. Approximately 20% (genotype 2 and 3) to 50% (genotype 1 and 4) of patients do not respond to current standard peg-IFN and ribavirin therapy. Three types of non-SVR can be distinguished: nonresponse, relapse and breakthrough. Non-responders are patients who failed to achieve a 2 log$_{10}$ IU/mL HCV RNA decline after 12 weeks of treatment or who never achieved undetectable HCV RNA during treatment of a minimum duration of 24 weeks (Figure 5). Retreatment with peg-IFN and ribavirin in HCV genotype 1 infected patients, who had a non-response to previous interferon-based therapy, resulted in SVR rates of only 4% to 14%. Relapse is defined as undetectable HCV RNA with a qualitative PCR-test at end of treatment, but detectable 24 weeks after stopping antiviral therapy. The relapse rate after treatment with peg-IFN and ribavirin is approximately 15-25%. Patients relapsing after treatment with standard IFN-based regimens respond to retreatment with peg-IFN and ribavirin in 32 to 53% of cases (Poynard). Breakthrough is defined as detectable HCV RNA at any time during antiviral therapy after previous undetectable HCV RNA. For patients in whom a SVR is not achieved, retreatment options were limited to reexposure to the same medications, with potential modification of the dose or duration of the regimen. These retreatment strategies are associated with clinically significant morbidity and generally have a limited chance of resulting in a successful outcome. Therefore, the development of effective regimens to retreat patients with chronic HCV infection who did not achieve an SVR after peg-IFN and ribavirin combination therapy is an urgent priority.

The future
New insights into HCV-specific virology have greatly improved our knowledge of potential antiviral targets. The lack of an adequate system to propagate HCV in laboratories formed a major hurdle in the quest for new therapies. The establishment of such a system capable of in vitro HCV replication was revolutionary. This discovery enabled high-throughput screening of compounds targeting specific steps essential in the HCV life cycle by pharmaceutical companies. Many new compounds are currently being developed for the treatment of chronic hepatitis C. These compounds can be broadly categorized into two classes: direct- and indirect-acting antivirals. Direct-acting antivirals (DAA) interfere with specific steps in the HCV replication cycle through a direct interaction with the HCV polyprotein and its cleavage products. Indirect-acting antivirals aim to inhibit replication by binding to host enzymes and thereby preventing participation in the HCV life cycle or by stimulating the host immune response resulting in antiviral activity.

**DIRECT-ACTING ANTIVIRALS**

**Entry Inhibitors**
Although many potential entry targets have been identified, only a few clinical trials have been performed using viral attachment as a target. A small number of molecules have been identified which interfere with viral attachment, only monoclonal and polyclonal antibodies have been tested in clinical trials. Unfortunately, no antiviral efficacy was observed.

**Translation Inhibitors**
The IRES directly regulates the assembly of translation initiation complexes on viral mRNA by recruiting cellular and viral proteins. Several potential antiviral approaches have been developed in various in vitro systems to inhibit HCV RNA translation. Three classes of drugs with different modes of action have been described: ribozymes, antisense-oligonucleotides and HCV IRES inhibitors. Ribozymes recognize and catalyze the cleavage of target RNA molecules. The second group comprises antisense DNA or RNA oligonucleotides. The sequence of these oligonucleotides is complementary to the target mRNA and they prevent the construction of regulatory sequences and/or structures important for efficient translation of HCV polyprotein (Hanecak). The antiviral activities of these three classes of drugs in phase 1 clinical trials were disappointing.

