Evolving drug therapies for chronic hepatitis C

Immunomodulation and beyond

Jilling Bergmann

Stellingen behorende bij het proefschrift

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Jilling Bergmann, 23 november 2011

1 Behandeling met een hoge dosis peginterferon alfa in HCV geïnfecteerde patiënten is over het algemeen veilig maar leidt niet tot verbetering van de behandeluitkomst in vergelijking met de standaard dosis peginterferon. (dit proefschrift)

2 Continue subcutane toediening van standaard interferon alfa in combinatie met ribavirine kan leiden tot een blijvende virologische repons in eerdere peginterferon non-responders. (dit proefschrift)

3 Endogene inductie van interferon alfa na toediening van een orale toll-like receptor agonist kan leiden tot een significante HCV RNA daling. (dit proefschrift)

4 Behandeling met narlaprevir (een NS3 proteaseremmer) gedurende 7 dagen leidt tot een meer dan 4 $\log_{10}$ HCV RNA daling in zowel naïeve patiënten als eerdere non-responders. (dit proefschrift)

5 Toevoeging van ritonavir leidt tot een verbetering van het farmacokinetisch profiel van narlaprevir door remming van cytochroom P450-3A4 waardoor tweemaal daags doseringen mogelijk zijn in plaats van driemaal daags. (dit proefschrift)

6 Over 10 jaar is chronische hepatitis C mogelijk een zeldzame aandoening in de Westerse wereld mits we bereid zijn om ruwweg € 75.000,- voor een antivirale behandeling neer te tellen.

7 Wetenschap lost nooit problemen op zonder 10 nieuwe problemen te creëren. (George Bernard Shaw)

8 Niemand heeft bezwaar tegen evidence-based medische richtlijnen zolang er maar te allen tijde van afgeweken kan worden.

9 De mens gebruikt de eerste helft van zijn leven om zijn gezondheid te ruïneren, terwijl hij de tweede helft op zoek gaat naar genezing. (Leonardo da Vinci)

10 Het beste argument tegen democratie is een conversatie van vijf minuten met een gemiddelde stemgerechtigde. (Winston Churchill)

11 Ik weet niets van muziek, in mijn vakgebied is dat ook niet nodig. (Elvis Presley)

Later in the evenin'
As you lie awake in bed
With the echoes from the amplifiers
Ringin' in your head
You smoke the day's last cigarette
Rememberin' what she said

Here I am, on the road again
There I am, up on the stage
Here I go, playin' star again
There I go, turn the page

(Turn the page, Bob Seger, 1972)

Voor Francesca, Sara en Maurits

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Evolving drug therapies for chronic hepatitis C

Immunomodulation and beyond

Therapeutische ontwikkelingen voor chronische hepatitis C

Immunomodulatie en meer

Proefschrift

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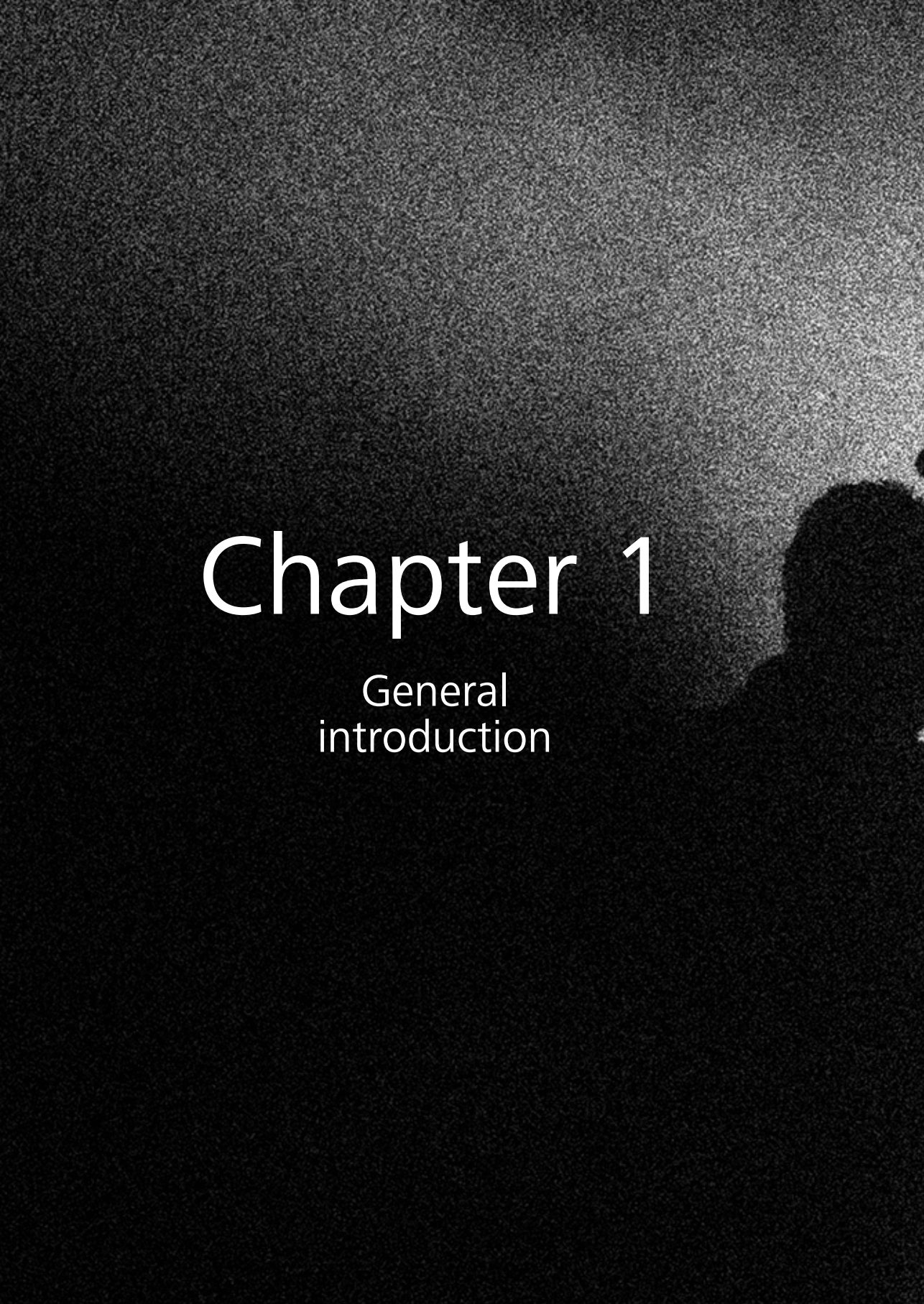

Chapter 1

General introduction

Introduction

Chronic hepatitis C infection is a major health problem and a leading cause of chronic liver disease. The hepatitis C virus was discovered in 1989 (1, 2). The virus is a small, enveloped, single-stranded, positive sense RNA virus and is a member of the hepacivirus genus in the family Flaviridae (3). Six major genotypes have been identified with several subtypes within each genotype (4). Viral replication occurs predominantly within hepatocytes in the liver but there is some evidence that it might also replicate outside the liver, in peripheral blood mononuclear cells, in lymphoid cells and in neurons (5, 6). Chronic hepatitis C infection can cause cirrhosis, digestive hemorrhage, liver failure and liver cancer.

Transmission of the hepatitis C virus (HCV) is mainly related to parenteral contact with blood and blood products. Blood transfusions and the use of shared, nonsterilized needles and syringes have been the main causes of the spread of HCV. After the introduction of routine blood screening for HCV antibody (1992 in most countries), transfusion-related hepatitis C virtually disappeared (7, 8). At present in the developed world, injection drug use is the most common risk factor. Body piercing, tattoos and sharing tooth brushes or razors with an infected person have also been described as risk factors for transmission (9, 10). High-risk sexual behavior of HIV-infected gay men might be a risk factor as well, whereas sexual transmission of HCV between monogamous partners is uncommon (11, 12). There is a small risk of transmission during delivery of a child from an infected mother (13).

An estimated 180 million people worldwide are infected with hepatitis C (14). The prevalence of chronic hepatitis C in Europe and North America is 1.0 to 1.7 percent. The prevalence is higher in Southern Europe, in Asia and in Egypt. In the Netherlands approximately 0.1 to 0.4% of the population is chronically infected (15). Hepatitis C is most prevalent among hemophiliacs who received plasma derived clotting factor concentrates before 1987 (after which time viral inactivation procedures were implemented), among intravenous drug users and among prison inmates (16).

Acute HCV infection is often subclinical and only 20-40% of the patients experience symptoms such as jaundice, nausea, abdominal pain or general unwellness (17). Acute icteric patients are more likely to spontaneously clear the infection than patients with a subclinical infection (18). The virus persists in 60-85% of infected patients, leading to chronic inflammation of the liver. During the early phase of chronic infection, patients are often asymptomatic or experience aspecific symptoms such as fatigue. It may take years or even decades before patients discover that they have chronic hepatitis C infection. Patients may be identified when routine blood tests show elevated liver enzyme values or if there are known risk factors. A small proportion of infected patients (1-2%) develop extrahepatic manifestations of hepatitis C, such as porphyria cutanea tarda, glomerulonephritis, or vasculitis due to cryoglobulinemia (19, 20).

Chronic hepatitis C leads to slow but progressive increasing degrees of fibrosis (21). Fibrosis is the deleterious but variable consequence of chronic inflammation. It is characterized by the deposition of an extracellular matrix component, leading to the distortion of the hepatic architecture with impairment of liver microcirculation and liver cell functions. Usually, HCV infection is lethal only when it leads to cirrhosis, the last stage of liver fibrosis. The rate of fibrosis progression is affected by some host-related characteristics such as the duration of the infection, alcohol abuse and factors like co-infection with hepatitis B virus (HBV) or HIV.

Shortly after the discovery of the hepatitis C virus, interferon alfa was approved as anti-HCV treatment. Although the mechanism of action of interferon has not been fully clarified, it has been shown that interferon has both a direct antiviral effect as well as important interactions with the adaptive and

innate immune reponses (22). Clearance of hepatitis C virus is associated with the development and persistence of virus specific responses by cytotoxic and helper T cells (23). The addition of the synthetic oral guanosine analogue ribavirin led to a significant improvement of treatment outcome. The mechanism of action attributed to ribavirin remains debatable. The goal of antiviral treatment is to prevent complications and death from HCV infection. Several types of virological responses may occur, labeled according to their timing relative to treatment. The most important is the sustained virological response (SVR), defined as the absence of HCV RNA from serum by a sensitive PCR assay 24 weeks following discontinuation of therapy. This is generally regarded as virological cure, although liver cancer has been identified years later, especially if cirrhosis existed at the time of achieving SVR (24). The elimination of circulating HCV RNA is durable in patients achieving SVR (25). SVR is also associated with a good overall clinical outcome and improved health-related quality of life (26).

The current standard of care (SOC) for chronic HCV infection is the combination of a pegylated interferon alfa and ribavirin. The covalent attachment of polyethylene glycol to the interferon molecule (pegylation) reduced the degradation and clearance, prolonged the half-life, and permitted once weekly, instead of thrice weekly, dosing . The choice of this regimen was based upon the results of three pivotal, randomized, clinical trials that demonstrated the superiority of this combination treatment over standard interferon alfa and ribavirin (27-29). The SVR rates following 24 or 48 weeks of treatment with peginterferon and ribavirin are close to 80-90% for genotype 2 or 3 patients, but only up to 50% in genotype 1 and 4 patients. Moreover, peginterferon and ribavirin cause many side effects, such as flu-like symptoms, cytopenia, dermatitis and depression. These side effects significantly hamper treatment outcome.

Several key host and viral factors have been identified as predictors of a favorable virological response and are being used for treatment decision making and personalization of treatment regimens (30). Factors associated with improved clinical outcome include young age (≤ 40 year), low body weight (≤ 75 kg) or low BMI (< 30 kg/m^2), absence of advanced fibrosis, steatosis and insulin resistance, as well as non-black ethnicity. A recent key discovery has shown that a single nucleotide polymorphism (rs12979860) around the gene that encodes interleukin 28B (IL28B) is strongly associated with SVR in genotype 1 patients (31-33). Subsequent genome-wide association studies have shown that this finding might also apply to genotype 2 or 3 infected patients, although to a lesser extent (34-36). Non-HCV genotype 1 and low baseline HCV RNA levels are viral factors associated with SVR (27, 29). Beneficial on-treatment factors include HCV RNA negativity at week 4 (rapid virological response; RVR) and a more than 2 log HCV RNA decline at week 12 (early virological response; EVR) (37, 38). RVR is considered the most valuable predictor of treatment outcome, independent of baseline factors, and enables response-guided therapy (39). Combining IL28B genotyping and RVR monitoring provides a high predictive value for SVR (40).

Optimizing current therapy

Based on viral decline during therapy, treatment duration might be adjusted to increase the chance of achieving SVR or to limit over-treatment of patients who are unlikely to achieve SVR. Viral kinetic studies have shown that there is a dose-dependent direct antiviral effect of interferon, justifying higher induction dosages of interferon, particularly during the first weeks of treatment (41, 42). Some studies have shown that induction dosages of peginterferon can improve treatment outcome (43). Prolongation of therapy duration might improve treatment outcome in a subgroup of patients (especially genotype 1) (44-46). The optimal dosage for ribavirin is not well established, although some studies support higher weight-based ribavirin dosages (47, 48). For patients unresponsive to peginterferon and ribavirin there are limited treatment options. Retreatment with peginterferon and ribavirin of previous non-responders has shown low virological response rates (SVR 7-16%) (49).

Development of new treatment options

Although some patients might benefit from optimized treatment schedules, viral eradication remains unachievable in a substantial part of patients. In addition, peginterferon and ribavirin related side effects affect drug compliance, treatment outcome and quality of life. In this perspective, there is a clear need for effective alternative or additional agents, especially as the burden of disease is expected to increase over the next decade (50). Potential novel antiviral targets are now being identified due to improved understanding of the HCV life cycle (51). During the past 10 years, numerous compounds with antiviral activity against HCV have been developed, which are referred to as direct acting antiviral agents (DAAs). Two protease inhibitors, telaprevir and boceprevir, have been evaluated in phase III studies and have shown improved antiviral efficacy in combination with peginterferon and ribavirin in naïve and treatment-experienced genotype 1 patients (52-55). These drugs are recently licensed by the FDA in the USA, whereas the registration in Europe is pending. This will radically change the landscape of antiviral treatment for HCV infection.

Aims

In this thesis we aim to examine safety and viral efficacy of optimized interferon-based strategies for treatment of chronic hepatitis C patients. We also investigated several new drugs in phase I clinical trials in order to further improve anti-HCV therapy.

Outline of the thesis

Chapter 1
Introduction.

Chapter 2
In this chapter we review current and developing therapies for chronic hepatitis C infection.

Chapter 3
In this study we investigate whether induction with high-dose peginterferon alfa-2b and prolongation of treatment duration can increase SVR in treatment-experienced HCV infected patients.

Chapter 4
In this randomized dose finding study we assess safety and viral efficacy of continuous subcutaneous high-dose interferon alfa-2b administration combined with ribavirin in treatment-experienced HCV infected patients.

Chapter 5
In this study we assess viral kinetics and immunological parameters during continuous subcutaneous high-dose interferon alfa-2b administration combined with ribavirin in order to identify factors associated with virological response in chronic HCV infected treatment-experienced patients.

Chapter 6
In this multicenter randomized double-blind and placebo-controlled phase I study we assess safety and antiviral activity of ANA773, a toll-like receptor agonist, in chronic HCV infected patients.

Chapter 7
In this study we assess immune activation of ANA773, a toll-like receptor agonist, in healthy volunteers and chronic HCV infected patients.

Chapter 8
In this multicenter randomized double-blind and placebo-controlled phase I study we assess safety and antiviral activity of JTK-652, an HCV infection inhibitor, in healthy volunteers and chronic HCV infected patients.

Chapter 9
In this multicenter randomized double-blind and placebo-controlled phase I study we assess safety and antiviral activity of narlaprevir, an HCV nonstructural protein 3 serine protease inhibitor, in chronic HCV infected patients.

Summary and discussion

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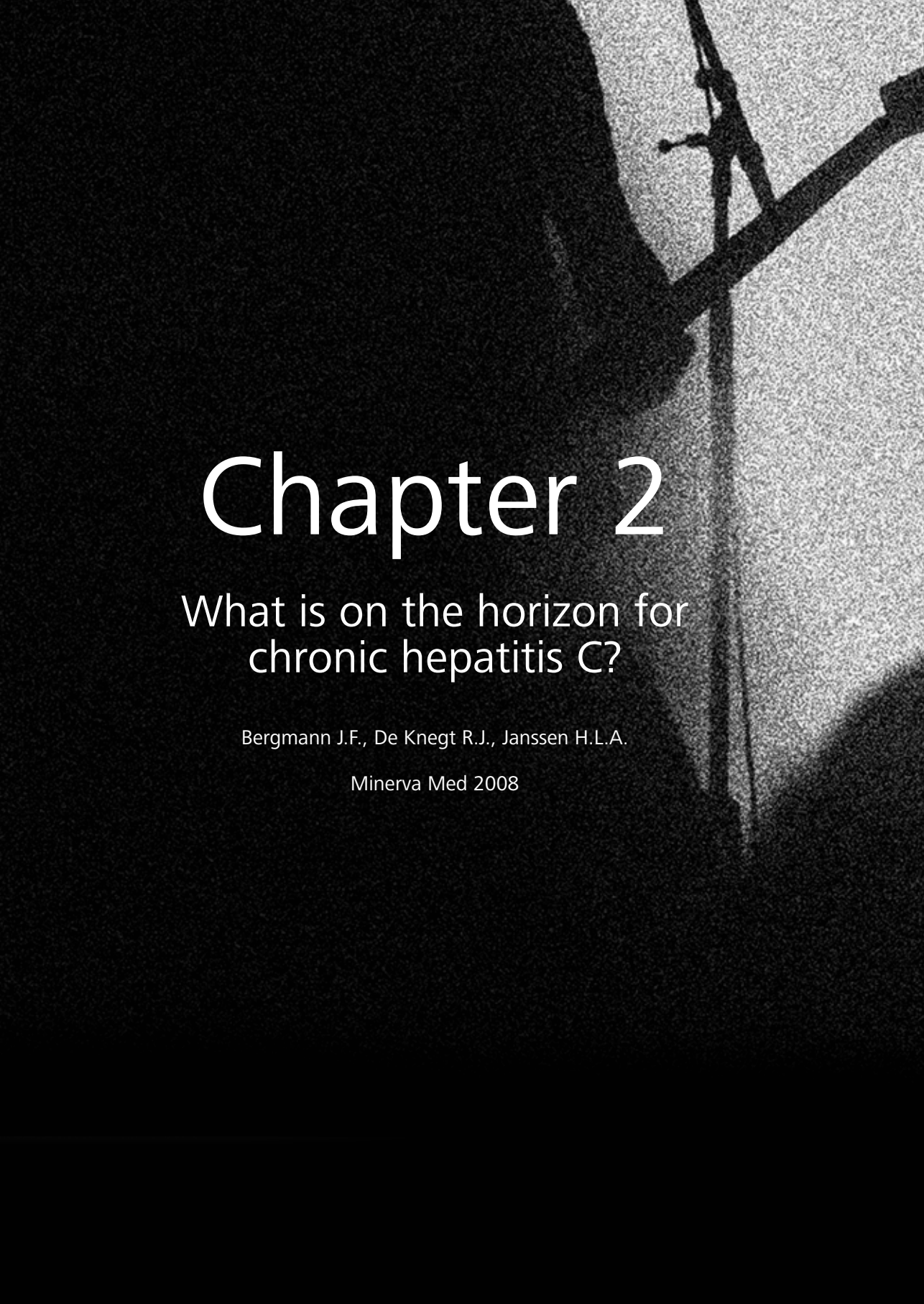

Chapter 2

What is on the horizon for chronic hepatitis C?

Bergmann J.F., De Knegt R.J., Janssen H.L.A.

Minerva Med 2008

Abstract

Current guidelines for chronic hepatitis C recommend peginterferon alfa and ribavirin combination therapy for 24 or 48 weeks, based on viral factors (genotype, viral load), host factors (stage of liver disease) and virological response during treatment. The main goal of treatment is eradication of hepatitis C virus (HCV) infection which is defined by HCV RNA negativity 24 weeks after end of treatment (i.e. sustained virological response, SVR). SVR can be achieved in up to 80% of patients. Most patients, however, experience adverse events during therapy which significantly affect drug compliance and treatment outcome. Several strategies have been evaluated in order to optimize outcome of current peginterferon-based therapy, including higher dosing of peginterferon and/or ribavirin, and adjusting therapy duration. Although some patients might benefit from these optimized treatment schedules, viral eradication remains unachievable in a substantial part of patients. In this perspective, there is a clear need for effective alternative or additional agents, especially as the burden of disease is expected to increase over the next decade. Potential novel antiviral targets are now being identified due to improved understanding of the HCV life cycle. Specifically targeted antiviral therapy for hepatitis C (STAT-C) is in clinical development and has already shown to increase SVR rate. At this moment, however, SVR can only be achieved when combining new molecules with peginterferon therapy. The role of ribavirin has been questioned, but available evidence suggests that ribavirin has significant impact on treatment outcome and should therefore remain part of antiviral therapy. More than a decade of interferon-based therapy and potential new agents will be reviewed.