**NS3/4A protease inhibitors**
The NS3 gene encodes a serine protease critical for viral replication and is thought to have a dual role in establishing chronic HCV infection. The protease mediates the cleavage of the HCV polyprotein into functional viral proteins required for replication and may also play a role in viral evasion of the immune system by preventing expression of IFN response genes. Peptidomimetic inhibitors of the NS3/4A protease belong to two chemical groups: macroyclic and linear a-ketoamide inhibitors. They bind tightly to the catalytic site of the enzyme and compete with its natural substrates, the polyprotein cleavage sites, thereby inhibiting polyprotein processing, i.e., the generation of mature viral proteins. BILN-2061, a macrocyclic protease inhibitor, was the first DAA to enter clinical trials. Development of this compound was halted because of cardiotoxicity in laboratory animals, but not before it demonstrated a rapid 3-log$_{10}$ decline in virus load in all HCV genotype 1 treated patients within 2 days. Almost simultaneously, in vitro studies described mutations...
In conclusion, viral resistance and viral relapse was investigated in phase 3 registration trials.\textsuperscript{115, 116-119} In phase 1b trials, telaprevir monotherapy (750 mg every 8 hours) for 14 days resulted in a median change from baseline of -4.41 log$_{10}$ IU/mL in plasma HCV RNA levels in patients infected with HCV genotype 1.\textsuperscript{120} Mean maximum changes in HCV RNA after 1 week boceprevir monotherapy (400 mg every 8 hours) was -1.61 log$_{10}$ IU/mL. Subsequently, various regimens combining telaprevir or boceprevir with peg-IFN, with or without ribavirin, as compared with peg-IFN and ribavirin alone, were assessed in phase 2 studies. Studies with boceprevir investigated treatment regimens with and without a 4-week lead-in of peg-IFN and ribavirin before the addition of boceprevir for 24 or 44 weeks.\textsuperscript{121} The rationale for the 4-week lead-in was to allow peg-IFN and ribavirin to reach steady-state concentrations before the addition of boceprevir such that backbone drug concentrations would be at an optimum and potentially reduce the likelihood for emergence of drug-resistant mutations by reducing viral levels.\textsuperscript{116, 117, 121}\textsuperscript{121} The most common types of serious side-effects reported for telaprevir were skin rashes, gastrointestinal events and anaemia. The main adverse events for boceprevir-treated groups were anaemia and dysgeusia. Treatment with a telaprevir- or boceprevir-based regimen significantly improved SVR rates in treatment-naïve and treatment-experienced patients with genotype 1 HCV, albeit with higher rates of discontinuation because of adverse events. Strikingly, SVR rates were lowest with the regimens that did not include ribavirin.\textsuperscript{122} These encouraging findings formed the starting signal of multiple phase 3 registration trials. The efficacy and safety of response-guided therapy using a three-drug regimen of telaprevir with peg-IFN and ribavirin in treatment-naïve chronically genotype 1 infected patients was assessed. In patients who received telaprevir, peg-IFN and ribavirin triple therapy a significant higher SVR percentage was achieved (70\%), compared to standard of care (44\%) (Figure 6). Extended RVR (eRVR, HCV RNA undetectable at week 4 and 12) was achieved in most patients (~70\%) due to the rapid HCV RNA decline induced by triple therapy. Patients who achieved an eRVR had SVR rates of ~90\%. As a result, using response-guided therapy, treatment could be shortened to 24 weeks compared to the standard 48 weeks in the majority of patients, without compromising the SVR rate.\textsuperscript{123} The addition of boceprevir to peg-IFN and ribavirin also resulted in significantly higher SVR rates in previously treated and untreated patients with chronic HCV genotype 1 infection, as compared with peg-IFN and ribavirin combination therapy.\textsuperscript{116, 117} In conclusion, phase 3 trials clearly demonstrated that the addition of telaprevir or boceprevir to peg-IFN and ribavirin increased the SVR rate in treatment-naïve and treatment-experienced patients. A major shortcoming associated with HCV-specific protease inhibitors is the low genetic barrier to resistance potentially resulting in therapy failure. The following main resistance substitutions have been reported for linear protease inhibitors, by order of increasing resistance in vivo: V36A/M/C, V170A/T, T54A/S, R155K/T/Q and A156S/T/V.\textsuperscript{125} In vitro data suggest cross-resistance among all first-generation NS3/4A protease inhibitors currently in development. Viral resistance analysis of macrocyclic protease inhibitors showed a partially different resistance profile compared to linear protease inhibitors. Macrocyclic inhibitors such as vaniprevir, danoprevir, TMC435 or BI201335 have been shown to also select variants bearing Q80R/K and D168A/Y/T/H substitutions besides the R155K/T/Q variant.\textsuperscript{124} Monotherapy with protease inhibitors selects resistant viral variants within a few days or weeks, depending on the level of drug exposure.\textsuperscript{120, 123} Data from subsequent phase 1b studies suggested that combining telaprevir or boceprevir with peg-IFN, with or without ribavirin, increased viral inhibition and decreased the emergence of viral resistance.\textsuperscript{120, 123, 124} Viral resistance and viral relapse was observed more frequently in patients who did not receive ribavirin during phase 2 studies with telaprevir suggesting a crucial role of ribavirin in preventing viral resistance.\textsuperscript{123} Selection of resistant associated variants and subsequent therapy failure can be partially prevented by combining DAAs with peg-IFN and ribavirin and/or by adding one or more non cross-resistant DAAs to the treatment regimen. Based on anti-retroviral monotherapy trials in HIV infected patients, there is a concern that patients who have been exposed to a DAA will continue to harbour resistant viruses, narrowing future treatment options. Whether resistant variants can persist at an increased level over longer time periods and the relevance for retreatment options for patients who failed DAA-based therapy is not yet known.