Introduction

In 1991, shortly after the discovery of the hepatitis C virus (HCV), interferon alfa was approved as anti-HCV treatment. Interferon alfa is a cytokine that has an important function in innate as well as adaptive antiviral immune responses. Attachment to specific receptors leads to activation of the intracellular pathway, involving the Janus-activated kinase and signal transducers and activators of transcription proteins. Activation of this pathway leads to the induction of multiple interferon-stimulated genes (1, 2). However, the SVR rate of interferon alfa monotherapy was below 20% (3). The addition of the synthetic oral guanosine analogue ribavirin led to a significant improvement of treatment outcome with SVR rates up to 45% (4, 5). Despite increased efficacy of combination therapy, the mechanism of action attributed to ribavirin remains debatable. Several mechanisms are suggested, including rapid and lethal mutation of virions or depletion of intracellular guanosine triphophate pools which is necessary for viral RNA synthesis, inhibition of host inosine monophosphate dehydrogenase activity, immune modulation, and minimal direct antiviral activity (6-8). Ribavirin monotherapy has shown decline of serum liver enzymes but without significant effect on HCV RNA levels (9). Further improvement of therapy was seen with the development of pegylated interferon (peginterferon): the covalent attachment of polyethylene glycol to the interferon molecule reduced the degradation and clearance, prolonged the half-life, and permitted less frequent once weekly dosing with sustained interferon levels (10). Initially, peginterferon monotherapy was investigated and several reports in 2000 and 2001 showed that the two available peginterferons (peginterferon alfa-2a and alfa-2b) could double the SVR rate achieved with non-pegylated interferon monotherapy and had similar response rates compared to interferon/ribavirin combination therapy (11-14). These findings paved the way for evaluation of peginterferon and ribavirin combination therapy and three pivotal trials laid the basis for today's standard of care. In these trials the overall SVR rate was 54%-56% and was comparable between weekly weight-based dosing of peginterferon alfa-2b (1.5 µg/kg) and fixed dosing of peginterferon alfa-2a (180 µg). A marked difference was seen in treatment outcome between various HCV genotypes, with an SVR rate for genotype 1 patients of approximately 45%, and for genotype 2 and 3 patients of approximately 80% (15-17). Several scientific institutions have developed guidelines stating that the currently recommended anti-HCV regimen consists of weekly subcutaneous injections of peginterferon and twice daily oral doses of ribavirin (18-20). Duration of treatment depends on genotype and should be 48 weeks for genotype 1 and 24 weeks for genotype 2 or 3 patients. The recommended dose of peginterferon alfa-2a is 180 µg per week and that of peginterferon alfa-2b is 1.5 µg per kilogram of body weight per week. The recommended ribavirin dose should be 1000 mg (bodyweight ≤ 75 kg) or 1200 mg (bodyweight > 75 kg) per day for genotype 1 or 4 patients and 800 mg for genotype 2 or 3 patients. For patients infected with genotype 5 or 6 the same schedule as for genotype 1 patients is recommended, although data for these patients are limited.

This generally accepted standard of care, however, has been further optimized in recent years. Several strategies including higher dosing of peginterferon and/or ribavirin, longer treatment and shorter treatment have been evaluated. Interferon-based treatment is associated with significant adverse effects. Shorter, more effective and safer treatments are needed. The current understanding of the HCV life cycle due to the availability of a sub-genomic replicon system and a full-length replicating HCV genome facilitates the development of specifically targeted antiviral therapy for hepatitis C (STAT-C) (21-23). Some specifically targeted therapies are now being evaluated in combination with peginterferon. In addition, several other new drugs have entered clinical trials (immune modulators, antifibrotics, therapeutic vaccines). Achieving SVR remains the ultimate goal for every individual patient, as improved clinical outcome has been shown in these subjects (24). This review focuses on clinically relevant therapeutic topics as we are moving into a new era of antiviral treatment for chronic hepatitis C.

Optimizing current therapy

The most important factors influencing outcome of peginterferon/ribavirin combination therapy include HCV genotype, HCV RNA load at start of therapy and during therapy, and stage of liver disease. Achievement of rapid virological response (HCV RNA negativity at week 4, RVR), however, is the single most important predictor of SVR, independently of baseline factors. Based on viral decline during therapy, treatment duration might be adjusted to increase the chance of achieving SVR or to limit overtreatment of patients who are more likely to achieve SVR. Viral kinetic studies have shown that there is a dose-dependent direct antiviral effect of interferon, justifying higher induction dosages of interferon, particularly during the first weeks of treatment. Already in the 90s induction regimens were evaluated and peginterferon induction studies are continuing until today. The optimal target dose for ribavirin is not well established. Several studies support higher weight-based dosing of ribavirin.

Longer treatment

Approximately 20% of genotype 1 infected patients experience a slow virological response, defined by HCV RNA positivity at week 12, but negativity at week 24. Recent randomized clinical trials support extension of therapy duration from 48 to 72 weeks in these patients (25-27). Berg et al. showed that slow responders had significantly higher SVR rates when treated for 72 weeks rather than 48 weeks (29% vs. 17%; p=0.04). Moreover, prolongation of therapy duration was considered safe and tolerable (25). The study by Sanchez-Tapias et al. randomized patients based on HCV RNA negativity at week 4 and showed that patients without RVR treated for 72 weeks achieved SVR in 45% compared to 32% in patients treated for 48 weeks (p=0.01). Incidence of adverse events was comparable between both groups, although discontinuation of therapy was significantly higher in patients treated for 72 weeks (26). A study by Pearlman et al. reported likewise findings; slow responders receiving 72 or 48 weeks of combination therapy showed 39% vs. 18% SVR (27). A retrospective analysis performed by Sanchez-Tapias of all participants in the European trials showed 77% vs. 31% SVR in patients achieving early virological response (> 2 $\log_{10}$ decline of HCV RNA at week 12; EVR), but without RVR (28).
Evidence for treatment prolongation in genotype 2 or 3 infection is limited. Overall, SVR is relatively high in these patients, although substantially reduced in patients with advanced liver fibrosis or cirrhosis. This subgroup might benefit from extension of treatment up to 48 weeks (17).

Shorter treatment

Considering shorter treatment for patients with genotype 1 depends on basline viral load and rapid virological response. Although the cut-off value of defining low vs. high baseline viral load is still debatable, it was shown in a prospective trial that patients with baseline HCV RNA levels < 600.000 IU/ml and RVR achieved 89% SVR with 24 weeks of therapy (29). This finding was confirmed for genotype 1 and 4 patients with low viral load (≤ 400.000 IU/ml) and RVR treated with peginterferon alfa-2a and 1000 or 1200 mg ribavirin (79% and 87% SVR in genotype 1 and 4, respectively) (30). Recently, both peginterferons were approved for 24 weeks of treatment in genotype 1 patients with low baseline viral load (< 800.000 IU/ml for peginterferon alfa-2a and < 600.000 IU/ml peginterferon alfa-2b) and RVR. In addition there are several other factors contraindicating shorter treatment, among which cirrhosis and HCV-HIV coinfection.

A considerable amount of studies has demonstrated excellent SVR rates in patients with genotype 2 or 3 infection after reducing treatment to 16, 14 or even 12 weeks of therapy with SVR rates varying from 76-100% (31-36). At this moment, the largest trial in genotype 2 or 3 patients investigating shorter treatment, however, showed significantly higher SVR rates with 24 weeks of treatment compared to 16 weeks (85% vs. 79%; p<0.001), mainly due to increased relapse rates after shorter treatment (31% vs. 18%,; p<0.001) (37). There is insufficient evidence supporting routinely shortening of therapy in

these patients. Patients with low baseline viral load (HCV RNA ≤ 400.000 IU/ml) and RVR have the highest probability of achieving SVR with a shorter treatment. In line with genotype 1 patients, shortening of therapy should not be considered for cirrhotics and HIV co-infected patients. A proposed algorithm for optimization of treatment duration based on RVR is shown in Table 1.

Table 1. Proposed algorithm for optimization of treatment duration based on genotype, baseline viral load, stage of liver disease and achievement of rapid virological response (17, 25-37)

HCV genotype	Baseline viral load*	Cirrhosis	RVR§	Treatment duration¶
Genotype 1 and 4	Low	No	Yes	24 weeks
	High	No	Yes	24 weeks
	Low	Yes	Yes	48 weeks
	High	Yes	Yes	48 weeks
	Low	Yes	No	72 weeks
	High	No	No	72 weeks
	Low	No	No	72 weeks
	High	Yes	No	72 weeks
Genotype 2 and 3	Low	No	Yes	16 weeks
	High	No	Yes	16 weeks
	Low	Yes	Yes	24 weeks
	High	Yes	Yes	24 weeks
	Low	Yes	No	48 weeks
	High	No	No	48 weeks
	Low	No	No	48 weeks
	High	Yes	No	48 weeks

* Cut-off values of 600,000 IU/ml can be used.

§ HCV RNA negativity at week 4 of treatment by qualitative assay.

¶ Shortening treatment duration may moderately reduce SVR rate compared to the currently recommended fixed treatment duration. Prolongation of therapy beyond 24 weeks (genotype 2 and 3) or 48 weeks (genotype 1 and 4) can be considered if the potential benefits outweigh the risk of developing (serious) adverse events.

Increased peginterferon dosing

Higher than standard dosing of interferon has already been studied extensively in the early non-pegylated interferon studies. Viral kinetic studies confirmed that early viral clearance was strongly associated with SVR and could be induced by a short aggressive period of high-dose daily interferon (38, 39). The added value of high dose induction of 4 or 12 weeks followed by standard interferon dosing, however, remained controversial and some stated that the potential benefit did not outweigh the concern of increased toxicity. Despite this, several investigators performed studies with peginterferon induction dosing, especially in patients previously unresponsive to interferon-based therapy. Gross et al. showed that weekly 3.0 µg/kg peginterferon alfa-2b yield superior SVR rates in interferon/ribavirin non-responders (17% vs. 12%; p=0.03) (40). A recent small trial by Diago et al. in genotype 1 non-responders showed an improved likelihood of achieving SVR with 360 µg/week peginterferon alpha-2a induction for 12 weeks followed by 36 weeks 180 µg/week compared to 48 weeks 180 µg/week (SVR 38% vs. 18%) (41). Although the increased efficacy of peginterferon induction seems

modest, most studies report comparable incidence of adverse events between induction and standard regimens (40-42). A four arm study in treatment-naïve genotype 1 patients with body weight >85 kg, evaluating weekly 180 or 270 µg peginterferon alfa-2a in combination with daily 1200 or 1600 mg ribavirin, showed that patients receiving the highest dose of both drugs achieved the highest rate of SVR (47%) and lowest relapse rate (19%), although this arm was less well-tolerated than the lower doses in the other arms (43). A combined peginterferon alfa-2a 360 µg/week induction (12 weeks) and prolongation regimen up to 72 weeks showed significant more SVR compared to a standard 48-week regimen in previous peginterferon alfa-2b non-responders (16% vs. 9%) (44). The issue of peginterferon induction therapy remains controversial but still relevant. As treatment options are limited for previous non-responders, toxicity is comparable to standard therapy and new agents are not yet available, there might be place for induction retreatment.

Increased ribavirin dosing

Although the definite mechanism of action of ribavirin remains to be determined, it is clear that the combination of ribavirin and peginterferon improves end of treatment response, reduces relapse rate and increases SVR rate. The most common side effect of ribavirin is hemolytic anemia leading to dose reductions, reduced quality of life and premature discontinuation. Initially, 1000 or 1200 mg of daily ribavirin was considered sufficiently for genotype 1 or 4 patients, and 800 mg for genotype 2 or 3 patients (17). A number of prospective and retrospective studies has now shown that weight-based dosing can improve SVR rates (15, 45-47). Substantial higher SVR rates were seen in genotype 1 patients receiving 16 mg/kg/day compared to 12 mg/kg/day, although this effect was not confirmed in genotype 2 and 3 patients (48). An interesting pilot study in genotype 1 patients receiving very high ribavirin dosages (range 1600-3600 mg/day) showed SVR in 9 out of 10 patients (49). In this study blood transfusion, erythropoietin treatment and oral iron supplements were used to cope with ribavirin-induced anemia. Supportive erythropoietin therapy has been studied extensively and can improve quality of life, maintain ribavirin dose and improve adherence (50-52). Moreover, a detailed cost-effectiveness study concluded that the concomitant use of erythropoetic growth factors during anti-HCV combination therapy is cost-effective for genotype 1, 2 and 3 (53). A recent study by Shiffman et al., however, could not detect a significant difference in SVR comparing standard therapy (13.3 mg/kg/day ribavirin) with or without epoetin alfa. A third arm with higher weight based ribavirin (15.2 mg/kg/day) and epoetin alpha did show a significant higher SVR rate of 49% ($p<0.05$) (54).

There is evidence for higher dosing of ribavirin, particularly for genotype 1 infected patients. The role of erytropoeietin should be further investigated because a direct positive effect on SVR has not yet been shown.

New immunomodulatory agents

A characteristic feature of the immune response in the majority of chronic HCV infected patients is that HCV-specific CD4+ and CD8+ responses are weak or absent in blood and liver, and are functionally impaired (55-57). Innate and adaptive immune responses need to be restored in order to clear HCV, suggesting that immune modulation will keep on playing a significant role in future anti-HCV therapy. Alternative interferons are being developed and sophisticated devices for interferon administration are being evaluated in order to improve treatment outcome. Moreover, a number of immunomodulatory agents with anti-HCV activity are being investigated in phase I and II clinical trials. An overview of novel agents in clinical development is shown in Table 2.

Table 2. Clinical development of antiviral agents for chronic hepatitis C

Antiviral agents	Phase of development
Immunomodulatory Agents	
Alternative Interferons	
Albinterferon alfa-2b	Phase II
Locteron	Phase II
Omega interferon	Phase II
Inosine Monophosphate Dehydrogenase Inhibitors	
Merimepodib	Phase II
Mycophenolate mofetil	Halted
Toll-Like Receptor Agonists	
Isatoribine	Halted
Resiquimod	Phase II
CPG10101	Halted
Therpeutic Vaccination	
IC41	Phase II
GI-5005	Phase II
Specifically Targeted Antiviral Therapy for HCV (STAT-C)	
Viral Entry Blockers	
HCIG	Phase II
HCV-AB 68	Phase II
Translation Inhibitors	
ISIS14803	Halted
Heptazyme	Halted
Serine Protease Inhibitors	
Ciluprevir	Halted
Telaprevir	Phase III
Boceprevir	Phase III
ACH-806	Halted
TMC435350	Phase II
Polymerase Inhibitors	
Valopicitabine	Halted
R1626	Phase II
Nesbuvir	Halted
Assembly and Release Inhibitors	
UT-231B	Halted
Celgosivir	Phase II
Antifibrotics	
Interferon gamma-1b	Halted
IDN-6556	Phase II

Alternative interferons

Albinterferon alfa-2b is a novel recombinant protein consisting of interferon alfa-2b genetically fused to albumin increasing its half-life by approximately 150 hours. This modification supports subcutaneous injection once every 2-4 weeks and might potentially improve its safety profile (58, 59). A recent phase II trial by Zeuzem et al. in 458 treatment-naïve patients with albinterferon alfa-2b in combination with ribavirin 1000-1200 mg/day, showed similar treatment outcome (approximately 55% SVR) with different dosing schedules, but with significantly more favourable quality of life scores in the albinterferon alfa-2b 900 µg every 2 weeks arm (60). In line with this, a controlled-release recombinant interferon alfa-2b called Locteron has shown a mean 4.7 log decline of HCV RNA at week 12 with 640 µg every 2 weeks by subcutaneous injection plus weight-based ribavirin. This phase IIa dose-ranging study showed strong anti-viral activity combined with an improved safety and tolerability profile compared to currently available interferons (61). A new type 1 interferon called omega interferon shows 60% homology in amino acid sequence with interferon alfa. This drug is designed for continuous delivery for three months by an implantable device. In 102 treatment-naïve genotype 1 patients 36% vs. 6% HCV RNA negativity was seen 12 weeks after 48 weeks of treatment with or without ribavirin, respectively, in an ongoing phase II trial (62). This unique delivery of omega interferon is expected to improve treatment outcome by providing a constant therapeutic level of active drug without peak levels associated with adverse events. A comparable rationale applies to an ongoing phase II trial in genotype 1 and 4 non-responders receiving continuous high-dose interferon alfa-2b administered subcutaneously by a portable pump, originally designed for insulin infusion, in combination with weight-based ribavirin (63).

Inosine monophosphate dehydrogenase inhibitors

The inhibition of inosine monophosphate dehydrogenase (IMPDH) is one of several proposed mechanisms of action of ribavirin. Therefore, several drugs that interfere with IMPDH have been evaluated. In the replicon system, merimepodib has shown potent antiviral efficacy by selective inhibition of the enzyme inosine 5'-monophosphate dehydrogenase which is essential for synthesis of guanine nucleotides. A dose-escalation trial in previous untreated patients did not show an additive effect of merimepodib and interferon compared to interferon monotherapy (64). More recently, however, a phase II trial in previous non-responders showed that peginterferon alfa-2b/ribavirin plus merimepodib could induce SVR, suggesting that further research is justified (65). Mycophenolate mofetil, widely used as an immunosuppressive drug, is another potent inhibitor of IMPDH. A study by Cornberg et al, however, was prematurely discontinued as mycophenolate mofetil in combination with interferon was considered ineffective in previous non-responders (66).

Toll-like receptor agonists

Toll-like receptors (TLRs) are essential mediators of innate immunity and are able to produce cytokines and interferons upon recognition of microbial components or viral pathogens. Agonists of TLRs 3, 4, 7, 8 and 9 have shown anti-HCV activity in the replicon system. Isatoribine is a selective agonist of TLR7 that has shown significant viral load reduction in a proof-of-concept study in 12 treatment-naïve HCV infected patients. Once daily intravenous administration for 7 days showed a low frequency of mild to moderate adverse events, as expected after immune activation (67). Further development, however, was suspended due to safety concerns in animals. Oral twice weekly administration of resiquimod, a TLR7 and TLR8 agonist, showed transient reduction of HCV RNA levels during 4 weeks of treatment. Adverse events in this phase IIa study were similar to interferon alfa (68). CPG10101, a synthetic oligodeoxynocleotide, is a TLR9 agonist that has shown a mean decline of 1.7 log HCV RNA during 4 weeks of therapy in the highest dose group. In this placebo-controlled phase Ib trial in 60 patients, CPG10101 was associated with a dose-related increase in markers of immune stimulation (69). The development of CPG10101, however, was suspended due to economic reasons.

Therapeutic vaccination

Current knowledge of the host immune response against HCV comprises components of innate, cellular and humoral immune responses. Development of neutralizing antibodies is the basis for a successful vaccine, but antibody generation and control of HCV viraemia is a complex process for several reasons. Lack of adequate study models hamper current development. The genetic variability of HCV is enormous, and therefore it is difficult to relate neutralizing activity to the virus present in a certain patient. The rapid generation of novel variants under immune selection further challenge candidate vaccines. The current focus lies on restoration of strong HCV-specific T-cell responses. IC41 is a synthetic peptide vaccine containing several HCV-specific cytotoxic and T-helper epitopes. In a phase II study, 60 treatment-experienced patients received 6 vaccinations of IC41. Vaccinations were tolerated well and HCV-sepcific T-cell responses were seen, despite persisting viraemia. A transient >1 log decline of HCV RNA was seen in only 3 patients, although associated with the strongest interferon gamma enzyme-linked immunospot assay values (70). The HCV vaccine candidate GI-5005 encodes for a core-NS3 fusion, and is designed to induce a cytotoxic T-cell response. This vaccine is currently being investigated in a phase II trial in combination with peginterferon and ribavirin in genotype 1 treatment-naïve and experienced patients (71). In addition, a number of candidate vaccines is being developed preclinically and in phase I trials.

Specifically targeted antiviral therapy for HCV

Current knowledge of the HCV life cycle has led to specifically targeted antiviral therapy for hepatitis C (STAT-C), in contrast with the immunomodulating working mechanism of interferon-based therapies. Hepatitis C has a positive RNA genome consisting of a single open reading frame of 9600 nucleoside bases which encodes a large precursor protein of approximately 3000 amino acids. Translation of this polyprotein is initiated by an internal ribosome entry site (IRES) at the 5' untranslated region and subsequently processed by viral and host proteases into at least 10 proteins: the core, envelope 1 (E1), E2, p7, nonstructural (NS) 2, NS3, NS4A, NS4B, NS5A, and NS5B (72). All subsequent proteins are considered potential targets for candidate drugs of which several have progressed into phase II clinical development.

Blocking viral entry

Our current understanding of the HCV entry mechanism is still limited and until now only polyclonal and monoclonal antibodies have been investigated in clinical trials. Polyclonal hepatitis C immune globulins (HCIG) have been shown safe in HCV-infected liver transplant recipients in order to prevent HCV infection. In a phase II study 18 patients received HCIG infusion over 14 weeks in a high or low dose. Although serum ALT levels normalized in most subjects in the high dose group, serum HCV RNA levels were not suppressed at either dose (73). A dose-escalation phase II study in 24 HCV-infected liver transplant recipients receiving HCV-AB 68, a monoclonal antibody, showed substantial decline of HCV RNA on day 2 post-transplantation, but the effect was lost after reducing the dosing frequency at day 7 (74).

Translation inhibitors

Several synthetic nucleic acids have been developed in order to attack specific target RNA sequences and prevent translation, including antisense oligonucleotides, ribozymes, small molecule inhibitors of IRES and small interfering RNA. The HCV 5' untranslated region is of specific interest as this is the most conserved region of the HCV genome. The ribozyme RPI.13919 (Heptazyme), designed to cleave the IRES from the 5' untranslated region of the HCV genome, showed modest antiviral activity in a phase II trial, but development was halted due to severe toxicity in primate studies (75). The phosphorothioate antisense oligodeoxynucleotide ISIS 14803 showed minimal antiviral efficacy with HCV RNA reduc-

tions of 1.2-1.7 log in 3 out of 28 patients. Although this compound moved on to phase II clinical trials, its development was discontinued due to lack of efficacy and safety concerns (76). A number of promising techniques like RNA interference is currently being developed preclinically. Major limitations include the predicted secondary structure of the target RNA sequence and delivery issues.