**Polymerase Inhibitors**

Successful HCV replication depends on a number of viral and cellular proteins. NS5B RdRp is an essential viral component for HCV RNA replication and therefore forms an important target for antiviral therapy. Two different classes of HCV polymerase inhibitors, nucleosides and non-nucleosides, can be distinguished. Each class inhibits HCV replication by a different mechanism; nucleosides are chain terminators, while non-nucleosides cause conformational changes disabling the HCV polymerase.\textsuperscript{127} Both classes of polymerase inhibitors are able to reduce HCV RNA levels substantially. Nucleoside analogs had relatively low antiviral activities in clinical studies (0.7–1.2 log$_{10}$ HCV RNA IU/mL decline), which were enhanced up to 3.7 log$_{10}$ only with very high doses. For non-nucleoside analogs low to medium antiviral activities (0.6–3.7 log$_{10}$ HCV RNA IU/mL decline) were observed in clinical studies.\textsuperscript{128, 129} Antiviral activity of non-nucleosides is restricted to certain genotypes. Nucleoside analogs display antiviral activity across genotypes and seem to have the highest genetic barrier to resistance. Mutations at the active site of NS5B most likely cause loss of polymerase function. Non-nucleoside inhibitors bind to allosteric sites on the surface of NS5B. Here, a high number of variants with multiple amino acid exchanges can occur without functionally altering the NS5B polymerase activity. It is therefore challenging to design an NS5B inhibitor with a high genetic barrier to resistance. Variants that are resistant to most non-nucleoside inhibitors were selected within a few days of treatment during phase 1 monotherapy trials.\textsuperscript{128, 129} Moreover, variants with reduced sensitivity to non-nucleoside NS5B inhibitors may be present at high frequencies already before initiation of direct-acting antiviral therapy. Toxicity and adverse event profile of non-nucleosides was promising during phase 1 and 2 studies, whereas toxicity of nucleotides (neutropenia, anemia) resulted in preliminary discontinuation of several clinical trials.
Figure 6. Sustained viral response rates in HCV genotype 1 infected patients treated with
telaprevir or boceprevir in combination with peg-IFN and ribavirin during phase 3 registration
studies.

Telaprevir, peg-IFN and ribavirin (left bar).
Boceprevir, peg-IFN and ribavirin (middle bar).
peg-IFN and ribavirin (right bar).

Differences of SVR percentages within a study is caused by different treatment durations of patients who
received a protease inhibitor.
* Patients from the ADVANCE-, ILLUMINATE- and SPRINT-2-study.
† Patients from the REALIZE- and RESPOND-2-study.
‡ Patients from the REALIZE study.

NSSA Inhibitors
The mechanism of action of the NSSA protein is unclear. NSSA does not have an enzymatic
function and its role in the HCV replicative cycle, although crucial, remains obscure. BMS-
790052, a small molecule inhibitor of the HCV NSSA protein, has been shown to bind specifically
to domain I of NSSA and to potently inhibit HCV, with effective concentrations of the order of
10-50 picomoles in the replicon model, and pan-genotype antiviral activity. In a phase I
clinical trial in patients chronically infected with HCV, administration of a single 100-mg dose of
BMS-790052 was associated with a 3.3 log reduction in mean viral load measured 24 hours
post-dose that was sustained for an additional 120 hours in two patients infected with genotype
1b virus. NSSA inhibitors selected resistant HCV variants bearing amino acid substitutions in
the NSSA protein. BMS-790052 has a low genetic barrier to resistance and selected viruses
bearing single amino acid substitutions at position M28, Q30, M21 or Y93, that confer high-
level and lower-level resistance to subtypes 1a and 1b, respectively. These results provided the
first clinical validation of a NSSA inhibitor, a protein with no known enzymatic function, as an
approach to the suppression of virus replication that offers potential as part of a therapeutic
regimen based on combinations of HCV inhibitors.

Assembly Inhibitors
Targets to inhibit virus assembly are based upon the assumption that a certain degree of
glycosylation is needed for envelope proteins to cross cellular membranes. Iminosugars
inhibit cellular glucosidases, resulting in the hyperglucosylation of envelope proteins, which
inhibits their ability to cross membranes. Few drugs have been tested in clinical trials and the
observed antiviral activity was moderate.