Serine protease inhibitors

After translation, the HCV polyprotein is processed by cellular and viral peptidases into structural and nonstructural proteins. Inhibition of this step in the HCV life cycle can lead to rapid viral decline. Ciluprevir (BILN 2061), a highly selective potent peptidomimetic inhibitor of HCV NS3/4A, was the first HCV serine protease inhibitor investigated in HCV-infected patients. A significant 2-3 log decline of viral load was seen after 2 days of oral administration in genotype 1 patients, and, to a lesser extent in genotype 2 and 3 patients (77-79). Although these studies provided proof of concept that protease inhibitors are effective in HCV-infected patients, development was halted due to cardial toxicity in primates receiving high doses for several weeks. The most likely agent to enter clinical practice soon is telaprevir (VX-950), a potent covalent, orally bioavailable, peptidometic inhibitor of NS3/4A with an alfa-ketoamide moiety anchoring at the active site. An initial phase I trial in genotype 1 patients showed a marked 4.4 log HCV RNA decline after 14 days of treatment with the 750 mg dose every 8 hours (80). Although the drug appeared well tolerated besides mild gastrointestinal symptoms, virological breakthrough occurred in a subset of patients due to selection of telaprevir-resistant variants. Low-level resistant mutations (V36A/M, T54A, R155K/T, and A156S) and high-level resistant mutations (A156V/T, 36+155, 36+156) have been detected, although resistant variants were replaced by wild-type virus after 3-7 months of telaprevir absence (81). A study in treatment-naïve genotype 1 patients showed a median 5.5 log HCV RNA decline after 14 days telaprevir in combination with peginterferon alpha-2a. This study not only showed the additive effect of peginterferon and telaprevir, but, more importantly, telaprevir-resistant variants remained sensitive to peginterferon alfa-2a (82, 83). Triple therapy, including ribavirin has been investigated in phase II clinical trials (PROVE trials). Final results of the PROVE-1 trial showed 35% SVR in the 12-week treatment arm, 61% in the 24-week treatment arm, 67% in the 48-week treatment arm and 48% in the control arm. Patients in the telaprevir arms received 12 weeks of the triple combination (84). The PROVE-2 trial had a similar design except for inclusion of a telaprevir plus peginterferon alfa-2a 12 week dual combination group. This study showed 36% SVR in the 12-week arm (no ribavirin), 62% in the 12-week arm (triple therapy), 68% in the 24-week arm and 48% in the control arm (85). Importantly, reduced efficacy at week 12 was seen without the administration of ribavirin. However, telaprevir administration is associated with frequent skin reactions, gastrointestinal adverse effects and anaemia leading to increased premature discontinuation of therapy in patients receiving telaprevir. A large phase III trial is currently underway in treatment-naïve HCV genotype 1 infected patients (ADVANCE trial) and is designed to definitely demonstrate the efficacy of telaprevir in combination with peginterferon alfa-2a and ribavirin (86). Another promising new agent is boceprevir (SCH503034), a peptidomimetic NS3/4A protease inhibitor, showing a 2.9 log HCV RNA decline after 14 days of combination therapy with peginterferon alfa-2b in genotype 1 non-responders (87). This compound moved into phase II and interim results of treatment in genotype 1 naïve patients have been presented (SPRINT-1). In this study patients received triple combination therapy with boceprevir 800 mg every 8 hours, peginterferon alfa-2b 1.5 µg/kg and ribavirin 800-1400 mg/day for 24 weeks or standard dual therapy with peginterferon and ribavirin, showing 78% vs. 34% early virological response at week 12, respectively, and SVR12 (virological response 12 weeks after treatment discontinuation) was 57% with triple therapy. Common adverse events included gastrointestinal symptoms and anaemia (88). Resistant mutations to SCH503034 have been identified in the replicon system, and include T54A, V170A and A156S (89). As with telaprevir, this compound is now being investigated in phase III trials, both in genotype 1 treatment-naïve patients (SPRINT-2) and

in patients who failed previous therapy (RESPOND-2) (90, 91). Another new drug, ACH-806 (GS-9132), an acylthiourea compound that inhibits binding of NS4A to the NS3 proteinase and thereby preventing formation of the active proteinase complex, showed an average 0.9 log HCV RNA drop during 5 days of treatment in a phase I trial (92). Further development of this drug was halted due to nephrotoxicity. A phase I study with TMC 435350, another NS3/4A proteinase inhibitor, has shown a median 3.9 log decline in HCV RNA after once daily dosing for 5 days in genotype 1 non-responders (93). This drug was well tolerated and is currently being investigated in a phase II trial in combination with peginterferon alfa-2a and ribavirin, in treatment-naïve and experienced patients (94).

Polymerase inhibitors

There is still limited understanding about the exact mechanisms of HCV replication. The NS5B RNA-dependent RNA polymerase seems to play an essential role in viral replication and inhibition of this enzyme offers a promising target for new antiviral drugs. For this purpose specific nucleoside analogues and non-nucleoside analogues have been developed. Valopicitabine (NM283) is a nucleoside analogue inhibitor of the HCV polymerase, that has shown a dose-dependent additive antiviral effect to that of peginterferon alfa in phase II trials (95, 96). Clinical development of this drug, however, was put on hold due to significant gastrointestinal toxicity at the 800 mg/day dose. R1626, a prodrug of the HCV polymerase inhibitor R1479, is a 4'azido-cytidine nucleoside analogue that showed a mean 5.2 log HCV RNA decline in treatment-naïve genotype 1 patients after 4 weeks of triple combination therapy vs. a mean 2.4 log decline with standard peginterferon alfa-2a plus ribavirin dual therapy. Up to 74% of patients treated with R1626 and peginterferon/ribavirin achieved RVR. Reversible mild to moderate haematological side effects, especially neutropenia, were associated with increased doses of this drug. No viral resistance has been observed until now after either two weeks of monotherapy or after four weeks of combination therapy (97). A non-nucleoside polymerase inhibitor nesbuvir (HCV-796) showed promising HCV RNA decline in various genotypes with a mean 3.3-3.5 log reduction combined with peginterferon alfa-2b vs. a 1.6 log drop with peginterferon monotherapy after 2 weeks (98). No selection of resistant variants was observed in this study. A phase II study, however, in which treatment-naïve and non-responder subjects received peginterferon alfa-2b, ribavirin and nesbuvir triple therapy, was halted due to presumed hepatotoxicity (i.e.: elevated liver enzymes in 8% of patients).

Assembly and release inhibitors

The process of HCV assembly and release is still hypothetical, although there is evidence that virus assembly occurs in the endoplasmatic reticulum. As viral proteins have been detected in the endoplasmatic reticulum and Golgi apparatus it is suggested that they are involved in transit and maturation steps. Iminosugars are believed to cross cellular membranes and concentrate in the endoplasmatic reticulum where they can inhibit protein glycosylation and potentially interfere with virus assembly (99). UT-231B, a glucosidase inhibitor, has been administered in a phase II trial in treatment-experienced patients, but no significant antiviral effect was shown (100). Currently a phase II trial with celgosivir (MX-3253), a prodrug of castanospermine that targets alfa-glucosidase, is ongoing in combination with peginterferon alfa-2b and ribavirin (101).

Antifibrotics

If eradication of HCV cannot be achieved, antifibrotic treatment might be a promising alternative strategy with the potential to improve patient survival. Interferon gamma-1b, a pleiotropic cytokine, has been suggested to reduce collagen production by hepatic stellate cells. A large placebo-controlled trial in 502 HCV infected patients, however, did not show any reversibility of fibrosis during 1 year of treatment (102). Oral IDN-6556 is a potent inhibitor of caspases, the proteases that execute apoptosis, and is suggested to improve hepatic fibrosis by this route. In a placebo-controlled phase II trial signi-

ficant lowering of ALT was seen over a 14-day dosing period. However, no change in HCV RNA load was seen and longer studies are needed to assess potential effects on liver inflammation and fibrosis (103). Another approach of reducing disease progression has been evaluated in the HALT-C trial. In this trial a 3.5 year course of 90 µg/week peginterferon alfa-2a maintenance therapy was evaluated in previous non-responders with advanced fibrosis or cirrhosis. Disease progression, however, was 34.1% in the peginterferon group and 33.8% in the control group, despite significant decline of HCV RNA and ALT levels in the treatment group. None of the primary outcome measures (death, hepatocellular carcinoma, liver decompensation, and increase in fibrosis) showed significant differences between both groups (104). Two other major trials evaluating maintenance therapy are currently ongoing: the COPILOT and EPIC3 trial. In the COPILOT trial, improved disease free survival with peginterferon maintenance therapy was exclusively seen in patients with portal hypertension (105).

Conclusion

Nearly two decades after the discovery of the hepatitis C virus, substantial progress has been made in developing adequate antiviral therapy. Success percentages up to 90% can be achieved in selected patients with optimized peginterferon alfa and ribavirin combination therapy. There lies however a great challenge in treating patients unresponsive to the current standard of care. In addition, the burden of disease will increase over the next decade. A wide range of potential targets for new antiviral drugs is being explored in human trials due to improved knowledge of the HCV replication cycle. A major concern of specifically targeted antiviral drugs is the emergence of antiviral resistance. The high error rate of the HCV polymerase and viral escape are issues to be taken into account when developing new anti-HCV drugs. With regard to that issue, lessons should be learnt from other viral diseases like HIV and HBV. Immune modulation will remain the mainstay of therapy in the near future, although adding a strong antiviral agent can further improve treatment outcome. Safety issues and additional costs might limit exploration of the ideal combination. Nevertheless, optimizing treatment should be resolutely pursued in proper translational and clinical trials.

Shorter, more effective and more tolerable therapies are possibly not yet within reach. However, summarizing recent achievements in the field of hepatitis C, it can be stated that revolutionary developments are ongoing which will boost clinical practice within the next decade.

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Chapter 3

Gamma-glutamyltransferase and rapid virological response as predictors of successful treatment with experimental or standard peginterferon alfa-2b in chronic hepatitis C non-responders

Bergmann J.F., Vrolijk J.M., Van der Schaar P., Vroom B.,
Van Hoek B., Van der Sluys Veer A., De Vries R.A., Verhey E., Hansen B.E.,
Brouwer J.T., Janssen H.L.A., Schalm S.W., De Knegt R.J.

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Abstract

Background: High-dose peginterferon alfa induction and prolongation of therapy may be an option to improve SVR rates among HCV non-responders, although higher and longer dosing of peginterferon alfa may intensify side effects.

Methods: We randomized 53 patients, who previously failed with standard interferon alfa +/- ribavirin, to a high-dose induction and extended regimen with peginterferon alfa-2b (3.0 µg/kg QW 12 weeks —> 2.0 µg/kg QW 12 weeks —>1.5 µg/kg QW 48 weeks) or standard regimen (1.5 µg/kg QW 48 weeks). All patients received daily weight-based ribavirin (800-1200 mg/day). The short-form 36 health survey was used to evaluate health related quality of life (HRQL).

Results: Intention-to-treat analysis showed no significant difference in SVR rate (44% vs. 37%, p=0.62) and relapse rate (9% vs. 31%, p=0.17) between experimental and standard treatment. Overall, patients with rapid virological response (RVR, HCV RNA negativity at week 4) achieved SVR in 80% (PPV). No significant dose-related differences in HRQL were seen between both groups. At baseline, genotype 2 or 3 (OR:7.4, 95%CI:[1.4,33.3], p=0.01) and gamma-glutamyltransferase (GGT) levels <2x ULN (OR:6.76, 95%CI:[1.5,31.3], p=0.009) were significantly associated with SVR. Multivariate logistic regression at week 4 showed that only baseline GGT < 2x ULN (OR:7.3, 95%CI:[1.4,38.5], p=0.01) and RVR (OR:15.6, 95%CI:[3.2,76.9], p<0.001) were independently predictive for SVR.

Conclusion: Retreatment with peginterferon alfa-2b and ribavirin for a minimum of 48 weeks should be considered in all patients unresponsive to previous interferon-based therapies. Baseline GGT values and RVR are highly predictive for retreatment outcome.

Introduction

Chronic hepatitis C virus (HCV) infection is a major cause of liver cirrhosis and hepatocellular carcinoma, and the most common indication for liver transplantation in Europe and the United States (1). The current standard combination therapy with peginterferon alfa and ribavirin yields sustained virological response (SVR) rates of 42-52% among genotype 1 and 76-84% among genotype 2 and 3 who are treatment-naïve (2-4). However, with standard peginterferon/ribavirin therapy the SVR rates among patients unresponsive to previous interferon alfa therapy with or without ribavirin are disappointing (4-18%) (5-8). Improvement of treatment outcomes among non-responders is aggressively pursued and two different strategies have been reported to improve SVR rates: induction dosing with peginterferon alfa (9-11) and prolonging therapy duration (12,13). A small pilot study reported that treatment with a combination of the two approaches (daily high-dose interferon alfa induction for six months and prolongation of therapy up to 76 weeks) can result in SVR rates as high as 67% in difficult-to-treat patients (i.e.: genotype 1, cirrhosis and/or previous non-response) (14). Two recent large trials showed overall SVR of 17% and 18% in previous interferon +/- ribavirin non-responders treated with double-dose peginterferon alfa-2b (3.0 µg/kg) (10,15). Moreover, double dose peginterferon was tolerated well and appeared not to be associated with significant more adverse events.

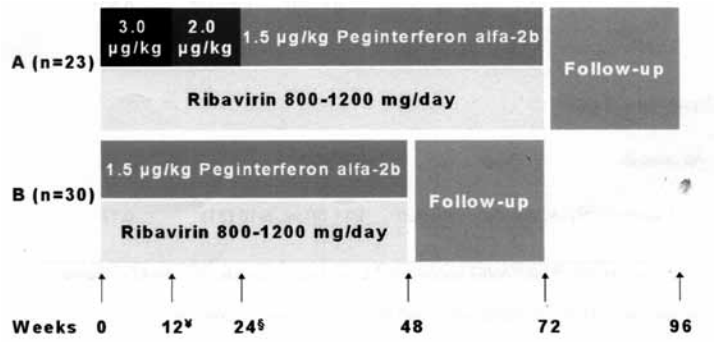
This is a prospective, randomized and controlled clinical trial. Fifty-three patients with chronic HCV infection who had failed to respond to standard interferon with or without ribavirin were randomly assigned to treatment with high-dose induction peginterferon alfa-2b combined with prolongation of treatment duration (72 weeks) or standard peginterferon alfa-2b, both in combination with weight-based ribavirin.

Patients and methods

Study design

This study was a prospective, randomized study of high-dose induction therapy combined with prolongation of therapy duration vs. standard therapy with peginterferon alfa and ribavirin for the treatment of patients with chronic hepatitis C who failed to achieve a sustained virological response to previous treatment with interferon alone or with ribavirin. The patients were recruited from the departments of Gastroenterology and Hepatology of seven Dutch hospitals. After obtaining written informed consent, patients were randomized to one of the following treatment groups (Figure 1):

Figure 1. Treatment schedule



¥ If < 2 $\log_{10}$ decline of HCV RNA at week 12 treatment was stopped.

§ If HCV RNA was positive at week 24 treatment was stopped.

Experimental regimen: 24-week-induction phase (weeks 1-12: peginterferon alfa-2b 3.0 µg/kg once weekly (QW) —> weeks 13-24: peginterferon alfa-2b 2.0 µg/kg QW) followed by 48 weeks of peginterferon alfa-2b 1.5 µg/kg QW. All patients received daily weight-based ribavirin (800 mg/day to 1200 mg/day) from week 1 to 72.
Standard regimen: 48 weeks of peginterferon alfa-2b 1.5 µg/kg QW. All patients received daily weight-based ribavirin (800 mg/day to 1200 mg/day) from week 1 to 48.
Randomization was stratified according to genotype (genotype 1/4 versus non-genotype 1/4), previous outcome (response-relapse vs. non-response) and treating hospital. Treatment was stopped if there was not at least a 2 $\log_{10}$ decline of HCV RNA at week 12 or if HCV RNA was detectable at week 24 by qualitative polymerase chain reaction (PCR).
Originally, recruitment of 55 patients into each treatment group was planned between December 2001 and June 2003. However, in October 2004 inclusion of patients was stopped due to significant lower numbers of patients included per center than originally expected and due to minimal benefit of high dose peginterfeorn reported in the literature. Until then 53 patients were randomized. Hereby the final intention-to-treat (ITT) analysis is presented of those 53 patients who had at least one dose of peginterferon alfa-2b.
The study protocol conforms to the ethical guidelines of the Declaration of Helsinki and was approved by the ethics committees of all seven hospitals.

Patient population

Patients in the outpatient clinic with chronic hepatitis C infection, elevated serum ALT activity documented on at least two occasions, liver biopsy findings consistent with active hepatitis and/or fibrosis and a history of non-response (HCV RNA positivity by qualitative assay during at least 3 months of interferon therapy with or without ribavirin) or relapse to at least three months of interferon therapy with or without ribavirin, were potentially eligible for the study. Patients were excluded if they had evidence of severe concomitant illness, if they had used any investigational drug or any systemic antiviral, antineoplastic or immunomodulatory treatment within 12 weeks prior to the first dose of the study, if they were pregnant or breast feeding, if they were under 18 or over 70 years of age, if they had decompensated liver disease or if they refused or were unable to give consent.

Liver histology

Liver biopsy samples taken within the previous 12 months before screening were analyzed before enrollment by a central pathologist who was unaware of the patient's identity. The severity of the liver disease was assessed by the METAVIR scoring system.

Assessment of HCV RNA

Serum samples were assayed for HCV RNA by quantitative PCR (Cobas Amplicor HCV Monitor test, Roche Diagnostics, Almere, The Netherlands; limit of detection 1000 copies/ml [600 IU/ml]) at baseline, at day 2, 7, 14, 28 and at week 12. Qualitative HCV RNA assays (Cobas Amplicor HCV test, Roche Diagnostics, Almere, The Netherlands; limit of detection 100 copies/ml [50 IU/ml]) were performed at week 24, 48 or 72 and 24 weeks after end of treatment (SVR). Genotypes were identified before the start of treatment by in-house sequence analysis. HCV RNA measurements and genotyping were performed in a central laboratory (Institute of Virology, Erasmus MC, Rotterdam, The Netherlands).

Health related quality of life

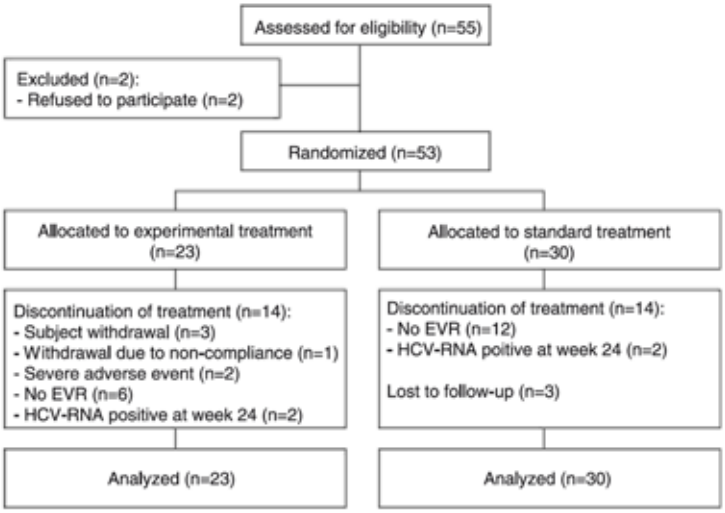
The impact of both treatment arms on the patients' health related quality of life (HRQL) was measured by the generic Short Form 36 (SF-36) questionnaire during screening (before randomization) and at week 4, 24, 48 and 72 or 96 (at end of treatment according to treatment regimen). The SF-36

questionnaire measures eight multi-items: physical functioning (PF), role physical (RP), role emotional (RE), bodily pain (BP), mental health (MH), vitality (V), social functioning (SF) and general health (GH) and was analyzed as recommended by the originators (16). The eight multi-items were also converted into a physical component summary scale (PCS) and a mental component summary scale (MCS) (17).

Statistics

Primary clinical endpoint was SVR rate, defined as HCV RNA 24 weeks after end of treatment. Secondary outcome measures were virological response at week 4 (rapid virological response, RVR), week 12 (early virological response, EVR), week 24 and week 48 or 72 (end of treatment response, ETR).
The study was designed to include 55 patients per treatment group, assuming SVR rates of 60% for experimental vs. 30% for standard treatment in order to achieve 80% power at the 5% level of significance. Comparisons between groups were done using the chi-square test for categorical variables. Logistic regression analysis of the endpoint SVR was performed using prognostic factors known at T=week 0 and T=week 4. The following potential predictors of SVR were studied: genotype (1, 4, 6, vs. 2, 3), medication (experimental vs. standard), fibrosis stage (F4 vs. non-F4), ALT (< vs. ≥ 2x ULN), GGT (< vs. ≥ 2x ULN), GGT/ALT ratio, rapid virological response (RVR; HCV RNA < vs. ≥ 1,000 geq/ml). Performance of the model was checked with the Hosmer-Lemeshow goodness of fit test. Repeated measurement models were used for analysis of HCV RNA, leukocyte count and platelet count over time with week of treatment included as a categorical variable and adjusted for baseline values of HCV RNA, leukocyte count and platelet count, respectively. All data were analyzed using SPSS (version 14.0.1 SPSS Inc., Chicago, IL) or SAS 9.1.3 PROC MIXED Restricted Maximum Likelihood estimation method. All tests for significance and resulting P values were two-sided, with a level of significance of 5%.

Figure 2. Trial profile (intention-to-treat population)



Note: Patients who withdrew from therapy and had negative HCV RNA were encouraged to return for follow-up. Patients lost to follow-up were considered non-responders. According to the protocol, patients without EVR (= early virological response) or patients who were HCV RNA postitive at week 24 were classified as non-responders and discontinued further therapy.

Table 1. Baseline characteristics in all patients and in both treatment groups

Characteristic	All patients (n = 53)	Experimental regimen (n = 23)	Standard regimen (n = 30)
Sex, M/F (% M)	40/13 (75)	17/6 (74)	23/7 (77)
Mean age in years (range)	47 (25-67)	48 (34-67)	47 (25-67)
Length (cm) ¥	174 ± 7	174 ± 8	174 ± 6
Weight (kg) ¥	79 ± 15	79 ± 13	79 ± 16
BMI (kg/m^2) ¥	26 ± 4	26 ± 4	26 ± 4
ALT (U/l) ¥	125 ± 90	115 ± 65	133 ± 106
HCV RNA log (geq/ml) ¥	6.67 ± 0.74	6.63 ± 0.83	6.70 ± 0.69
HCV genotype, n (%)			
Genotype 1	28 (53)	13 (57)	15 (50)
Genotype 2	5 (9)	3 (13)	2 (7)
Genotype 3	10 (19)	3 (13)	7 (23)
Genotype 4	9 (17)	4 (17)	5 (17)
Genotype 6	1 (2)	-	1 (3)
Histological diagnosis, n (%) §			
Fibrosis stage 0	5 (11)	2 (9)	3 (12)
Fibrosis stage 1	8 (17)	4 (18)	4 (16)
Fibrosis stage 2	9 (19)	6 (27)	3 (12)
Fibrosis stage 3	10 (21)	3 (14)	7 (28)
Fibrosis stage 4	15 (32)	7 (32)	8 (32)
Previous treatment IFN, n (%)	18 (34)	8 (35)	10 (33)
Previous treatment IFN/RBV, n (%)	35 (66)	15 (65)	20 (67)
Previous relapse, n (%)	10 (19)	6 (26)	4 (13)
Previous non-response, n (%)	43 (81)	17 (74)	26 ((87)

¥ Mean ± standard deviation. § n = 47. Histological diagnosis was judged by a central pathologist based on liver biopsy prior to the start of treatment. BMI = body mass index; ALT = alanine aminotransferase; geq = genome equivalent; IFN = interferon alfa; RBV = ribavirin.