Combination of direct-acting antivirals
Efficacy, safety and tolerability of these new compounds form obstacles to registration and
improved treatment regimens. Aside from their safety and efficacy profiles, the success of
these new agents depends on their ability to inhibit a broad range of viral variants and prevent
emergence of drug-resistant mutants. Anticipated advantages of DAA-based regimens are a
shorter, better tolerated therapy with greater SVR rates. Experience with anti-HIV drugs and
other antivirals predicted that development of viral resistance was expected to occur. Minor
populations of pre-existing, resistant variants have a fitness advantage over wild-type virus
in the presence of DAAs and become the dominant viral species. Rapid selection of resistant
variants can be explained by the high HCV replication rate and the low accuracy of the RNA
dependent RNA polymerase. Therefore, levels of resistance, pre-existent resistant variants
and fitness of the mutants are key parameters in the field of HCV research. Another similarity
between HIV and HCV treatment is the dire necessity of combination regimens with distinct
mechanisms of action to avoid drug resistance. The first study to investigate the efficacy and
safety of a combination regimen of a nucleoside polymerase inhibitor (R7128) and an NS3
inhibitor (R7227/ITMN191) without the addition of peg-IFN and ribavirin was encouraging. In
this proof-of-principle study, patients were given both DAA agents for up to 2 weeks. HCV RNA
concentrations decreased by 5.2 log IU/mL and were undetectable by the end of the study in
63% of patients without the occurrence of viral breakthrough. The results of subsequent clinical
studies investigating DAA combination therapy to suppress replication for longer durations and
to ultimately achieve a SVR without peg-IFN and/or ribavirin administration are promising.
It remains to be seen how many, and which patients may benefit from IFN-free regimens.

INDIRECT-ACTING ANTIVIRALS

Immune stimulation
The last decade, IFN-based treatment formed the cornerstone of hepatitis C treatment. peg-
IFN will probably remain the backbone of HCV therapy in the near future, as its antiviral
and immunomodulatory activities seem critical to achieve an SVR. Many new advances in
IFN drug design and administration systems are being developed. Novel approaches include
an implantable device for sustained long-term delivery of IFN, controlled-release of a
biodegradable delivery system of recombinant IFN (Locketer, Omega) or prolonging the half-
life of IFN (Albumin IFN-n2b). These novel approaches aim to offer improved efficacy,
more convenient, less frequent dosing and enhance tolerability compared to regular peg-IFN.
Stimulation of the immune system with interferon-λ or Toll-like receptor (TLR) agonists’ are
potentially promising alternative indirect antivirals to regular interferon-alpha therapy.
Interferon-λ (IL-29) is a member of the type III interferon family with functional similarities to type I IFNs, which include IFN-α and IFN-β. Like IFN-α and IFN-β, IFN-λ is induced in response to viral infections such as hepatitis C and has demonstrated antiviral activity in vitro, including inhibition of HCV RNA replication in the replicon model. IFN-λ interacts with the structurally unique IFN-λ receptor complex to stimulate an intracellular response through phosphorylation of the Janus kinase/signal transducer and activator of transcription pathway (similar to the mechanism of action of IFN-α) and leads to the upregulation of ISGs and an antiviral effect.

Unlike the widely distributed IFN-α receptor, expression of the IFN-λ receptor is more restricted. Although all cell types in the liver express the IFN-α receptor, the IFN-λ receptor is found only in hepatocytes. Similarly, although all peripheral blood leukocytes, including B, T, and natural killer cells, neutrophils, and monocytes, express the IFN-α receptor, messenger RNA of the IFN-λ receptor is not expressed in hematopoietic cells with the exception of B lymphocytes. The limited distribution of the IFN-λ receptor suggests the potential for reduced adverse events with IFN-λ-based therapy in comparison with IFN-α-based therapy along with preservation of the antiviral effect in HCV.

TLRs recognize specific pathogen-associated molecular patterns. Binding of TLRs to invading microorganisms triggers the immune system and initiates a host innate and adaptive immune response. Stimulating TLRs by TLR agonists could enhance viral-specific immunomodulatory response. Signaling through Toll-like receptors induces the production of type 1 T-helper cells, promotes cytokine and chemokine production, and stimulates natural killer cells. So far, 10 TLRs have been identified. TLR7 and TLR9 have been targeted by several compounds with modest antiviral activity. Endogenous stimulation of the innate and adaptive immune system by TLR agonists remains promising as there are no resistance issues in contrast to DAAIs. Moreover, an improved systemic side-effect profile of TLRs compared to IFN-based therapy could be expected.