Results

Patient characteristics

A total of 55 patients were assessed for eligibility. Out of these, two patients refused to participate, whereas 53 patients who received at least one dose of peginterferon alfa-2b were included in the intention-to-treat analysis (Figure 2). Forty men and 13 women with chronic hepatitis C (mean age: 47 years; range 25-67 years) who previously failed to achieve a sustained virological response (including 13 interferon alfa monotherapy non-response, 5 interferon alfa monotherapy relapse, 30 interferon alfa/ribavirin non-response and 5 inteferon alfa/ribavirin relapse) were included. Of these patients, 23 were assigned to receive the experimental peginterferon alfa-2b schedule and 30 to receive the standard peginterferon alfa-2b schedule. Genotypes were equally divided among the treatment arms. In total 38 patients (72%) were infected with HCV genotype 1, 4 or 6, and 15 patients (28%) were infected with HCV genotype 2 or 3. Baseline characteristics of these patients are shown in Table 1. In

Table 2. Virological response rates at different timepoints and relapse rates (intention-to-treat population)

	n/n (%)	P
Sustained virological response (SVR)		
All patients	21/53 (40)	
Experimental vs. standard regimen	10/23 (44) vs. 11/30 (37)	0.62
Genotype 1,4,6, vs. genotype 2,3	9/38 (24) vs. 12/15 (80)	<0.0001
Previous treatment IFN vs. IFN/RBV	12/18 (67) vs. 9/35 (26)	0.004
Previous relapse vs. non-response	6/10 (60) vs. 15/43 (35)	0.14
Mild fibrosis¥ † vs. advanced fibrosis§†	10/22 (45) vs. 9/25 (36)	0.51
Baseline GGT < 2x ULN vs. ≥ 2x ULN€	17/28 (61) vs. 3/23 (13)	0.0005
RVR vs. non-RVR	16/20 (80) vs. 5/33 (15)	<0.0001
Rapid virological response (RVR)‡		
All patients	20/53 (38)	
Experimental vs. standard regimen	10/23 (43) vs. 10/30 (33)	0.45
Genotype 1,4,6, vs. genotype 2,3	7/38 (18) vs. 13/15 (87)	<0.0001
Baseline GGT < 2x ULN vs. ≥ 2x ULN€	15/28 (54) vs. 4/23 (17)	0.008
Early virological response (EVR)*		
All patients	32/53 (60)	
Experimental vs. standard regimen	14/23 (61) vs. 18/30 (60)	0.95
Genotype 1,4,6, vs. genotype 2,3	18/38 (47) vs. 14/15 (93)	0.002
Baseline GGT < 2x ULN vs. ≥ 2x ULN€	23/28 (82) vs. 8/23 (35)	0.001
Relapse rates, n (%)¶		
All patients	6/27(22)	
Experimental vs. standard regimen	1/11 (9) vs. 5/16 (31)	0.17

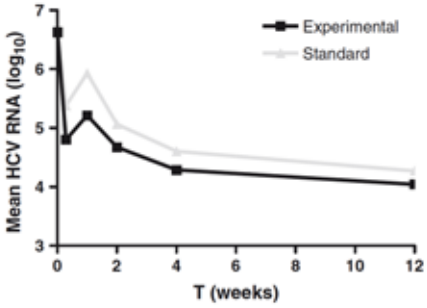
¥ METAVIR score ≤ 2. § METAVIR score ≥ 3. † Liver biopsie available in n = 47. € Baseline GGT available in n = 51. ‡ Polymerase chain reaction negative at week 4. * Polymerase chain reaction negative or at least a 2-$\log_{10}$ reduction at week 12. ¶ Relapse rate after achieving end of treatment response. IFN = interferon-alfa; RBV = ribavirin.

both treatment groups there were no differences with respect to genotype, baseline viral load, fibrosis stage or body weight. In total, 19 out of 23 patients in the experimental arm and 27 out of 30 patients in the standard arm completed the full study period according to protocol. In the experimental group one patient was withdrawn at week 3 due to non-compliance and one patient withdrew at week 8 for personal reasons. Another two patients withdrew at week 22 and 56, respectively, but both achieved SVR. In the standard group one patient was lost to follow-up after week 12. Another two patients were lost to follow-up after achieving ETR. All patients who did not complete follow-up were considered non-responders.

Liver histology

Assessment of liver biopsy samples was performed in 47 patients (89%). The amount of fibrosis in both treatment arms did not differ significantly. There were 10 (46%) patients in the experimental arm and 15 (60%) patients in the standard arm with advanced fibrosis (METAVIR score ≥ 3).

Figure 3. Mean decline of HCV RNA during first 12 weeks according to type of treatment



Note: Repeated measurement analysis showed a trend towards stronger decline of HCV RNA at day 2 and day 7, but the level of significance could not be reached (p = 0.10 and p = 0.06, respectively).

Virological response

Overall SVR rates were slightly higher in the experimental group than in the standard group (44% vs. 37%), however, no significant difference was detected between groups (p=0.62; Table 2). Rapid virological response (RVR) rates (defined as HCV RNA negativity at week 4) were 43% vs. 33%, respectively (p=0.27) and early virological response (EVR) rates (defined as at least a 2-$\log_{10}$ reduction of HCV RNA at week 12) were 61% vs. 60%, respectively (p=0.95). The end of treatment response (ETR) rates were 48% vs. 53% respectively (p=0.69). The relapse rate after ETR was 9% in the experimental arm vs. 31% in the standard arm (p=0.17). One patient in the standard arm experienced virological breakthrough after approximately 36 weeks of treatment.

Subgroup analyses of SVR rates showed no significant benefit of experimental treatment (Table 2). However, patients previously treated with interferon alfa monotherapy appeared to respond better to experimental therapy than standard therapy (88% vs. 50%; p=0.09).

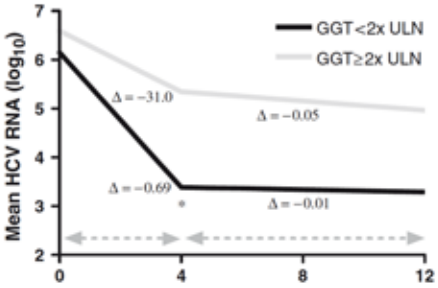
Viral kinetics

A typical biphasic decline of HCV RNA was seen during the first week of treatment (Figure 3). A stronger decline of HCV RNA was seen in the experimental treatment arm during the first 4 weeks; however, repeated measurement analysis showed no significant difference between both treatment groups (p= 0.10 at day 2 and p=0.06 at day 7).

Predictive factors of sustained virological response

At start of treatment (week 0) logistic regression showed that genotype 1, 4, 6 (OR: 0.14, 95% CI: 0.03-0.69, p=0.01) and baseline GGT < 2x ULN (OR: 6.76, 95% CI: 1.47-31.25, p=0.009) were significantly associated with SVR. However, logistic regression at week 4 showed that only baseline GGT < 2x ULN (OR: 7.25, 95% CI: 1.36-38.50, p=0.01) and achievement of RVR (OR: 15.63, 95% CI: 3.23-76.92, p<0.001) were significantly associated with SVR. Log transformation of baseline GGT/ALT ratio also showed a significant association with SVR in univariate logistic regression (p=0.003), but the multivariate logistic regression model performed better with baseline GGT activity alone vs. GGT/ALT ratio (log likelihood 44.5 vs. 47.0). Type of medication, fibrosis stage and basline ALT levels were not significantly associated with SVR. As baseline GGT values were found to be independently predictive of outcome, we compared these levels with HCV RNA decline at week 4 and week 12 (Figure 4). A significant stronger decline of HCV RNA at week 4 was seen in patients with baseline GGT < 2x

Figure 4. Mean decline of HCV RNA at week 4 and week 12 according to baseline GGT values



* p<0.0001. Overall, a significant stronger decline in HCV RNA during the first 4 weeks of retreatment was seen if baseline GGT < 2x ULN (repeated measurement analysis).

ULN (p<0.0001). Patients with baseline GGT < 2x ULN achieved RVR in 54% (15 of 28 patients) vs. 17% (4 of 23) if GGT ≥ 2x ULN (p=0.008) and SVR in 61% (17 of 28) vs. 13% (3 of 23), respectively (p=0.0005). It should be noted that in 2 patients baseline GGT values were not available. In patients with both baseline GGT < 2x ULN and RVR a very high SVR of 93% (14 of 15 patients) was seen.

Positive and negative predictive values at weeks 4 and 12

Overall, 20 patients achieved RVR; of these patients 16 achieved SVR (positive predictive value [PPV]: 80% [95% CI: 61%-92%]). Thirty-three patients did not achieve RVR and of these patients 28 did not achieve SVR (negative predictive value [NPV]: 85% [95% CI: 74%-92%]). The PPV of EVR was 66% [95% CI: 49%-82%] (21 of 32 patients) and the NPV was 100% [95% CI: 87%-100%] (21 of 21 patients).

Adverse events, dose reduction and discontinuation

During treatment adverse effects were seen that were typical of treatment as seen with interferon-based therapy. One or more dose reductions of peginterferon alfa-2b were needed in eight patients. Three patients needed a dose reduction in the experimental arm and five patients in the standard arm. In both groups three patients needed one or more dose reductions of ribavirin.

Discontinuation due to serious adverse events (SAE) was needed in two patients in the experimental arm. One patient attempted to commit suicide after 54 weeks of treatment (while being virus negative) and one patient died because of sepsis due to cholecystitis, after being treated for eight weeks.

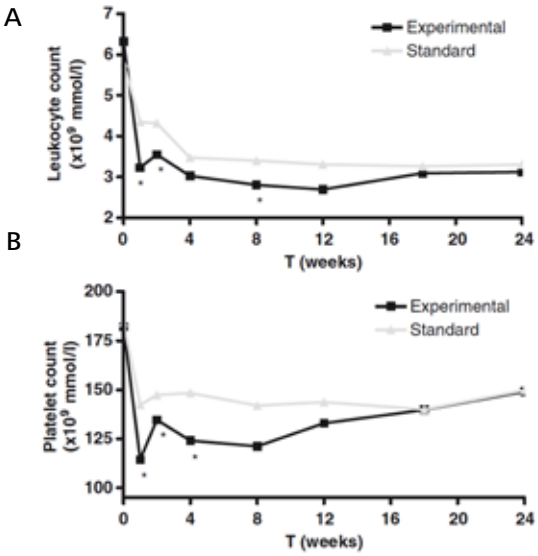
Laboratory abnormalities

A significant decline of leukocytes and thrombocytes was seen in all patients during treatment. Leukocyte count was significantly lower with high-dose induction at week 1 (p=0.016), week 2 (p=0.04) and week 8 (p=0.04). After week 8 there were no significant differences in leukocyte count between both groups (Figure 5). Platelet count was significantly lower with high-dose induction at week 1 (p=0.001), week 2 (p=0.04) and week 4 (p=0.01). After week 4 there were no significant differences in platelet count between both groups.

Health related quality of life

Both physical component summary scales (PCS) and mental component summary scales (MCS) de-

Figure 5. Mean decline of leukocyte count (A) and platelet count (B) during first 24 weeks of treatment (induction phase)



* p<0.05. Significantly stronger decline of leukocyte and platelet count during experimental treatment when compared to standard treatment by repeated measurement model.

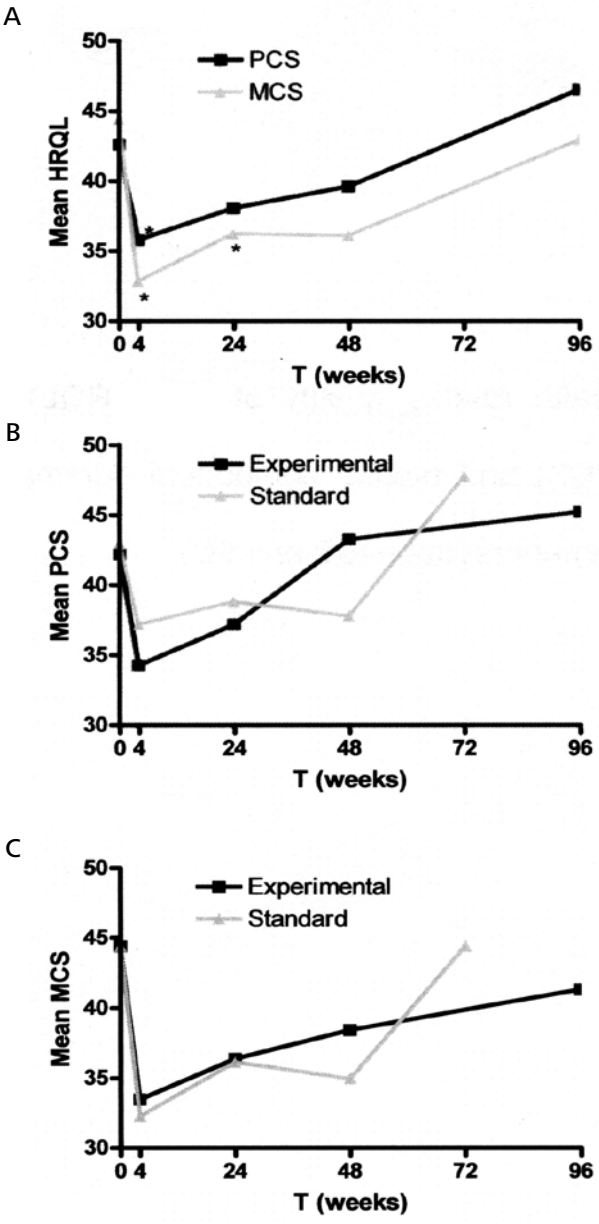
clined significantly at week 4 in all patients (p = 0.01 and p<0.001, respectively) (Figure 6A). At week 24 PCS scores were no longer significantly declined (p = 0.38) compared to baseline, but MCS scores still were (p = 0.03). MCS scores remained significantly lower until week 48 (p = 0.17). At week 96 all summary scales returned to baseline values. Between both treatment groups there were no significant differences in changes of PCS or MCS scores (Figure 6B and 6C).

Discussion

Optimal antiviral treatment for patients with chronic hepatitis C unresponsive to interferon-based therapies is a challenge. In this study we investigated whether induction with high-dose peginterferon alfa and extension of treatment duration from 48 to 72 weeks can lead to increased SVR rates in previous non-responders to interferon with or without ribavirin. Our main finding suggests that retreatment should be considered, especially when favourable predictive factors are present. It should be noted that the groups were small and that stratification for three factors in combination with premature discontinuation of the study has led to unequal randomization. Although the study was eventually underpowered to detect a significant difference between both regimens, it can be considered a pilot study showing that in this heterogenous population the clinical relevance of retreatment is high since an acceptable percentage of SVR was found in patients for whom currently no other treatment is available.

The overall SVR rate of 40% was slightly higher than previously reported. Several studies reported SVR rates of 16-29% when treating non-responders with peginterferon alfa and ribavirin (18-20). In all of these studies, however, standard dose peginterferon was used. Gross et al. (10) have shown that double dose peginterferon alfa-2b vs. standard-dose yield superior SVR rates in interferon/ribavirin non-responders (17% vs. 12%, p = 0.03). In our study no beneficial effect of high dose peginterferon alfa-2b was seen, although double dose was not used during the full treatment period.

Figure 6. Mean decline of health related quality of life (HRQL) according to physical component summary scales (PCS) and mental component summary scales (MCS) (A). PCS and MCS according to treatment regimen (B and C)



* p<0.05. Significant decline of PCS and MCS when compared to baseline value by standard t-test with correction for multiple comparisons (Bonferroni correction).

HRQL = health related quality of life; PCS = physical component summary scale; MCS = mental component summary scale.

Prolongation of treatment has been investigated to improve SVR rates by reducing relapse rate. Recently an improvement of SVR was seen when treating slow-responders with peginterferon alfa-2a plus ribavirin for 48 or 72 weeks (32% vs. 45%, p = 0.01) (13). Retreatment with standard peginterferon alfa-2a for 48 weeks in patients with genotype 2 or 3 with relapse to 24 weeks of peginterferon alfa-2a showed an SVR rate of 63% (21). On the contrary, Berg et al. (22), reported no difference in SVR rates (53% vs. 54%) among treatment-naïve genotype 1 patients who received peginterferon alfa-2a plus ribavirin for 48 or 72 weeks. In our study the relapse rate in the experimental group was lower (9%) than in the standard group (31%), suggesting a decrease in relapse rate.
Adverse effects were observed but there was no significant difference between both groups, although two serious adverse events in the induction group led to treatment discontinuation. Also Gross et al. did not see a significant increase in the incidence of adverse effects with double-dose peginterferon alfa-2b (10). Leukocyte and platelet count declined significantly stronger in the high-dose group, potentially causing more infections and/or bleedings. However, these side effects were not seen more often and some investigators have shown that lower values of both leukocyte and platelet count during peginterferon treatment do not necessarily lead to more side effects (23,24). A significant decrease in quality of life was seen during treatment compared to baseline values and it took more time for the mental component summary scales to return to baseline values compared to the physical component summary scales. A dose-dependent effect could not be detected.

Rapid virological response was predictive of an SVR, offering a useful variable in predicting outcome of retreatment. Baseline GGT levels < 2x ULN were highly associated with SVR. In treatment-naïve patients it has previously been shown that baseline GGT levels were strong predictors of SVR (25). The amount of steatosis might be reflected by GGT levels; as hepatic steatosis might hamper achievement of SVR, a significant lower SVR rate with GGT levels ≥ 2x ULN can be explained. Patients with active alcohol abuse were not eligible for this study, so alcohol was not considered to play a role. A low GGT/ALT ratio was another significant predictor of SVR, previously described by Mihm et al. (26) in treatment naïve patients. This ratio in our study, however, was only dependent on GGT activity and was less strong than baseline GGT levels alone.

In conclusion, peginterferon alfa-2b/ribavirin combination therapy should be considered for all patients unresponsive to standard interferon alfa monotherapy or interferon alfa with ribavirin. In this pilot study an overall SVR of 40% was achieved. Retreatment of genotype 2 and 3 non-responders for 48 weeks or more may even lead to 80% SVR. The presumed beneficial effect of high-dose induction in combination with prolongation of treatment was not seen, although a trend for a reduced relapse rate was observed. With regard to safety aspects a stronger decline in leukocyte and platelet count was seen in the experimental group, but the clinical consequences are probably not significant. Rapid virological response and baseline GGT values are important independent variables in order to predict outcome of retreatment. Definite determination of the role of peginterferon alfa induction and prolongation of therapy in previous non-responders with a minimum of 300 patients is warranted but in view of the rapid development of new antivirals such a study would probably not be relevant.

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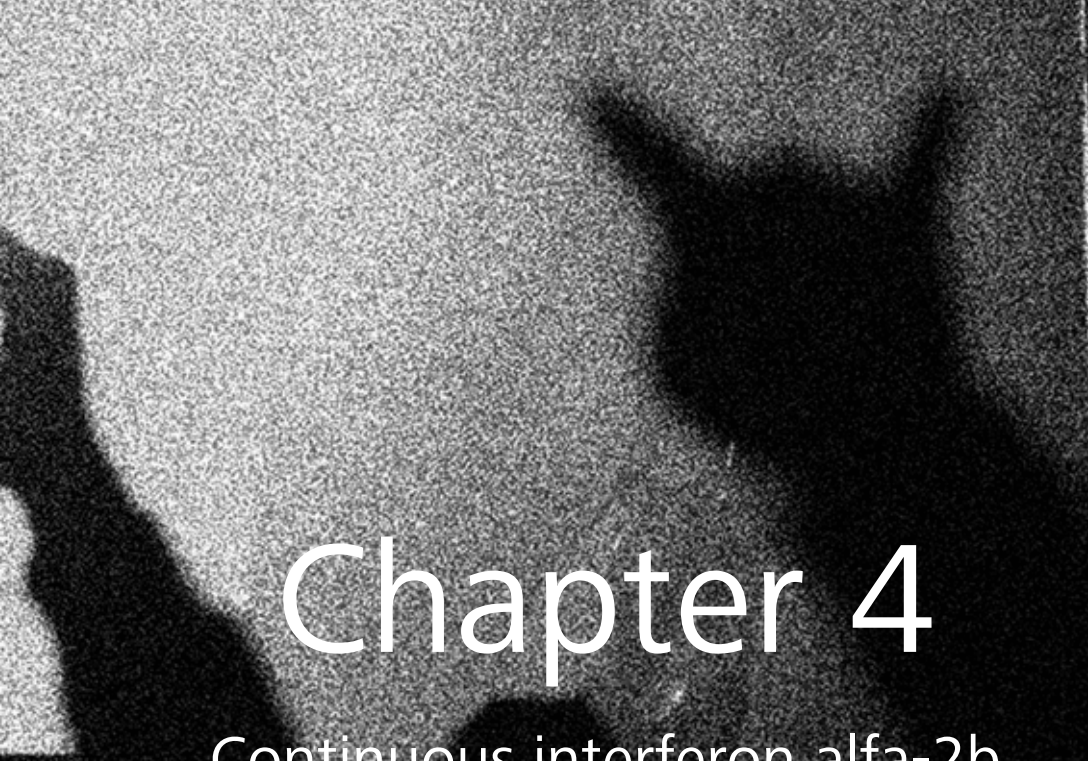


Chapter 4

Continuous interferon alfa-2b infusion in combination with ribavirin for chronic hepatitis C in treatment-experienced patients

Bergmann J.F.*, Roomer R.*, Boonstra A., Hansen B.E., Haagmans B., Kwadijk-de Gijsel S., Van Vuuren A.J., De Knegt R.J., Janssen H.L.A.

*contributed equally

Antiviral Therapy (in press)

Abstract

Background: Sustained virological response (SVR) rates in previous non-responders to peginterferon alfa and ribavirin for chronic hepatitis C (HCV) remain low (~10%). We hypothesize that continuous subcutaneous delivery of fully potent interferon alfa-2b via an external pump will lead to stable blood concentrations and thereby prevent sub-therapeutic trough levels associated with viral breakthrough. The aims of the study were to assess safety, tolerability and virological response in patients who previous peginterferon alfa/ribavirin non-responders.

Methods: We randomized 30 HCV genotype 1 (n = 24) and 4 (n = 6) patients to receive 6, 9 or 12 MU interferon alfa-2b daily by continuous subcutaneous administration using an insulin pump (Medtronic MiniMed 508) in combination with ribavirin (1000-1600mg) for 48 weeks.

Results: The magnitude of viral decline in the 12 MU group after 4 weeks of treatment was 2.67 log HCV RNA compared to 1.21 and 1.27 log HCV RNA in the 9 and 6 MU group respectively (p=0.001). In the intention-to-treat analysis SVR rate was 20% (6/30). The per-protocol SVR rate was 25% (6/24) of which 4 out of 6 patients in the high-dose arm achieved SVR.
Adverse events appeared dose-dependent, were mostly mild to moderate and typical of interferon therapy. Five patients developed irritation and/or abscesses at the injection site. Six serious adverse events were reported in 5 patients.

Conclusions: Continuous delivery of interferon alfa-2b can induce a strong dose-dependent viral suppression. This could be an effective approach in conjunction with, or as lead-in therapy prior to treatment with a direct antiviral agent.

Introduction

Chronic hepatitis C virus (HCV) infection is one of the leading causes of cirrhosis, hepatolocellular carcinoma and end stage liver disease (1). The current standard of care for chronic HCV infection is a 24 or 48 week regimen with peginterferon alfa and ribavirin. This treatment regimen leads to sustained viral responses in 42-82% of patients depending on host factors and viral genotype (2-4). Unfortunately, 50-60% of patients with genotype 1 and 4 do not respond to this treatment regimen, which has led to a continuous increase in the pool of unresponsive patients. Retreatment sustained virological response (SVR) rates of these patients range between 4 and 15%. Increasing this percentage is considered to be a great challenge (5-10).

Pegylation of interferon alfa has improved the pharmacokinetic profile of conventional interferon by maintaining constant blood levels. This enabled once-weekly dosing and resulted in higher response rates. However, it has been shown that the volume of distribution due to pegylation is considerably restricted, decreasing biological activity and potentially decreasing treatment efficacy (11). Continuous exposure to interferon alfa could potentially overcome this hindrance by providing sustained and constant levels of a fully potent protein. We hypothesize that constant levels could induce a stable viral suppression and prevent side effects associated with peaks after injection as well as subtherapeutic drug levels associated with troughs.

The continuous infusion of interferon alfa has been studied in several small phase 1 studies. A significant decrease in serum ALT was observed in one study and continuous infusion of interferon was safe and well tolerated. However, these studies were small and treatment length was inadequate (12-14).

In this pilot study we aim to investigate efficacy, safety and feasibility of continuous subcutaneous infusion of interferon alfa-2b in combination with weight-based ribavirin (15.2 mg/kg) for 48 weeks in patients who previously failed to respond to peginterferon and ribavirin.

Methods

This study, referred to as the SCIN-C study (Subcutaneous Continuous Interferon alfa-2b infusion in Chronic Hepatitis C Previous Non-responders) was an investigator initiated study, sponsored by the Foundation of Liver and Gastrointestinal Disorders (SLO, Rotterdam, the Netherlands). Financial support and infusion devices were obtained from Medtronic Inc. (Minneapolis, Mn, USA).

Study design

The study was a single center, randomized, open label, dose finding study with 3 treatment arms. We randomly assigned 24 genotype 1 and 6 genotype 4 patients in a 1:1:1 ratio to receive 6, 9 or 12 MU interferon alfa-2b per day by continuous subcutaneous infusion in combination with daily ribavirin (Figure 1). Stratified random assignment was used to balance genotype distribution. Ribavirin dosage was weight-based: 1000 mg for patients ≤ 65 kg, 1200mg for 65-80 kg, 1400 mg for > 80-100kg and 1600mg for > 100kg. Patients visited the outpatient clinic at time of screening, at baseline, during treatment (week 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 28, 32, 36, 40, 44 and 48) and during post-treatment follow up (week 52, 60 and 72). In the event of grade 3 adverse events interferon alfa-2b dose was reduced by 1.5 MU/day. In case of an ANC < 750/µl (absolute neutrophil count) or a platelet count < 50000/µl interferon alfa-2b dose was reduced to 75% of the initial dose. This dose reduction was not performed in the first 12 weeks of therapy or in case of HCV RNA positivity after 12 weeks of therapy, considering the negative impact of dose reductions, on virological response. In case of ANC < 375/µl or a platelet count < 25000/µl interferon alfa-2b dose was reduced to 50% of the initial dose regardless of viral load or duration of therapy. When hemoglobin concentration dropped below 5.0

mmol/L interferon alfa-2b dose was reduced to 75%, in case of a drop below 4.0 mmol/l ribavirin dose was reduced to 10 mg/kg daily and patients were treated with blood transfusions.

Patients

Patients were considered eligible for enrollment in this study if they were between 18 and 60 years of age, had chronic hepatitis C infection genotype 1 or 4, were unresponsive to previous peginterferon/ribavirin therapy and had persistently elevated serum ALT or histological evidence of continuing or progressive fibrosis. Non-response to previous therapy was defined as null response (a less than 2 log drop at week 12 during previous therapy), partial response (HCV RNA positivity at week 24), breakthrough (viral breakthrough during therapy) or relapse (viral relapse after therapy). Duration of previous treatment was required to be at least 3 months. All patients had detectable HCV RNA by a polymerase chain reaction (PCR) assay. Patients were excluded if they had signs of decompensated liver disease, evidence of hepatocellular carcinoma (hepatic imaging performed within 3 months prior to screening), other acquired or inherited liver diseases, co-infection with HIV or chronic hepatitis B, significant pulmonary, cardiovascular or renal dysfunction, malignancies in the previous 5 years, history of seizure disorder, uncontrolled thyroid disease, psychiatric disorders, the presence of immunological disorders, pregnancy, breast feeding, and/or active substance abuse (I.V. drugs or > 80 grams of alcohol per day).

Continuous subcutaneous infusion of interferon alfa-2b

For subcutaneous infusion of interferon alfa-2b the MiniMed® insulin pump (Medtronic, Minneapolis, Mn, USA) was used. At time of screening, patients received instructions regarding pump handling and operation. At baseline these instructions were repeated and patients were asked to demonstrate how to handle the Minimed® pump. If patients encountered problems during treatment regarding pump handling, they were instructed to call one of the investigators. Patients received 5 reservoirs with interferon alfa-2b for every 2 weeks on therapy. Reservoirs were replaced every 3 days.

Assessment of safety and feasibity

Safety was assessed by physical examination, laboratory tests and recording of adverse events at every visit during and after therapy. At every outpatient clinic visit patients were asked if any problems regarding to pump handling had occurred. Furthermore the daily interferon alfa-2b dose administration was checked using an automated dosing registry within the pump and patients registered the daily interferon dose and ribavirin dose on drug accountability forms.

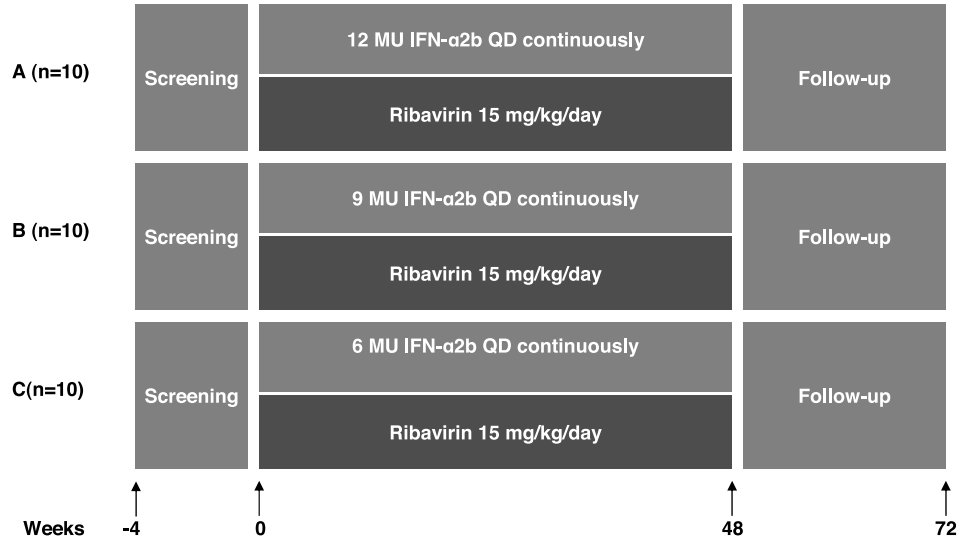
Assessment of virological response

HCV RNA was measured at every visit from baseline until week 12, at week 24, 36, 48 and at every visit during follow up using the VERSANT® HCV RNA 3.0 quantitative assay (branched DNA, lower limit of quantification 615 IU/ml). In case of unquantifiable HCV RNA a qualitative assay (Cobas Amplicor / Cobas TaqMan HCV test v1.0, lower limit of detection < 15 IU/ml) was used for the detection of HCV RNA. Treatment was discontinued in patients with detectable HCV RNA at week 24. Virological endpoints were viral decline during therapy and HCV RNA negativity at week 48 and after 24 weeks of follow up.

Interferon level determination

Interferon alfa-2b concentrations were determined by application of a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) method (BMS216INST, Bender Medsystems Diagnostics GmbH, Vienna, Austria).

Figure 1. Study design



Treatment was discontinued if HCV RNA was detectable at week 24 of treatment using the COBAS TaqMan HCV RNA assay (LLOD < 15 IU/ml).

IL28B genotype determination

The IL28b SNP rs12979860 variants were determined using competitive allele-specific PCR (KASP; KBioscience Hoddesdon, UK).

Statistical analysis

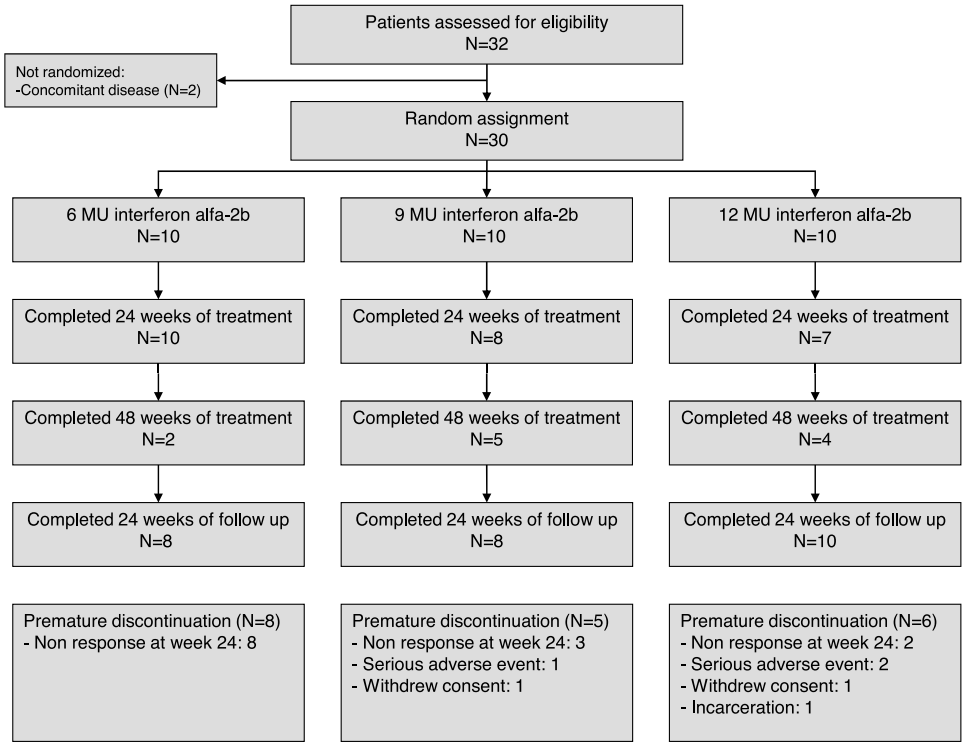
Chi square and Fisher's exact tests were used to compare adverse events and virological responses between 3 different treatment arms. Both parametric and nonparametric tests were performed to compare continuous variables. Linear regression was used to analyze viral decline and virological responses during treatment. Continuous variables are expressed as means ± SD or medians (range) where appropriate. All statistical tests were two-sided, and a P value < .05 was considered to be statistically significant. SPSS version 15.0 was used for all statistical analysis (SPSS Inc., Chicago, IL, USA).

Results

Patients

Patients were enrolled between July 2007 and November 2008. A total of 32 were assessed for eligibility and 30 patients were randomly assigned to 1 of 3 treatment arms (Figure 1). End of follow up data are available in 26 patients (87%) (Figure 2). Baseline characteristics of patients are summarized in Table 1. No significant differences between treatment arms were observed. Patients were predominantly Caucasian (93%) and were infected with HCV genotype 1 (80%). Twenty patients were classified as null or partial responders during previous therapy and 10 patients experienced viral breakthrough during or viral relapse after their previous treatment. IL28B SNP rs12979860 was determined in 29 patients. As expected most patients had the intermediate or poor response variants CT and TT and only 3 patients had the favorable genotype CC. No significant differences between the 3 treatment arms were found.

Figure 2. Study flow diagram



Safety and tolerability

Adverse events, hematologic abnormalities, dose reductions and treatment discontinuations are listed in table 2. All patients had at least one adverse event. In the 12 MU group 5.8% of adverse events were classified as severe compared to 3.3% and 2.2% of adverse events in the 9 and 6 MU groups, respectively (p=0.230). Twenty-four infections occurred in 17 patients. Injection site reactions occurred in 21 patients. Four of these reactions were defined as small skin abscesses at the injection site, all of which improved rapidly after drainage. Severe injection site reactions mostly occurred in the first months of this study probably due to less experience regarding replacement of the infusion sets. In 5 patients injection site reactions were defined as severe and in 2 patients as a serious adverse event (SAE) due to hospital admission. One of these 2 hospitalized patients was diagnosed with cellulitis and antiviral treatment was discontinued temporarily. Some weeks later this patient developed a hyperglycemia induced seizure, the patient's second SAE, and treatment was discontinued permanently. This patient suffered from diabetes mellitus and had not been compliant with insulin therapy. Other SAE's included community acquired pneumonia, diarrhea and an upper respiratory tract infection. In total 6 SAE's occurred in 5 patients; 4 in the 12 MU and 1 in the 9 MU group (p=0.027). All SAE's resolved after temporary or permanent discontinuation of treatment. Four out of 5 patients with SAE's had cirrhosis. Dose reductions due to hematologic abnormalities or adverse events were performed in 5 out of 30 patients (17%): 3 patients from the 12 MU group and 2 patients from the 9 MU group. In 6 patients treatment was discontinued prematurely (4 patients from the 12 MU group and 2 patients from the 9 MU group, p=0.12). Reasons for discontinuation were the occurrence of an SAE in 3 patients, withdrawal of consent in 2 patients and due to incarceration in 1 patient.

Table 1. Baseline characteristics

Variable	Total N = 30	6MU N = 10	9MU N = 10	12MU N = 10	P-value
Male (%)	22 (73)	7 (70%)	6 (60%)	9 (90%)	0.303
Age (years	46.9	46.1	48.2	46.5	0.685
Caucasian/Black/Asian	27/1/2	8/1/1	9/0/1	10/0/0	0.399
Genotype 1 vs. 4	24/6	8/2	8/2	8/2	1.000
Cirrhosis (%)	13 (43)	3 (30)	3 (30)	7 (70)	0.123
IL28B genotype (rs12979860)					
CC/CT/TT	3/20/6	1/6/2	1/6/3	1/8/1	0.863
BMI in kg/m^2 (mean)	27.5	26.6	28.7	27.2	0.664
Response to previous therapy					0.980
-Non response week 12*	9	2	4	3	
-Non response week 24**	11	4	3	4	
-Breakthrough	3	1	1	1	
-Relapse	7	3	2	2	
Mean log HCV RNA	5.45	5.25	5.67	5.46	0.397
Albumin in g/l	42.9	42	44.7	42	0.371
Bilirubin in µmol/l	12.0	10.2	12.5	13.2	0.790
Prothrombin time in seconds	12.2	11.7	12.1	12.7	0.267
ALT in U/l	88.3	88.9	88	88	0.717
GGT in U/l	113.5	151.3	81.9	107.2	0.245
Hemoglobin in mmol/l	9.3	9.2	9.5	9.4	0.889
ANC in cells / µl mean	3.1	3.0	2.8	3.3	0.571
Platelet count in thousands / µl	177	183	192	158	0.249

* Defined as null responder.

** Defined as partial responder.

Interferon levels

Interferon levels increased dose-dependently reaching peak levels between 48 hours and 1 week of treatment followed by continuous steady state levels during the remaining treatment period. Mean interferon levels at week 4 were 344 pg/ml, 264 pg/ml and 225 pg/ml in the 12, 9 and 6 MU group respectively. Between patients a great inter individual variability was observed. A weak negative correlation was found between interferon levels and viral load (r=-0.297, p<0,001).

Viral kinetics

The magnitude of viral decline in the 12 MU group after 4 weeks of treatment was 2.67 log HCV RNA compared to 1.21 and 1.27 log HCV RNA in the 9 and 6 MU groups respectively (p=0.001, figure 3). After 12 weeks viral decline was 3.57 log HCV RNA in the 12 MU group compared to 2.8 log HCV RNA in the 9 MU group and 1.91 log HCV RNA in the 6 MU group (p = 0.075). In the multivariate linear regression analysis interferon alfa-2b dose (p = 0.004) and the IL28b variant (p = 0.017) were significantly associated with viral decline at week 4.

Table 2. Dose reductions, discontinuations, adverse events and laboratory abnormalities

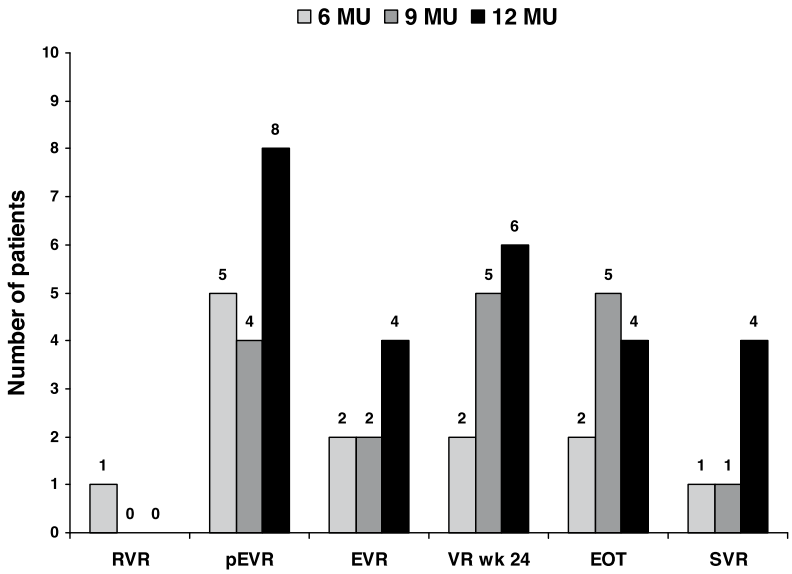
Variable	Total N = 30	12 MU N = 10	9MU N = 10	6MU N = 10
Dose reductions	5	3	2	0
Discontinuations	6	4	2	0
Serious adverse events	6	5	1	0
Infections	24	6	13	5
Adverse events				
- Fatigue	28	8	10	8
- Myalgia	20	8	6	6
- Headache	20	6	9	5
- Nausea	18	7	8	3
- Loss of appetite	18	7	5	6
- Fever	17	5	6	6
- Injection site reaction	17	7	5	5
- Pruritus	13	5	6	2
- Weight loss	13	6	4	3
- Alopecia	13	6	4	3
- Depression	13	4	6	3
- Abdominal pain	12	5	5	2
- Diarrhea	12	4	3	5
- Emotional disorder	11	3	4	4
- Dyspnea	11	6	4	1
- Cough	11	3	6	2
- Concentration impairment	9	5	1	3
- Arthralgia	9	3	4	2
- Rash	9	4	4	1
- Dry skin	9	4	4	1
- Dizziness	9	2	2	5
- Irritability	7	2	1	4
- Back pain	7	2	3	2
- Vomiting	6	3	3	0
- Dry mouth	6	2	3	1
- Obstipation	6	3	3	0
- Epistaxis	6	4	2	0
Other	33	11	9	13
Hematologic abnormalities				
- Anemia*	14	4	5	5
- Neutropenia**	15	5	5	5
- Thrombocytopenia***	6	2	1	3

* Defined as Hb < 6.2 mmol/l.

** Defined as ANC < 750/µl.

*** Defined as Platelet count < 50000/µl.

Figure 3. Viral decline during therapy



The magnitude of viral decline in the 12 MU group after 4 weeks of treatment was 2.67 log HCV RNA compared to 1.21 and 1.27 log HCV RNA in the 9 and 6 MU group respectively (p = 0.001).

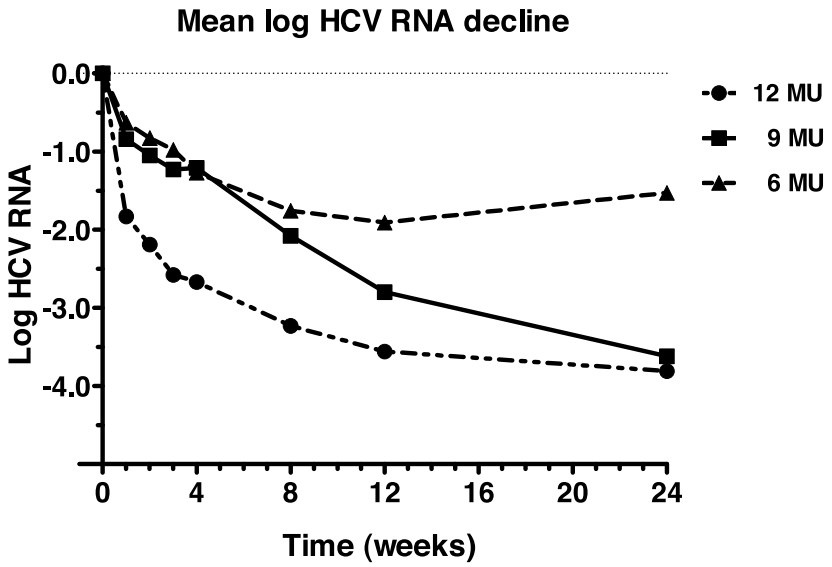
Viral response

In the intention-to-treat analysis 6 out of 30 (20%) patients achieved SVR. Three out of 20 (15%) previous null or partial responders (1 from the 9 MU group and 2 from the 12 MU group) achieved SVR. The remaining 3 patients who achieved SVR were patients with a viral breakthrough during, or relapse after previous antiviral therapy (1 from the 6 Mu group and 2 from the 12 MU group). Of the 6 SVR patients 3 had cirrhosis (all from the 12 MU group). Five out of 24 (21%) genotype 1 patients and 1 out of 6 (17%) genotype 4 patients achieved SVR.

In the per-protocol analysis the SVR rate was 6 out of 24 (25%). Of the 6 patients (67%) treated per-protocol in the 12 MU group, 4 achieved SVR compared to 1 out of 10 (10%) and 1 out of 8 (12.5%) in the 6 and 9 MU group respectively (p = 0.042).