In a recent genome-wide association study, a single nucleotide polymorphism (rs12979860) 3 kilobases upstream of the IL28B gene, was shown to be strongly associated with spontaneous viral clearance upon acute HCV infection, in particular in asymptomatic patients. This study showed that the C/C genotype enhances resolution of HCV infection among individuals of both European and African ancestry. These results are the strongest and most significant genetic effect associated with natural clearance of HCV, and implicate a primary role for IL28B in resolution of HCV infection. The exact contribution of IL28B to viral clearance remains to be elucidated. Other genome-wide association studies in chronic HCV genotype 1-infected patients treated with peg-IFN and ribavirin have revealed that IL28B SNPs are also associated with successful outcome or non-response to antiviral therapy with peg-IFN and ribavirin. The IL28B C/C genotype was strongly associated with lower level of hepatic ISG expression. The results suggest that IL28B genotype may explain the relationship between hepatic ISG expression and HCV treatment outcome. Furthermore, the previously unresolved issue of racial differences in virological response, can now largely be ascribed to different allele frequencies between populations.

Currently, treatment of chronic hepatitis C is being increasingly individualized. Pre-treatment viral characteristics (baseline viral load, genotype) and on-treatment viral response at week 4, 12 and 24 determine the treatment duration and are important predictors for therapy outcome and duration. Predictive genetic variants, such as IL28B, will possibly be added to predict treatment response and guide HCV treatment duration in the future. Other host markers (IP-10 and inosine triphosphatase [ITPA]) may also bear some correlation with disease progression or response to treatment. In general, however, there is currently no sufficient evidence to recommend testing of these markers in routine clinical practice in order to decide treatment allocation.

Ribavirin analogues

Ribavirin has been an indispensable adjunct to IFN-based therapy, and seems destined to remain an important component of regimens that incorporate novel compounds, as suggested by the results of the previously discussed telaprevir and boceprevir trials. However, haemolytic anaemia caused by ribavirin is often encountered during standard of care treatment as a result, dose reductions, discontinuations or treatment with erythropoietin or blood transfusion may be required. Recent trials showed that dose reduction or discontinuation of ribavirin is not desirable as SVR rates become significant lower.

In an attempt to limit adverse hematological effects associated with ribavirin, analogs of this compound are under development. Taribavirin (viramidine) is a ribavirin analogue that has been tested in 2 phase 3 trials. Significantly fewer patients within the taribavirin group developed anaemia given at 20–25 mg/kg compared with the ribavirin group. Weight-based dosing with taribavirin provides a safe and effective treatment alternative to ribavirin for chronic HCV infected patients. Currently, no other substitute for ribavirin is in an advanced stage of clinical development.

Vaccines

Unlike HBV infection, the development of an effective HCV vaccine remains an unsolved challenge. The world of HCV vaccine development has witnessed an exponential growth in the number of vaccine candidates being tested in preclinical models. Whereas efficacy of therapeutic vaccine remains poor, the initially tested candidates were clearly not tailored to induce the most robust T-cell responses. Currently, induction of strong, long-lasting and cross-reactive T-cell responses by vaccines is the main focus of intensive research. The next years will be pivotal in determining whether these new HCV vaccines can change the course of infection or prevent it altogether.
OUTLINE OF THIS THESIS

The main focus of this thesis was to improve our understanding of new antiviral therapies for chronic hepatitis C. The treatment of chronic hepatitis C is changing rapidly with a wide range of new antiviral options and an increasingly individualized antiviral therapy. Together with these promising direct and indirect acting antivirals, many challenging questions arise.

I. Drugs interacting with the hepatitis C virus life cycle

The HCV life cycle offers a number of possible targets for viral inhibition, and many specifically targeted drugs are in clinical development. Addition of direct acting antivirals to the current standard of care is aimed to improve the SVR rate with the potential of shorter treatment duration. The early clinical development of new antiviral compounds for chronic hepatitis C is characterised by 4 key parameters: safety, tolerability, pharmacokinetics and efficacy. These 4 parameters were extensively studied in 5 phase 1 studies including an HCV infection inhibitor JTK-692 (chapter 2), NS3 protease inhibitors narlaprevir with or without ritonavir (chapter 3), IDX320 (chapter 4), PHX1766 (chapter 5) and the non-nucleoside NS5B inhibitor IDX375 (chapter 6).