Virological response rates are shown in figure 4. At week 4, one patient (from the 6 MU group) had undectectable HCV RNA. In the 12, 9 and 6 MU group respectively, 4, 2 and 2 patients had undetectable HCV RNA at week 12 (p = 0.384). Eight out of 10 patients from the 12 MU group became HCV RNA negative during therapy compared to 5/10 (50%) and 2/10 (20%) in the 9 and 6 MU group respectively (p=0.027). Two patients of these patients were HCV negative before week 24 (week 8 and 12) but had to stop treatment due to an SAE. Importantly, 10 out of 20 patients with a previous null or partial response to antiviral therapy became HCV RNA negative during therapy (HCV RNA <15 IU/ml).

Figure 4. Virological responses during therapy per treatment arm



RVR: rapid virological response, defined as HCV RNA < 15 IU/ml at week 4; pEVR: partial early virological response, defined as > 2 log drop of HCV RNA at week 12; EVR: complete early virological response, defined as HCV RNA < 15 IU/ml at week 12; VR24: virological response at week 24, defined as HCV RNA < 15 IU/ml; EOT: end of treatment response, defined as HCV RNA < 15 IU/ml at end of treatment; SVR: sustained virological response, defined as HCV RNA < 15 IU/ml at 24 weeks after treatment discontinuation.

All patients with the IL28b rs12979860 CC variant became HCV RNA negative whereas only 50% of patients with the CT (10/20) and TT variants (3/6) became HCV RNA negative (p=0.423). In patients who achieved SVR IL28B rs12979860 variant was CC in 2 patients (one in each of the 6 and 9 MU groups) and CT in 4 patients (all from the 12 MU group). The third CC patient had a cEVR (complete early virological response), however treatment was stopped in this patient in week 21 due to an SAE. In the 12 MU group, virological responses were independent of IL28b genotype.
None of the patients who had less than a 2 log decline of HCV RNA at week 4 achieved SVR and thus the negative predictive value (NPV) was 100%. Thirteen out of 27 patients (48%) had detectable HCV RNA at week 12 of antiviral therapy and none of these patients achieved an SVR (NPV 100%).

Discussion

This pilot study is the first to investigate safety and efficacy of continuous infusion of high daily doses of interferon alfa-2b in combination with ribavirin for a full 48 weeks in chronic hepatitis C patients who previously failed therapy. The rationale of continuous subcutaneous infusion of interferon alfa-2b

was firstly to optimize response by maintaining constant high interferon alfa blood concentrations enabling continuous viral suppression and secondly prevention of side effects associated with interferon peaks which may occur with weekly injections of peginterferon alfa.

Our most important finding was that delivery of interferon alfa-2b in combination with ribavirin resulted in a strong dose-dependent viral suppression. At week 4 two-thirds of patients from the 12 MU group had unquantifiable HCV RNA and at week 12 all patients from this group had unquantifiable HCV RNA. Furthermore, 50% of previous null and partial responders became HCV RNA negative during treatment. Six out of 30 patients (20%) achieved SVR. The per-protocol SVR rate was 25% with 4 out of 6 patients (67%) from the 12 MU group achieving SVR.

This strong viral suppression of high-dose continuous subcutaneous interferon infusion could play an important role within the recently proposed concept of lead-in therapy prior to triple therapy with peginterferon, ribavirin and a direct antiviral agent. In these studies investigating direct antiviral agents, a rapid virological decline during a peginterferon/ribavirin lead-in was crucial to achieve SVR and prevent virological breakthrough or relapse (15-18). Short-term high-dose continuous interferon infusion could help to achieve this necessary rapid viral decline and therefore this concept of continuous interferon alfa could play an important role in the new era of direct antiviral agents. Especially patients with a known null or partial response to previous therapy with peginterferon alfa and ribavirin could benefit from this treatment concept.

In addition, this study reports on the predictiveness of SNPs near the IL28b gene in previous non-responders. As expected most patients had the rs12979860 CT and TT variants that are associated with decreased responsiveness to peginterferon/ribavirin therapy whereas only 3 patients had the favorable CC variant of this SNP. An important finding was that the strong viral suppression in patients treated with 12 MU of interferon alfa-2b was independent of IL28b genotype. These results suggest that the lack of innate interferon responsiveness seen in this group of patients can be overcome by high-doses of continuous interferon alfa-2b infusion. Patients with the CC genotype had a more pronounced virological response compared to CT and TT patients. Response rates between patients with CT and TT variants were comparable.

Side effects were typically interferon related and severity increased as dose increased. Weekly peaks of side effects did not occur, however intensity of side effects appeared comparable to treatment with peginterferon and ribavirin. Five patients developed skin abscesses and severe injection site reactions. These reactions occurred only in the early phase of the study and could be prevented later on in the study by adequate instructions for replacement of the injection cannula (infusion sets). Most serious adverse events occurred in cirrhotic patients, for this reason caution is warranted in this patient group. To prevent infectious complications antibiotic prophylactic therapy should be considered. Dose reductions and discontinuation occurred only in the 9 and 12 MU groups. The most common reasons for dose reductions were adverse events and hematologic abnormalities.

Interferon levels were measured throughout the study, all patients reached steady state levels in the first weeks of treatment. The pharmacokinetic profile of continuous infusion of interferon alfa differs from that of treatment with peginterferon alfa-2a or alfa-2b and ribavirin, which causes peak and trough concentrations. A study investigating pharmacokinetics of peginterferon alfa2a and alfa-2b showed that undetetectable trough concentrations of peginterferon alfa-2b can occur in some patients (19). This phenomenon did not occur in our study.

A great interindividual variability of interferon alfa concentrations exists between patients. For this reason it is difficult to use these concentrations for prediction of treatment outcome. To our knowledge, to date, no data have been published on the predictiveness of (peg)interferon alfa concentrations on treatment outcome.

Approximately 50-60% of genotype 1 patients are non-responsive to treatment with peginterferon alfa and ribavirin for chronic hepatitis C. Several treatment options to cure these difficult-to-treat patients have been investigated. Retreatment of genotype 1 patients who previously failed peginterferon alfa and ribavirin therapy with peginterferon alfa-2b and weight based ribavirin lead to SVR rates up to 11% (8). Comparable SVR rates were achieved with retreatment of true non-responders with consensus interferon and ribavirin (5). Studies investigating peginterferon induction therapy could not demonstrate an increase in SVR rates. However, early virological response rates were increased in patients receiving induction therapy (6-7, 9-10). The highest SVR rates were achieved when combining a peginterferon alfa-2a/ribavirin induction regimen with an extension of the treatment duration to 72 weeks (7). Sixteen percent of these patients, who were non-responsive to previous therapy with peginterferon and ribavirin, achieved SVR.

In our study, treatment with interferon alfa-2b and ribavirin by continuous infusion led to an intention-to-treat SVR rate of 20%, this was nearly twice as high compared to the SVR rate of retreatment with peginterferon alfa and ribavirin reported so far (8). A limitation of this study is the lack of a control arm. However, retreating previous null or partial responders to peginterferon and ribavirin with the same regimen, will have limited chances of SVR.

In conclusion, continuous delivery of high doses of interferon alfa-2b with a pump device can be carried out successfully in this difficult-to-treat population. If side effects are managed adequately, continuous delivery of interferon alfa-2b can induce a strong dose-dependent viral suppression leading to improved SVR rates. Short-term continuous interferon treatment could also be an effective approach in conjunction with or as lead-in therapy prior to treatment with a direct antiviral agent, especially in difficult-to-treat populations.

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The authors would like to thank Lena van Santen, Lucille Maarschalkerweerd, Melek Polat, Edith Bouwmeester, Gaalda Ramdjan and Irene Brings from the clinical research bureau for their assistance in study coordination and data management. In addition the authors would like to thank Bill van Antwerp and Rachael Scherer from Medtronic inc.

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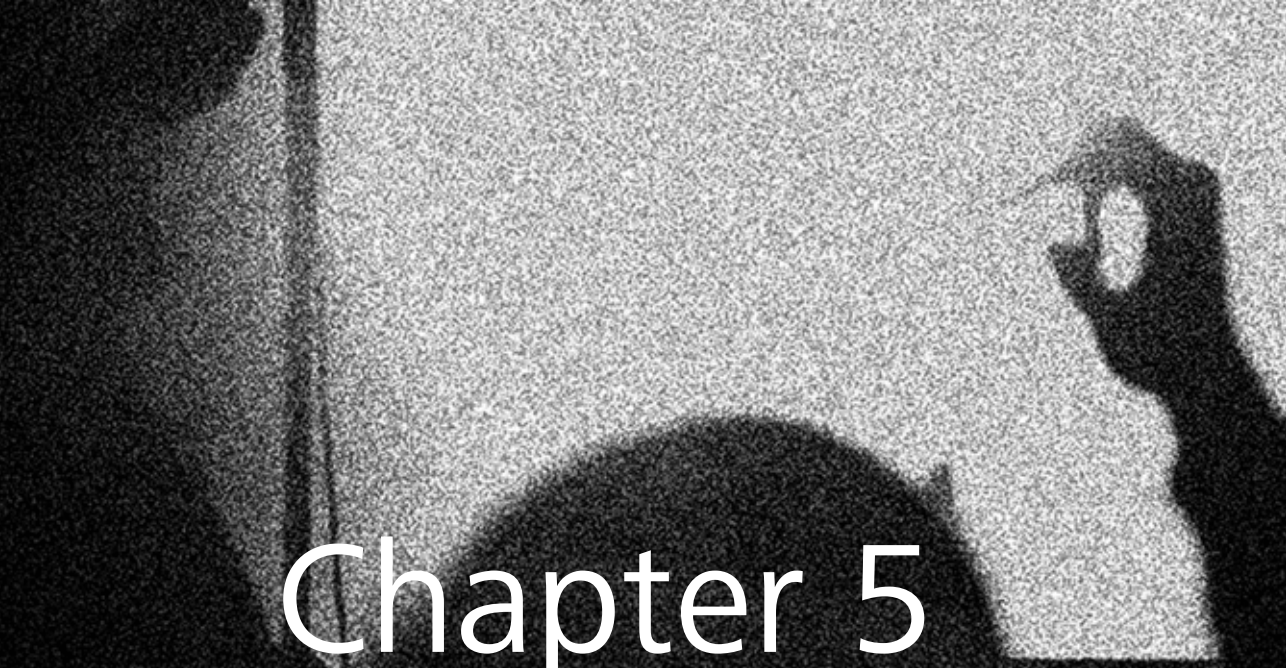


Chapter 5

Viral kinetics and immune activation during continuous subcutaneous administration of interferon alfa-2b in treatment-experienced patients chronic hepatitis C patients

Bergmann J.F., Roomer R., Haagmans B., Hansen B.E., De Knegt R.J., Janssen H.L.A., Boonstra A.

Manuscript in preparation

Abstract

Background: Retreatment outcome of chronic HCV-infected patients with peginterferon/ribavirin is generally poor after previous non-response or relapse.

Aim: To compare viral kinetics and markers of immune activation between treatment-experienced HCV infected patients receiving two different dosages of continuously administered interferon alfa-2b, which resulted in a significant difference in viral response at week 4.

Methods: Patients received 12 MU interferon alfa-2b (n=10) or 9 MU (n=10) per day by continuous subcutaneous administration combined with weight-based ribavirin. HCV RNA levels, interferon alfa levels, the enzymatic activity of 2,5-oligoadenylate synthetase (2,5-OAS) and levels of neopterin and beta2-microglobulin were measured in serum at T = 0, 4, 8, 12, 24, 48, 72, 96 hours and at week 1, 2, 3, 4 after start of treatment. Sustained virological response was based on HCV RNA negativity 24 weeks post-treatment.

Results: A typical biphasic viral decline was demonstrated with a significant stronger median HCV RNA decline at day 2 (-1.57 log vs. -0.59 log; p=0.006) and week 4 (-2.58 log vs. -0.84 log; p=0.017) in patients receiving 12 MU interferon per day versus 9 MU interferon per day, respectively. The significant stronger HCV RNA decline in the 12 MU dose group was reflected by higher neopterin peak levels at day 2 and higher steady levels up to week 4 (3.75 ng/ml vs. 2.40 ng/ml; p=0.027). No significant differences were found between the two dose groups with regard to serum interferon levels, 2,5-OAS activity and beta2-microglobulin levels. All patients that achieved SVR (4 patients in the 12MU dose group vs. 1 patient in the 9 MU dose group) had a more than 2 log viral decline at week 4.

Conclusion: A strong HCV RNA decline at week 4 can be induced by high dose continuous interferon therapy in patients who failed a previous course of peginterferon and ribavirin. A more than 2 log viral decline at week 4 is essential for achieving SVR. Strong viral suppression is associated with high neopterin levels.

Introduction

Chronic hepatitis C virus (HCV) infection is a major public health problem leading to liver fibrosis, cirrhosis and hepatocellular carcinoma (1). An estimated 180 million people are infected worldwide (2). It is expected that HCV related mortality will continue to increase over the next two decades (3). The current standard of care with pegylated interferon alfa and ribavirin results in sustained virological response (SVR) in approximately 40-50% of genotype 1 infected patients (4-6). Phase III clinical trials with protease inhibitors (telaprevir and boceprevir) have shown that SVR rates can increase up to 80% in treatment-naïve patients and up to 65% in treatment-experienced patients (7-10). These direct antiviral agents show strong, rapid viral declines and are expected to enter clinical practice soon, but need to be combined with peginterferon (and ribavirin), stressing the continued importance of achieving immunological control during anti-HCV therapy.

Viral kinetic analyses have provided insight into different response types during (peg)interferon-based therapy and are important for prediction of SVR (11). Responders to therapy show a typical biphasic decline of HCV RNA with a rapid first phase lasting for approximately 24-48 hours followed by a slower second phase (12-15). Subsequently, marked elevation is seen of several serum markers that are known to reflect the induction of antiviral activity and systemic immune activation, including 2,5-oligoadenylate synthetase (2,5-OAS) activity, neopterin and beta2-microglobulin levels (16-19). The levels of these interferon-induced markers are indicative of the antiviral efficacy of peginterferon and have been implicated as factors determining the clinical outcome of therapy (18, 20).

In the present study, we sought to analyze differences between interferon-inducible markers in treatment-experienced patients with chronic hepatitis C who were treated with continuous subcutaneous interferon infusion in our earlier randomized SCIN-C trial (21). The rationale for continuous interferon delivery by an external pump included achievement of sustained and constant levels of the interferon protein, resulting in increased antiviral activity and biologic potency. This randomized trial showed a significant stronger mean decline in viral load at week 4 in patients receiving continuously 12 MU standard interferon per day combined with weight based ribavirin (-2.67 log HCV RNA) versus 9 MU (-1.21 log HCV RNA) and 6 MU per day (-1.27 log HCV RNA), respectively. In the current analysis we sought to explain differences in viral decline between patients in the two highest dose groups (12 MU and 9 MU standard interferon per day) based on early viral kinetics, markers of immune activation and interferon levels.

Methods

Study design

The characteristics of the chronic hepatitis C patients who participated in this study have been described in detail before (21). This was a single center, randomized, open label, dose finding study and was performed in accordance with Good Clinical Practice and the World Medical Association Declaration of Helsinki, after approval by the institutional review board We randomly assigned 24 genotype 1 and 6 genotype 4 patients in a 1:1:1 ratio to receive 12, 9 or 6 MU standard interferon alfa-2b per day by continuous subcutaneous infusion in combination with daily weight-based ribavirin (approximately 15 mg/kg). Patients were treated for 48 weeks, but discontinued therapy at week 24 when HCV RNA positive. All patients provided written informed consent before participating in any study-related activity. For the ancillary study the two cohorts of chronic HCV infected patients receiving 12 MU or 9 MU interferon per day were evaluated for viral kinetics and markers of immune response (Figure 1). All patients in the main study were asked to participate in the ancillary study which included extra serum sample collections during the first week at T = 4, 8, 12, 24, 48, 72, 96 hours after onset of therapy.

Informed consent for extra sample collections during the first week of therapy was obtained in 7 out of 10 patients in the 12 MU dose group and 8 out of 10 patients in the 9 MU dose group.

Patients

Patients were considered eligible for enrollment in this study if they were between 18 and 60 years of age, had chronic hepatitis C infection genotype 1 or 4, were unresponsive to previous peginterferon/ribavirin therapy and had persistently elevated serum ALT or histological evidence of continuing or progressive fibrosis. Non-response to previous therapy was defined as null response (a less than 2 log drop at week 12 during previous therapy), partial response (HCV RNA positivity at week 24), breakthrough (viral breakthrough during therapy) or relapse (viral relapse after therapy). Duration of previous treatment was required to be at least 3 months. All patients had detectable HCV RNA in serum by a polymerase chain reaction (PCR) assay. Patients were excluded if they had signs of decompensated liver disease, evidence of hepatocellular carcinoma (hepatic imaging performed within 3 months prior to screening), other acquired or inherited liver diseases, co-infection with HIV or chronic hepatitis B, significant pulmonary, cardiovascular or renal dysfunction, malignancies in the previous 5 years, history of seizure disorder, uncontrolled thyroid disease, psychiatric disorders, the presence of immunological disorders, pregnancy, breast feeding, and/or active substance abuse (I.V. drugs or > 80 grams of alcohol per day).

Viral assessment

Serum samples were collected at T = 0, 4, 8, 12, 24, 48, 72, 96 hours and at week 1, 2, 3, 4 after start of treatment. Sustained virological response was based on HCV RNA negativity 24 weeks post-treatment. HCV RNA measurements were performed by the VERSANT® HCV RNA 3.0 quantitative assay (branched DNA, lower limit of quantification 615 IU/ml). In case of undetectable HCV RNA 24 weeks post-treatment a qualitative assay (Cobas Amplicor / Cobas TaqMan HCV test v1.0, lower limit of detection < 15 IU/ml) was used for the detection of HCV RNA.

Interferon level determination

Interferon levels were assessed at the same timepoints as HCV RNA levels. Interferon alfa-2b concentrations were determined by quantitative sandwich enzyme-linked immunosorbent assay (ELISA) method (Bender Medsystems Diagnostics GmbH, Vienna, Austria).

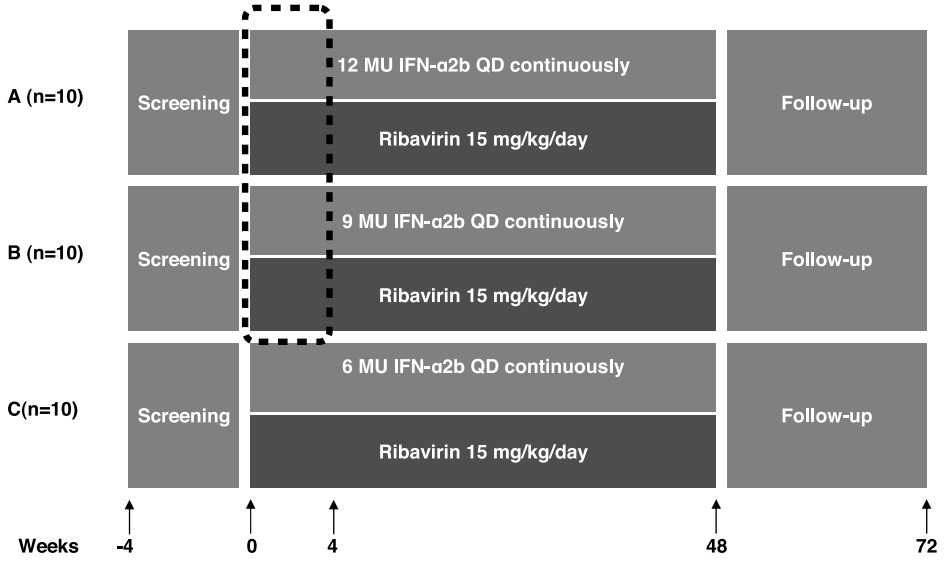
Serum markers of immune activation

Activity of 2,5-oligoadenylate synthetase (2,5-OAS) activity, neopterin and beta2-microglobulin levels were assessed at the same timepoints as HCV RNA levels and interferon levels. Neopterin and beta2-microglobulin levels were analyzed by ELISA (DRG Diagnostics, Marburg, Germany). Activity of 2,5-OAS was analyzed by radioimmunoassay (Eiken, Tokyo, Japan).

Statistics

No formal sample size calculations for this study were performed. The determination of the sample size was based on empirical considerations rather than statistical justification. The sample size of 10 patients in each dose group was considered appropriate for this type of study. Both parametric and nonparametric tests were performed to compare continuous variables. Continuous variables are expressed as means or medians where appropriate. All statistical tests were two-sided, and a p-value less than 0.05 was considered to be statistically significant. SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) and SAS 9.2 (location) were used for all statistical analyses.

Figure 1. Study design SCIN-C



The study period of this ancillary analysis is marked in black.

Figure 2. Viral decline according to dose

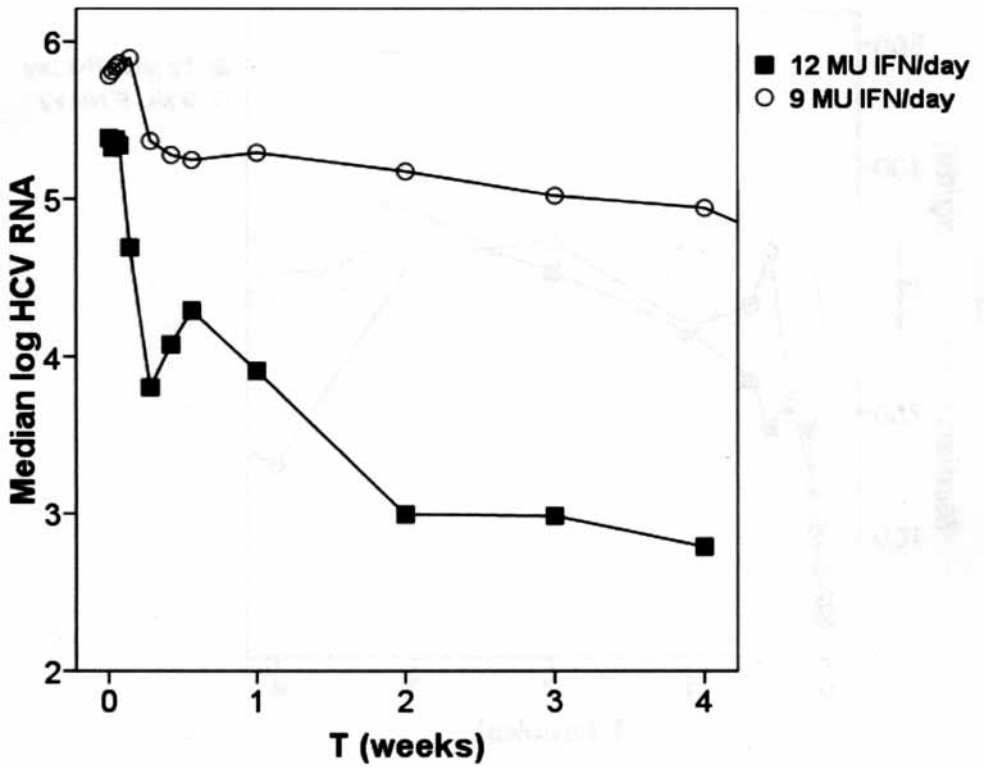
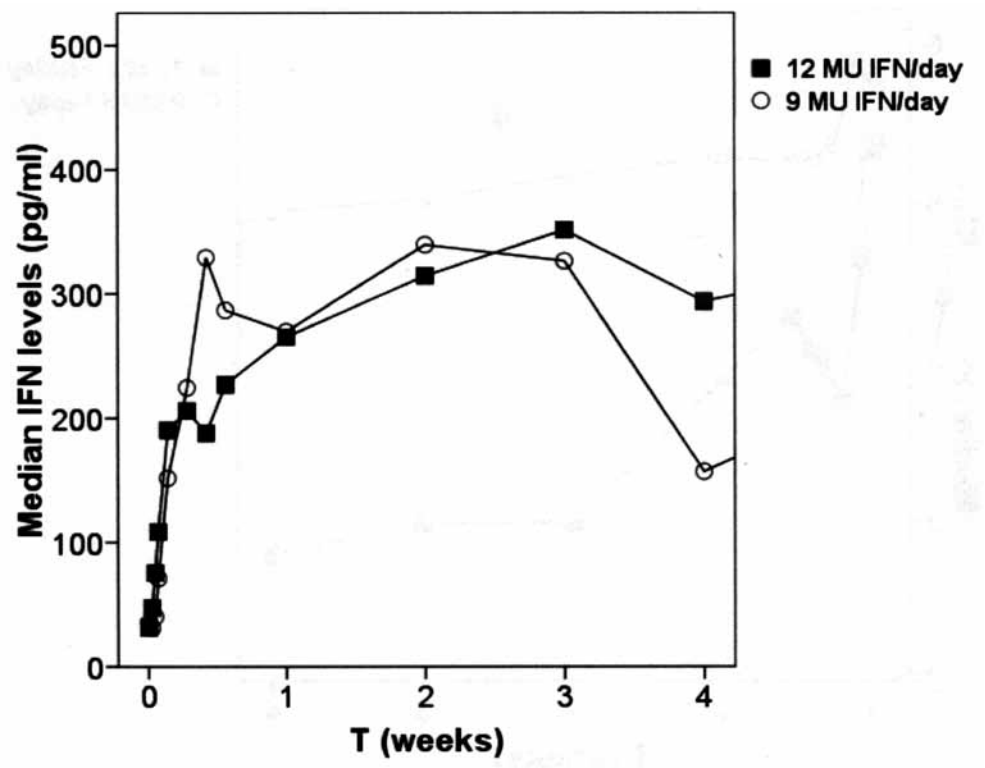


Figure 3. Interferon levels according to dose



Results

Patients

Baseline patient characteristics have been described previously and were comparable between both groups (21). In the 12 MU dose group 90% of patients were male versus 60% in the 9 MU dose group. The mean age was 46.5 versus 48.2, respectively. Median baseline viral load was 5.39 log versus 5.78 log HCV RNA, respectively. In the 12 MU dose group one patient discontinued therapy after 2 weeks. Adverse events were typically interferon/ribavirin related and have been described previously (21).

HCV RNA levels

A typical biphasic viral decline was seen in most patients. The strongest decline was seen in patients receiving 12 MU interferon per day (Figure 2). Approximately 48 hours after onset of therapy the strongest first phase decline was seen, followed by a much slower second phase decay (both dose groups). The median HCV RNA drop at day 2 was 1.57 log in 12 MU dose group versus 0.59 log in the 9 MU dose group ($p=0.006$). The median HCV RNA drop at week 4 was 2.58 log in the 12 MU dose group versus 0.84 log in the 9 MU dose group ($p=0.017$). SVR was achieved in 4 patients in the 12 MU dose group and in 1 patient in the 9 MU dose group. All patients achieving SVR after 48 weeks of therapy had more than 2 log decline of HCV RNA at week 4.

Figure 4. Interferon levels in individual patients (pg/ml)

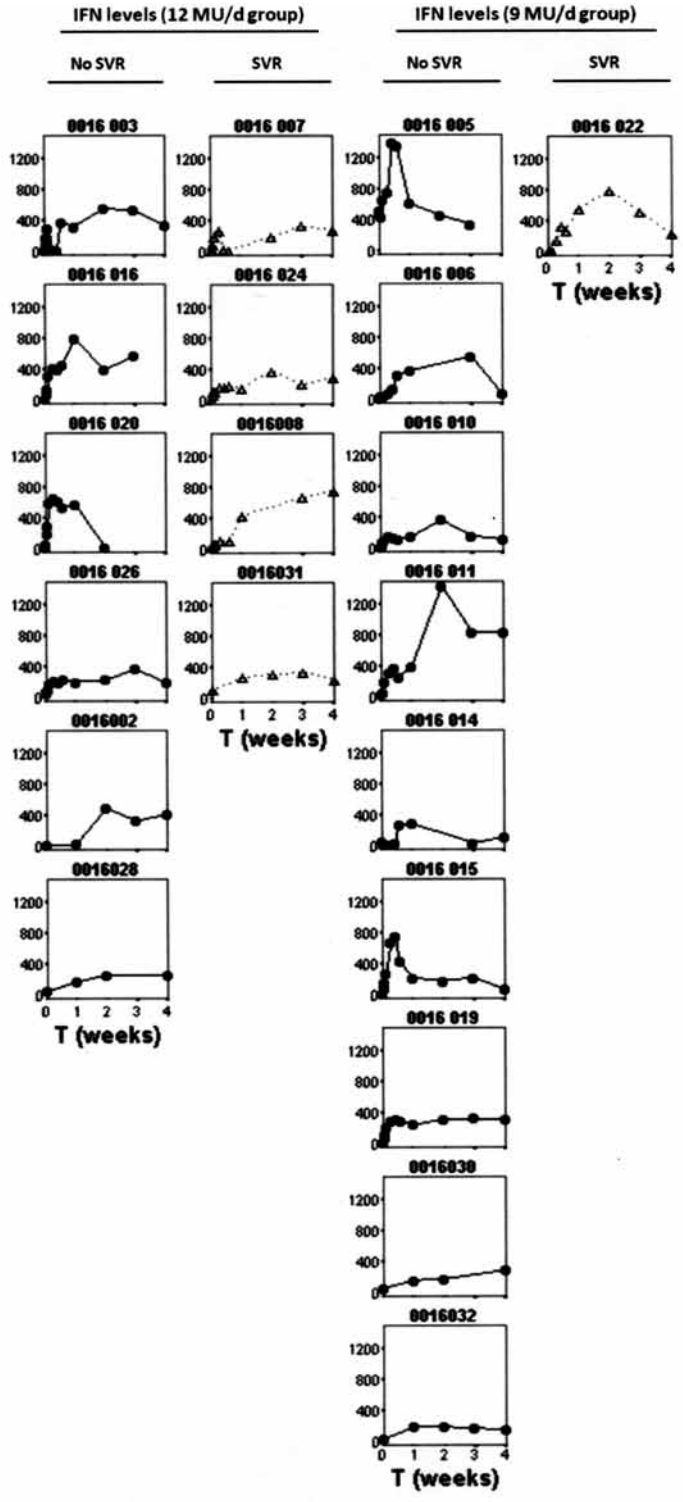


Figure 5A. 2,5-OAS activity according to dose

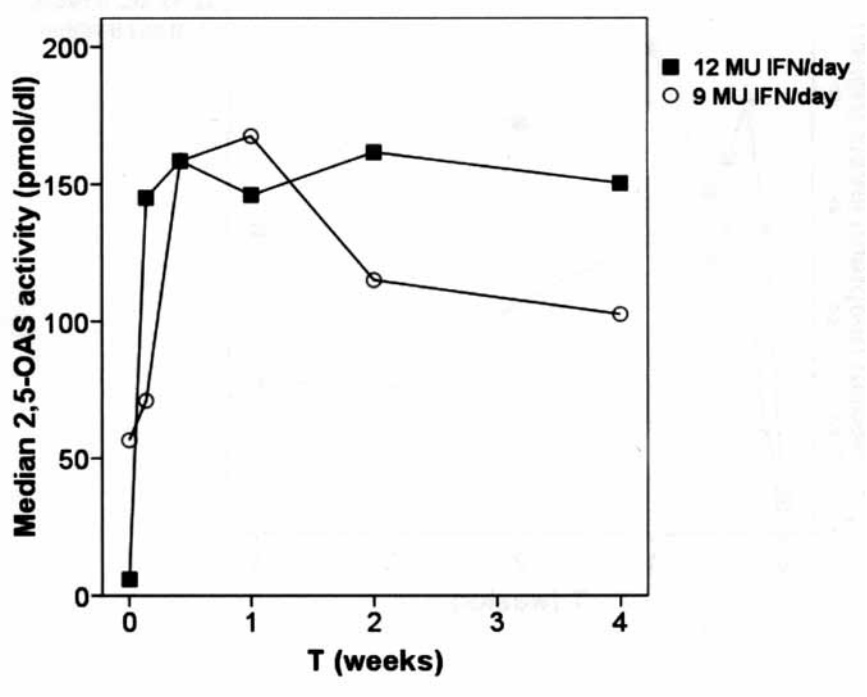


Figure 5B. Neopterin levels according to dose

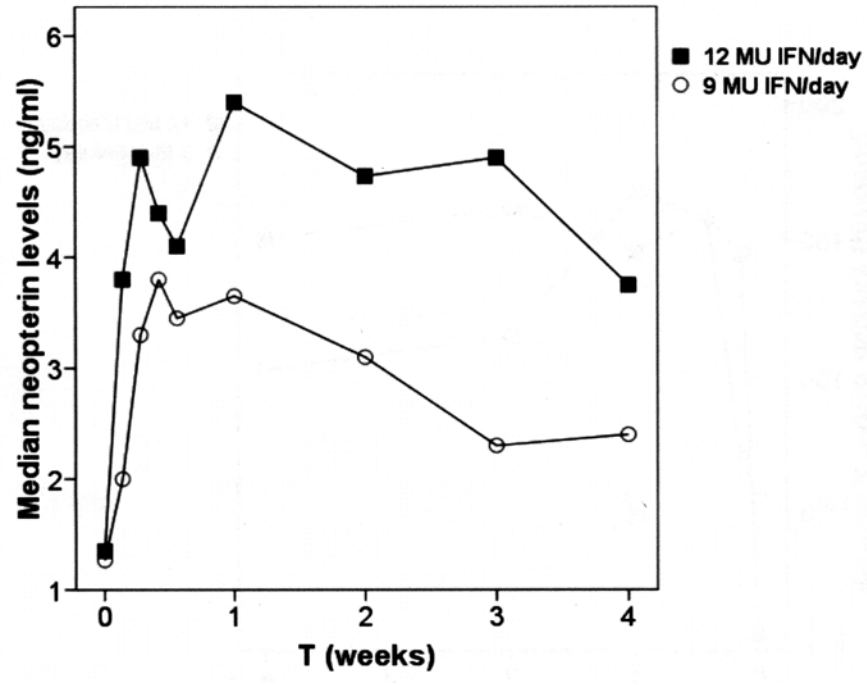


Figure 5C. Beta2-microglobulin levels according to dose

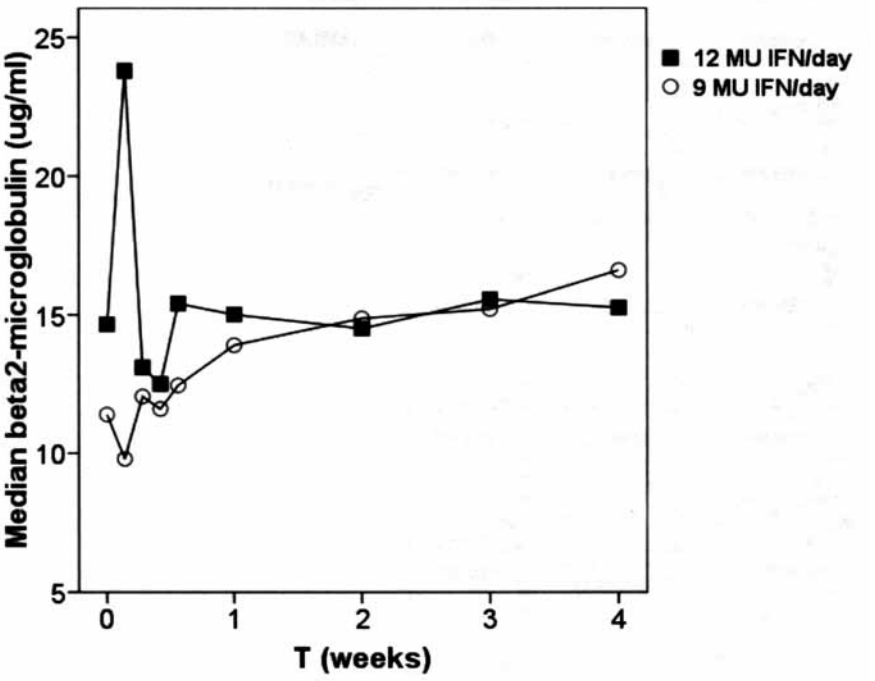
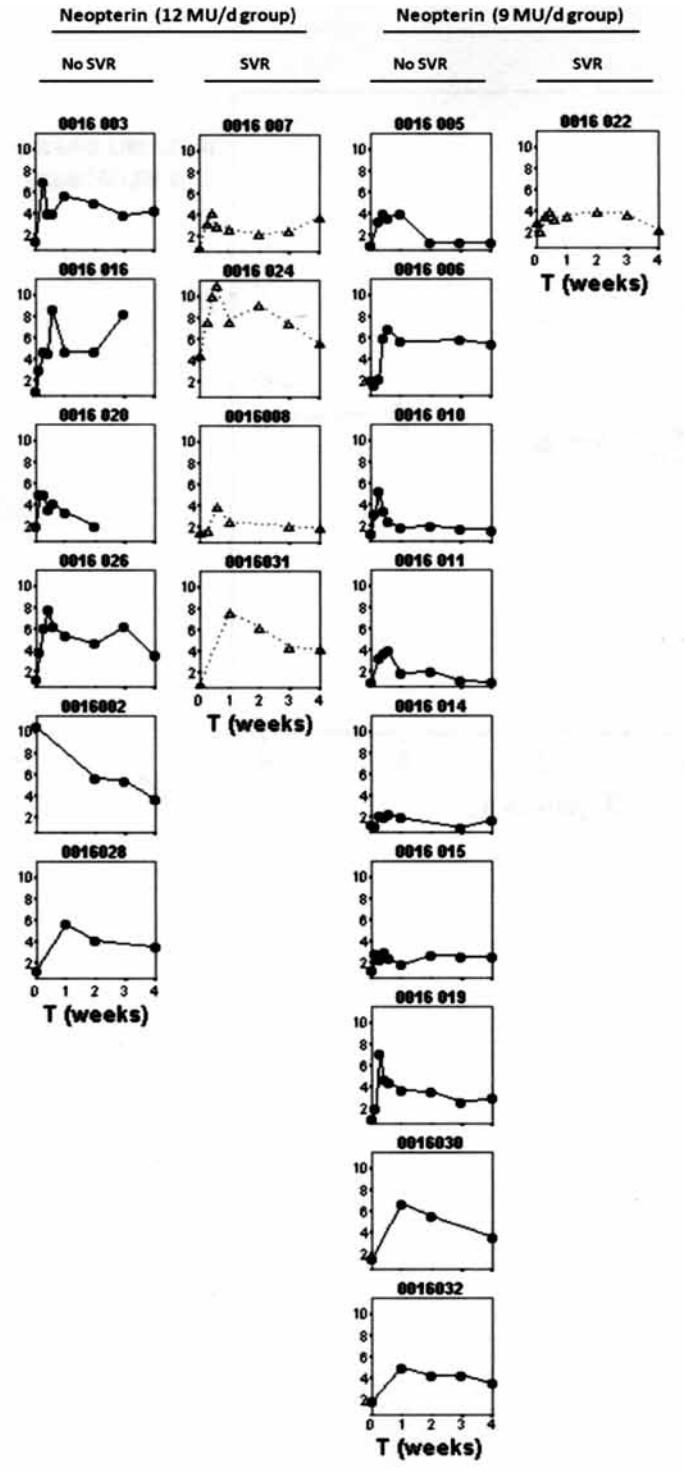


Figure 6. Neopterin levels in individual patients (ng/ml)



Interferon levels

Serum interferon alfa levels peaked between T = 48 hours and week 1 followed by steady-state (Figure 3). A stronger interferon peak was seen in patients receiving 9 MU interferon/day at day 3 (329 pg/ml versus 188 pg/ml, p=0.49), but interferon levels continued to rise in the 12 MU group and maximum levels were reached between week 2 and 4. The highest serum interferon levels at week 4 were reached in patients receiving 12 MU interferon/day with median interferon levels of 294 pg/ml, versus 157 pg/ml in patients receiving 9 MU interferon/day (p=0.093). Although interferon levels were quickly measurable in all patients, there was some inter-individual variability. We could not detect any differences in interferon patterns between individual patients in the 12 MU dose group and the 9 MU dose group and between patients within these group that went on to achieve SVR (Figure 4).

Induction of 2,5-OAS, neopterin and beta2-microglobulin

Most patients had undetectable or low levels of 2,5-OAS, neopterin and beta2-microglobulin at baseline and no significant differences were seen between the two dose groups at start of treatment. Activity of 2,5-OAS peaked between T = 24 hours and week 1 (Figure 5A). Patients receiving 12 MU per day showed steady state of 2,5-OAS activity after this peak, while in patients receiving 9 MU per day this peak was followed by slow decline resulting in lower 2,5-OAS activity at week 4 (158 vs. 102 pmol/dl; p=0.43). Neopterin level increased within 24 hours in most patients but higher peak levels between day 2 and 3 were seen in patients receiving 12 MU per day (Figure 5B). Following the neopterin peak, a gradual decline was seen of neopterin levels, resulting in significant higher levels at week 4 in the 12 MU dose group (3.75 vs. 2.40 ng/ml; p=0.027). Beta2-microglobulin levels increased moderately in all patients (Figure 5C).

The only significant difference that was seen, with regard to these markers of immune activation, were higher neopterin levels in the 12 MU dose group. However, on an individual basis we could not detect a specific pattern in neopterin levels between patients receiving 12 MU or 9 MU interferon and those patients within these group that went on to achieve SVR (Figure 6).

Discussion

In this study we show that a strong HCV RNA decline at week 4 can be induced by continuous subcutaneous administration of 12 MU interferon alfa-2b per day combined with weight-based ribavirin in patients who failed previous peginterferon/ribavirin therapy. A significant stronger HCV RNA decline during the first 4 weeks was seen in patients receiving 12 MU interferon per day versus 9 MU interferon per day. All patients that achieved SVR (5 out of 20) had a more than 2 log viral decline at week 4.

The original pilot study was designed to investigate safety and efficacy of high dose interferon by continuous subcutaneous administration (21). We hypothesized that constant high levels of unmodified interferonmight improve viral suppression and avoid adverse events associated with serum interferon peaks during thrice weekly (or daily) administration of standard interferon or once weekly administration of peginterferon.

Initial viral kinetic studies of HCV showed that administration of interferon produces a biphasic decline in viral load (12, 14, 22, 23). The first phase is dose dependent and shows a rapid decrease in serum HCV RNA concentration of 0.5-2.0 log. The slope of the first phase of viral decline reflects interferon sensitivity. The second phase begins 24-48 hours after onset of treatment and reflects immune-mediated clearance of HCV infected cells. Pegylation of interferon improved viral response rates compared with standard interferon due to an improved pharmacokinetic profile, allowing once weekly dosing with stable blood concentrations throughout the dosing interval (4, 6). A similar biphasic viral decline

was seen with peginterferon during the first weeks of therapy (24). Viral rebound between 48 and 72 hours after start of treatment occurs in some patients and has been associated with decreased serum peginterferon concentrations (25, 26). In our study, this typical biphasic HCV RNA decline was seen in most patients receiving continuous interferon. However, a significant difference in viral kinetics was seen between patients receiving 12 MU interferon per day versus 9 MU interferon per day. This difference was predominantly caused by a stronger first phase decline at day 2, but also due to a steeper second phase in patients receiving 12 MU interferon per day. Based on interferon levels we could not explain the difference between both dosing groups. Especially during the first phase of viral decline higher peak interferon levels in serum were seen in the 9 MU dose group, in contrast with what would be expected. Notably, viral rebound after the first phase of viral decline was seen in the 12 MU dose group despite continuous interferon administration. Interestingly, a relation was seen between neopterin levels (as marker of immune activation) and viral decline. Higher neopterin peak levels were achieved at day 2 in the 12 MU dose group compared to the 9 MU dose group, followed by higher levels during the first 4 weeks of therapy. The viral rebound after day 2 in the 12 MU dose group was also reflected by temporarily decreased neopterin levels. Although neopterin is regarded as a biomarker for activation of the cellular immune system, and its concentration is elevated in several diseases including chronic HCV infection, its role as an antiviral effector molecule is still unclear (27). Importantly, despite the observation that patients receiving 12 MU interferon demonstrated more potent viral decline as compared to patients receiving 9 MU, no differences were found with regard to 2,5-OAS activity and beta2-microglobulin levels between the two dose groups. This is surprising since serum levels of these markers are known to reflect systemic antiviral activity induced by interferon. In order to identify in detail which antiviral effector molecules, such as members of the interferon stimulated genes (ISG) family, are differentially induced by the 12 MU dose and not the 9 MU dose, future studies will include gene profiling during the course of treatment by microarray analysis of peripheral blood collected from patients participating in this study (28).

This is the first study to evaluate viral kinetics in HCV infected previous non-responders during continuous subcutaneous administration of high dose interferon in combination with ribavirin. We demonstrated a biphasic viral decline, as described before during treatment with unmodified and pegylated interferon. The significant stronger HCV RNA decline in the 12 MU dose group was reflected by higher neopterin levels.

Understanding viral kinetics in hepatitis C remains of utmost importance as viral decay at week 4 is still the most important predictor of achieving SVR. Current new triple therapies including direct antiviral agents show much stronger early viral decline than seen with peginterferon/ribavirin. However, peginterferon induced immune control will remain a cornerstone of anti-HCV therapy. Continuous high dose interferon administration combined with ribavirin and a direct antiviral agent might further improve triple therapy outcome, especially in previous non-responders or relapsers.