II. Viral phylogeny, kinetics and resistance

Epidemiological studies have shown that HCV genotype 1 is the predominant genotype worldwide and particularly present in developed countries. Not surprisingly, the development of direct acting antiviral agents is mainly focused on HCV genotype 1. Recent studies emphasized that the prevalence of HCV genotype 4 in Europe has increased in the past few decades. The study presented in chapter 7 describes the spread of HCV genotype 4 in the Netherlands by using a molecular epidemiological approach. This study showed that HCV genotype 4d rapidly spreads among HIV-positive MSM and becomes more prevalent in European individuals reporting IDU with and without HIV coinfection. Since HCV genotype 4 infection is difficult to treat and already cause an estimated 10% of chronic HCV infections worldwide, effective therapy against HCV genotype 4 infection will increasingly be needed.

A major issue for the development of new therapeutic approaches is the emergence of drug-resistant variants leading to therapy failure. The high genetic heterogeneity of HCV and its rapid replication poses a high risk for selection of resistant variants after administration of direct acting antivirals. In chapter 8, a longitudinal clonal analysis throughout the narlaprevir phase 1 study is described. This study was performed to investigate the development of resistant variants during and after narlaprevir mono- and combination therapy with peg-IFN, followed by standard of care.

Whether resistant variants can persist in the long term as major or minor quasispecies in nonresponsive patients or those with viral breakthrough or relapse after DAA therapy is not well understood. The aim of the study presented in chapter 9 was to characterize HCV quasispecies harbouring resistance mutation in telaprevir-treated patients at baseline and long term follow-up. This was assessed by using clonal sequencing and ultra-deep pyrosequencing (UDPS). Whether treatment-induced viral resistance has impact on the viral population and therapy outcome upon re-treatment with the same protease inhibitor is presented in chapter 10. TMC435 (a macrocyclic NS3/4A protease inhibitor) monotherapy was administered to 6 patients who previously failed interferon-based therapy. Approximately 1.5 years later, these patients were re-treated with TMC435 combined with peg-IFN and ribavirin. The emergence of viral resistance during TMC435 monotherapy and re-treatment was studied by population, clonal and UDPS at baseline, during therapy and follow-up.

The case report reported in chapter 11 illustrates the disadvantages of the current standard of care and the dire necessity for improved treatment strategies. In addition, it elucidates possible complications such as toxicity, viral resistance, and durability of an SVR, that can be anticipated when new antiviral agents are added to peg-IFN and ribavirin. Viral analysis suggests the potential for DAA treatment to significantly reduce genomic diversity and to produce viral populations functionally different from those present before treatment.

III. Immune modulating therapy

The effect that interferon therapy for HCV infection has on cellular immune responses is currently unknown. There is currently no consensus as to the role that (HCV-specific) T cells play in inducing a SVR to therapy. Recent studies have suggested that the proliferative capacity or magnitude of HCV-specific CD8+ and CD4+ T cells before treatment is important. Other investigators have suggested that responses induced during therapy correlate with SVR. Some have shown no association.

Chronic systemic ‘latent’ viral infections such as Cytomegalovirus infection are known to leave a fingerprint in the total T-cell population, identified by an increased percentage of highly differentiated effector-memory type T-cells. In chapter 12, we explored whether chronic viral infections with a ‘persistent’ viremia, such as chronic hepatitis B or C, that are characterized by local organ-specific inflammation, also impact the total peripheral T-cell population or other virus specific T-cells that do not target hepatitis viruses before, during and after antiviral therapy. Peripheral blood mononuclear cells were collected from patients with chronic hepatitis B, chronic hepatitis C and healthy controls and analyzed using fluorescence-activated cell sorting flow cytometer.

Toll-like receptors (TLRs) are a family of pathogen-recognition receptors. Binding of TLRs to invading microorganisms triggers the immune system and initiate a host innate and adaptive immune response. Stimulating TLRs by TLR agonists enhances viral-specific immunomodulatory response. ANA773 is an oral produg of a TLR7 agonist, developed for the treatment of patients with chronic HCV infection. The phase 1 study describing the development of the indirect acting antiviral ANA773, a TLR-7 agonist, is described in chapter 13.

In the general discussion (Chapter 14) the main findings of the studies presented in this thesis and the implications for chronic hepatitis C treatment and research in the future are discussed.
REFERENCES