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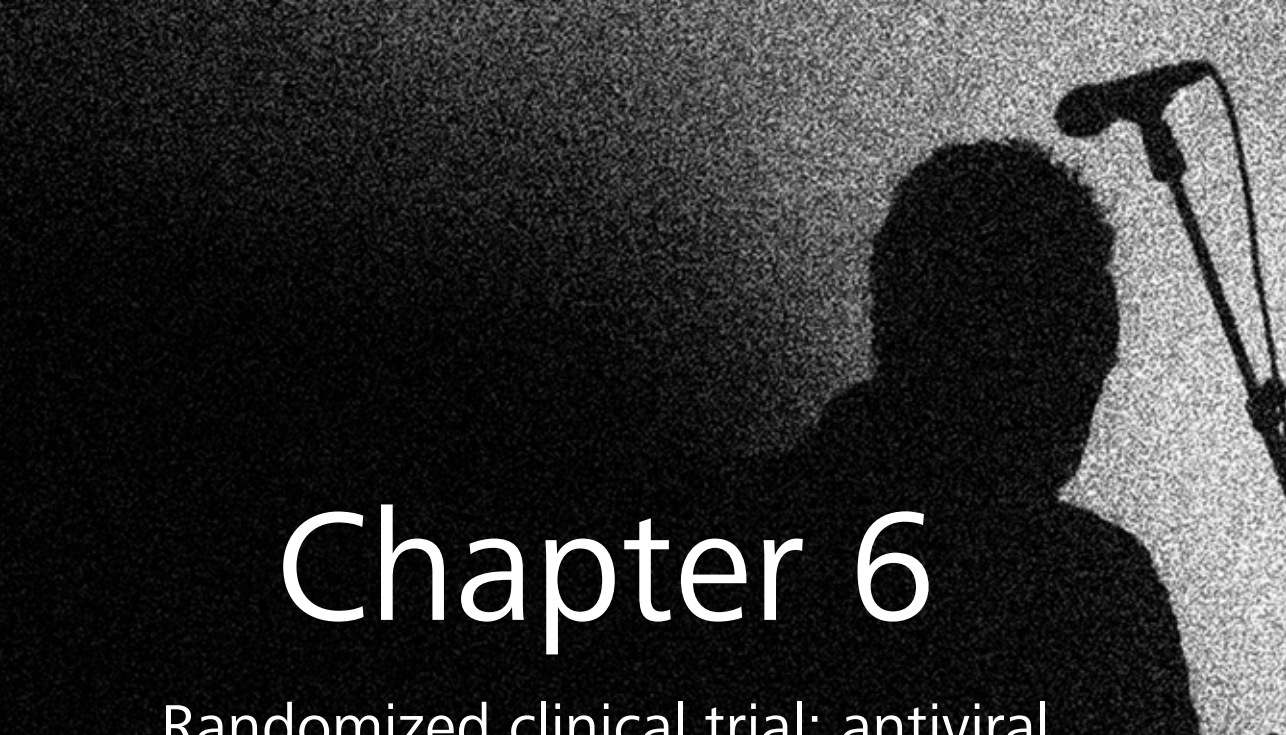

Chapter 6

Randomized clinical trial: antiviral activity of ANA773, an oral inducer of endogenous interferons acting via TLR7, in chronic HCV

Bergmann J.F.*, De Bruijne J.*, Hotho D.M., De Knegt R.J., Boonstra A., Weegink C.J., Van Vliet A.A., Van de Wetering J., Fletcher S.P., Bauman L.A., Rahimy M., Appleman J.R., Freddo J.L., Janssen H.L.A., Reesink H.W.

*contributed equally

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Abstract

Background: ANA773 is an oral prodrug of a small-molecule toll-like receptor (TLR)7 agonist. Preclinical and healthy volunteer clinical studies with ANA773 have demonstrated induction of endogenous interferon alfa of multiple subtypes, which supports the potential utility in the treatment of chronic hepatitis C virus (HCV) infection.

Aim: To examine safety, tolerability, pharmacodynamics, pharmacokinetics and antiviral activity of ANA773.

Methods: ANA773 was investigated in a double-blind, placebo-controlled study in 34 patients chronically infected with HCV of any genotype. Patients were treatment-naïve or had relapsed following previous interferon-based treatment. This dose escalation study was composed of four dose groups (800, 1200, 1600 and 2000 mg). In each group, 6-8 patients received ANA773 and 2 received placebo. Patients were dosed with ANA773 every-other-day for either 28 days (800, 1200 or 1600 mg) or 10 days (2000 mg).

Results: Mild to moderate adverse events were reported, with an increase in frequency and intensity with increasing dose. No serious AEs were reported and there were no early discontinuations. There were dose-related increases in various markers of interferon alfa response. The mean maximum change in serum HCV RNA level from baseline was -0.34, -0.29, -0.40, -0.97 and -1.26 $\log_{10}$ in the placebo, 800, 1200, 1600 and 2000 mg cohorts, respectively. At the 2000 mg dose, ANA773 significantly (p=0.037) reduced serum HCV RNA levels (range: 0.14 to -3.10 $\log_{10}$).

Conclusions: ANA773 was generally well tolerated and resulted in a dose-related interferon-dependent response leading to a significant decrease in serum HCV RNA levels in the 2000 mg dose group.

Introduction

Chronic hepatitis C virus (HCV) infection is a major cause of liver cirrhosis and hepatocellular carcinoma. HCV-related end-stage liver disease is now the main indication for liver transplantation in North America and Western Europe (1). Estimates suggest that there are 170 million HCV infected patients worldwide and 3 to 4 million people are newly infected each year (2). Approximately 80% of patients who become infected with HCV develop chronic hepatitis C (3). Combination therapy with pegylated interferon alfa and ribavirin results in sustained virologic response (SVR) in only approximately 40-50% of patients with genotype 1 (4-6). Thus, there remains an urgent unmet medical need to offer new therapies that may eradicate HCV infection (7). The most likely agents that will enter clinical practice soon are the protease inhibitors telaprevir and boceprevir. Phase III clinical trials of these drugs combined with peginterferon and ribavirin in genotype 1 patients have shown SVR rates up to 80% in treatment-naive patients and up to 65% in treatment-experienced patients (8-11).

Immune responses against HCV are considered to play an important role in controlling and clearing chronic viral HCV infections (12, 13). Toll-Like Receptors (TLRs) are a family of pathogen-recognition receptors that activate the innate immune response (14). Stimulation of TLRs either directly or indirectly leads to (i) the release of multiple cytokines, including type I and type II interferons, (ii) the induction of pathways and enzymes that destroy intracellular pathogens and (iii) the stimulation of antigen-presenting cells which may result in the activation of adaptive immunity. To date, ten functional TLRs have been identified in humans. Among these, TLR7 is of particular interest because small molecule agonists for this receptor have been identified (15), and like TLR9 agonists, induces type III interferons. Furthermore, there is precedent for this target in viral diseases. In a clinical proof-of-concept study, a TLR7 agonist, isatoribine, was shown to have an anti-viral effect in the treatment of chronic HCV infection (16). In addition, topical imiquimod is approved for the treatment of genital papillomavirus infections (17). Unfortunately, imiquimod is poorly tolerated when administered orally (18) and is both extensively and rapidly metabolized when administered systemically, limiting systemic exposure of the drug.

ANA773 is an oral prodrug of a TLR7 agonist, developed for the treatment of patients with chronic HCV infection. The active metabolite of ANA773 induces multiple subtypes of endogenous interferon alfa, induces interferon-dependent anti-HCV activity and functionally activates natural killer (NK) cells in vitro. Oral and intravenous administration of ANA773 induced systemic interferon alfa production in Cynomolgus monkeys, with interferon-dependent responses being observed in both the periphery and the liver. The alternate-day dosing schedule in these studies were shown to induce desirable levels of T-cell activation and proliferation, and has been selected as the dosing schedule for clinical evaluation. A single and multiple dose escalation study (200-1600 mg/day every-other-day up to 4 administrations) of oral ANA773 in 41 healthy volunteers showed a dose-related increase of interferon alfa and markers of interferon response. The occurrence of treatment related adverse events in this study were seen predominantly in the higher dose groups (1200 and 1600 mg/day) and included pronounced interferon-like side effects such as pyrexia, chills, myalgia, headache, nausea and malaise, which confirm the relation with the study drug. Importantly, ANA773 was well-tolerated in 13 week toxicology studies at doses that produced robust immune induction (19).

Here, we report the safety profile, pharmacokinetics, antiviral activity and the immunological effects of ANA773 administered orally at four dose levels (800, 1200, 1600 and 2000 mg/day every-other-day) to 34 treatment-naive and treatment-experienced HCV infected patients.

Materials and methods

Study design

This randomized, placebo-controlled, double-blind, multiple dose escalation, phase Ib study was conducted at three sites in The Netherlands from October 2008 until August 2009. This study was conducted in accordance with Good Clinical Practice and with the World Medical Association Declaration of Helsinki after approval by the institutional review board at each center. All patients provided written informed consent before participating in any study-related activity. Initially, HCV patients of any genotype were randomized into three sequential cohorts (800, 1200 and 1600 mg). In the 800, 1200 and 1600 mg groups, 6 patients received oral ANA773 and 2 received placebo every-other-day for 28 days (14 doses). As doses in the first 3 patient dose groups were well tolerated and encouraging antiviral responses were observed at the highest dose level of 1600 mg, a fourth dose group was added to investigate a higher ANA773 dose of 2000 mg. In this dose group, 10 patients (8 active, 2 placebo) were administered 2000 mg of ANA773 or placebo every-other-day for 10 days (5 doses). The shorter treatment period was considered sufficient since immunological and antiviral effects of ANA773 were seen shortly after start of treatment of patients in the previous lower dose groups.

During the first 3 doses of ANA773 (800, 1200 and 1600 mg group), and during all 5 doses in the 2000 mg group, the study was conducted as an inpatient study for careful assessment of safety and tolerability. Study medication (100 mg capsules) and placebo capsules were supplied by Anadys Pharmaceuticals, Inc., San Diego, USA. Patients were allowed to start standard of care (peginterferon + ribavirin) immediately after the study period at the discretion of the patient.

Patients

Key inclusion criteria included male and female patients between 18 to 65 years, with body mass indexes of 18 to 35 kg/m^2, HCV RNA level $\geq 75 \times 10^3$ IU/ml, and clinical laboratory evaluations consistent with chronic hepatitis C infection as defined by the protocol. Treatment-naive or relapse patients were allowed. Relapse was defined as undetectable HCV RNA at completion of a previous IFN-based treatment, but positive HCV RNA during follow-up. Key exclusion criteria included decompensated liver disease, findings consistent with Child Pugh B/C liver cirrhosis, and co-infection with HIV or HBV. Previous non-responders to interferon-based therapies were excluded due to expected unresponsiveness to immunomodulatory therapy. Patients receiving antiviral therapy or immunomodulatory therapy within 90 days prior to administration of the first dose of study medication were excluded. Patients with chronic stable haemophilia or on stable methadone substitution treatment were eligible.

Safety assessment

Patients who received at least 1 dose of ANA773 were considered evaluable for safety. Safety was evaluated by adverse events registration and clinically significant changes from pre-treatment baseline in laboratory values, vital signs, electrocardiogram tracings, and findings that were recorded during physical examinations. Dose escalation to the next dose group was based upon the safety and tolerability of ANA773 in all patients in the previous dose group following completion of the 28-day treatment period as determined by the Ethics Committee.

Pharmacokinetic assessment

Plasma pharmacokinetic samples for assay of the active metabolite of ANA773 and 2 other metabolites were collected at the following times relative to ANA773 dosing: at pre-dose (0 hour), 0.5, 1, 1.5, 2, 3, 4, 8, 12 and 24 hours after dosing on Day 1 in all dose groups. In the 2000 mg group, plasma pharmacokinetic samples were also drawn on Day 9 on the same time points. Pharmacokinetic analyses were performed by a central laboratory.

Viral assessment

Siemens INNO-LiPA HCV II assay was used to assess geno(sub)typing of all patients. Serum samples for HCV RNA analysis were obtained on Day 1 before the first morning dose, followed by sample collection on Day 2, 3, 4, 5, 6, 13, 21, 27, 28, 34 and 41 (800, 1200 and 1600 mg groups) or on Day 2, 3, 4, 5, 6, 7, 8, 9, 10 and 16 (2000 mg group). HCV RNA measurements were performed by a central laboratory using the Roche COBAS AmpliPrep/COBAS TaqMan HCV Test with a lower limit of quantification (LLQ) of 43 IU/ml. A > 1.0 $\log_{10}$ reduction in viral load during treatment was considered a clinically relevant decline.

Immunological parameters

Cytokine serum samples (interferon alfa) were obtained on Day 1, 2, 3, 5, 13, 27, 28, and 34 (800, 1200 and 1600 mg groups). In the 2000 mg group, cytokine serum samples were obtained on Day 1, 3, 5, 7 and 9. Samples were analyzed by Enzyme-Linked ImmunoSorbent Assay (ELISA) at Alta Analytical Laboratory, San Diego, USA. Serum RNA samples (OAS1 and ISG15) were obtained on Day 1, 2, 3, 4, 5, 6, 13, 21, 27, 28, 34 (800, 1200 and 1600 mg groups) and on Day 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 16 (2000 mg group) by Anadys Pharmaceuticals, San Diego, USA, using branched DNA (bDNA) assay. Neopterin and 2,5-OAS activity samples were collected at Day 1, 2, 3, 4, 5, 6, 13, 21, 27, 28, 34, 41 (800, 1200 and 1600 mg groups) and at Day 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 16 (2000 mg group). Neopterin samples were analyzed by ELISA at Alta Analytical Laboratory, San Diego, USA. 2,5-OAS activity was analyzed by radioimmunoassay (RIA) at PRA International, Assen, The Netherlands.

Statistical analysis

The determination of the sample size was based on empirical considerations rather than statistical justification. No formal sample size calculations for this study were performed. The sample size of 24 patients in 3 dose groups with 8 patients per dose group (800, 1200 and 1600 mg groups) and an additional 10 patients in the 2000 mg group were considered appropriate for this type of study. Safety data were tabulated and summarized by descriptive statistics. Pharmacokinetic parameters were summarized by descriptive statistics at each dose level and by study day. Analysis of variance (ANOVA) was used to compare the dose normalized Cmax and AUC values across dose groups. The log transformed HCV RNA values were summarized by descriptive statistics according to dose group and study. ANOVA was used to compare the difference between each time point and baseline, the nadir and baseline, and between the end-of-treatment and baseline across dose groups (including placebo). Immunological parameters for serum cytokines (interferon alfa), RNA (OAS and ISG15), neopterin and 2,5-OAS activity were summarized by descriptive statistics according to dose group and study day. The software used for all summary statistics and statistical analyses were SAS version 9.1.3 (SAS Institute, Inc.) and comparable desktop parameters. For the calculation of some pharmacokinetic parameters WinNonlin (Pharsight, Inc.) version 5.0.1 was used. For the calculation of correlation between variables the Spearman non-parametric test was used. Any p-values calculated as a result of the statistical analysis were interpreted in accordance with the exploratory nature of this study.

Results

Demographic and baseline characteristics

A total of 54 patients were screened of whom 33 patients were considered eligible. One patient participated twice in the study under two different subject numbers. This was allowed per protocol as the patient received placebo during his first treatment period. The different subject numbers were treated as if they were two individuals and therefore the total number of approved and included subjects was 34. There were no drop outs or withdrawals; all patients completed the study as per protocol. Eight-

Table 1. Demographics

	800 mg (n = 6)	1200 mg (n = 6)	1600 mg (n = 6)	2000 mg (n = 8)	Placebo (n = 8)
Male, n (%)	6 (100)	6 (100)	4 (67)	7 (88)	7 (88)
Age, mean (range)	50 (40-55)	44 (32-51)	51 (46-55)	50 (33-61)	52 (38-64)
White, n (%)	6 (100)	5 (83)	4 (67)	8 (100)	8 (100)
BMI, mean (range)	27.1 (20.2-33.1)	26.7 (21.4-31.4)	26.0 (20.0-30.0)	26.3 (18.3-34.6)	25.8 (20.2-33.7)
Cirrhosis, n (%)	1 (17%)	1 (17%)	0 (0)	0 (0)	1 (13)
Baseline $\log_{10}$ HCV RNA, mean (SD)	6.42 (0.80)	6.04 (0.46)	6.43 (0.61)	6.06 (0.92)	6.24 (0.80)
HCV genotype					
1, n (%)	5 (83)	5 (83)	3 (50)	0 (0)	4 (50)
2, n (%)	0 (0)	0 (0)	0 (0)	2 (25)	0 (0)
3, n (%)	1 (17)	1 (17)	1 (17)	4 (50)	3 (38)
4, n (%)	0 (0)	0 (0)	2 (33)	2 (25)	1 (13)
Previous treatment					
Naive, n (%)	2 (33)	3 (50)	2 (33)	7 (88)	4 (50)
Relapse, n (%)	4 (67)	3 (50)	4 (67)	1 (12)	4 (50)

BMI: body mass index.

een patients (53%) were treatment-naïve; the remaining 16 patients (47%) had relapsed from a prior full course of interferon alfa based therapy. Patients were infected with genotype 1 (n = 17), 2 (n = 2), 3 (n = 10) or 4 (n = 5). Of the 34 patients, 30 (88%) were male and 4 (12%) were female. Thirty-one (91%) patients were white/caucasian. The mean age was 49.5 years (range, 32-64 years) and mean BMI was 26.35 kg/m^2 (range, 18.3-34.6 kg/m^2). Demographic and other baseline characteristics of the randomized patients are shown in Table 1.

Safety

Safety data including serious adverse events, premature discontinuations and hematologic effects are shown in Table 2. Overall, multiple oral doses of ANA773 were safe and generally well tolerated at all dose levels tested up to 2000 mg. There were no deaths or other SAEs during the study. The majority of AEs were of mild or moderate intensity. The total number of AEs was highest in the 1600 mg (66 AEs) and 2000 mg (58 AEs) dose group. The number of AEs reported by the placebo-treated patients was only slightly less (49 AEs). A total of 167 AEs were considered possibly or probably related to the study medication (71% of the total number of AEs). The most frequently reported related AEs were headache, myalgia, fatigue, abdominal pain and dizziness. The occurrence of headache appeared to increase with dose, whereas for the other frequently reported related AEs a relation with dose was not observed. In addition, nausea (total of 6 events) considered related to the study medication occurred in the highest dose groups only. At the 1600 mg dose level 2 patients experienced flu-like symptoms (e.g. headache, myalgia, chills, malaise) at the beginning of their dosing period. However, these symptoms rapidly disappeared during continued dosing, even when they were initially of moderate to severe intensity. At the 2000 mg dose level 3 patients treated with ANA773 experienced flu-like symptoms, mainly at the first 2 dosing occasions. One of these patients started to use paracetamol as prophylactic treatment and the remaining doses were well tolerated. Individual dose reductions, as

Table 2. Safety results

	Total (n = 34)	800 mg (n = 6)	1200 mg (n = 6)	1600 mg (n = 6)	2000 mg (n = 8)	Placebo (n = 8)
Serious adverse events (n)	0					
Discontinuations (n)	0					
Adverse events (n)	235	31	31	66	58	49
Patients with AE (%)[1]						
- Headache	53	33	33	67	75	55
- Fatigue	41	50	33	67	38	25
- Myalgia	38	33	17	50	50	38
- Abdominal pain	32	17	17	50	13	63
- Dizziness	24	50	17	50	0	13
- Back pain	21	0	17	50	13	25
- Constipation	18	0	0	50	0	38
- Nausea	18	0	0	33	50	0
- Dyspepsia	15	0	0	33	25	13
- Malaise	15	17	0	33	25	0
- Chills	12	0	17	33	13	0
- Diarrhoea	12	17	0	17	0	25
- Pain in extremity	12	17	0	17	13	13
- Pyrexia	12	0	0	0	38	13
Hematologic abnormalities						
- Anemia[2]	0					
- Leukocytopenia[3]	0					
- Trombocytopenia[4]	0					

[1] AEs listed that occurred in > 10% of all patients.

[2] Hemoglobin level < 6.2 mmol/l.

[3] Leukocyte count < 1.5×10^9/l.

[4] Platelet count < 50×10^9/l.

were allowed per protocol, were not necessary. Trends for decreases in leukocytes (nadir -4.06 x 109/l on Day 6), thrombocytes (nadir -38.4 x 10^9/l on Day 6) and lymphocytes (nadir -1.23 x 10^9/l on Day 2) were observed for the highest dose level (2000 mg ANA773), but were not considered clinically significant. No other clinically significant abnormalities in vital signs, ECG, clinical laboratory values and physical examination were observed.

Pharmacokinetics

Pharmacokinetic parameters of the active metabolite of ANA773 are shown in Table 3. ANA773 was rapidly absorbed in all patients. At all dose levels the active metabolite of ANA773 appeared in plasma within 0.5 h (the first sampling time point). Maximum mean plasma concentrations of the active metabolite of ANA773 were observed at 1 h post-dose. Thereafter the plasma concentrations declined rapidly. In most patients, low plasma concentrations of the active metabolite of ANA773 were still observed at the last sampling time point 24 h post-dose. The mean plasma concentrations of the active metabolite of ANA773 increased with increasing dose. For the highest dose group (2000 mg),

Table 3. Pharmacokinetic parameters of plasma ANA773 active metabolite

Dose (mg)	Day	Cmax (ug/ml)	Tmax (h)	AUCinf (ug.h/ml)	T1/2 (h)
800	1	10.69 (7.57-15.3)	0.509 (0.50-1.00)	24.96 (21.9-29.1)	3.254 (2.09-3.96)
1200	1	11.99 (8.10-16.9)	1.000 (0.50-1.50)	31.53 (26.2-34.7)	2.901 (1.87-3.75)
1600	1	13.94 (9.17-19.0)	1.000 (0.50-2.00)	40.76 (33.3-49.4)	3.375 (2.66-3.85)
2000	1	15.87 (10.9-23.9)	1.000 (0.50-1.50)	51.06 (36.7-69.4)	2.995 (1.55-4.29)
2000	9	15.50 (13.1-19.3)	1.000 (0.50-1.50)	46.42 (35.0-60.4)	3.237 (2.67-3.65)

For all parameters except tmax the geometric mean (range) is presented; for tmax the median (range) is presented.

Table 4. Individual HCV RNA data for patients with a maximal viral decline of > 1.0 log_{10} IU/ml

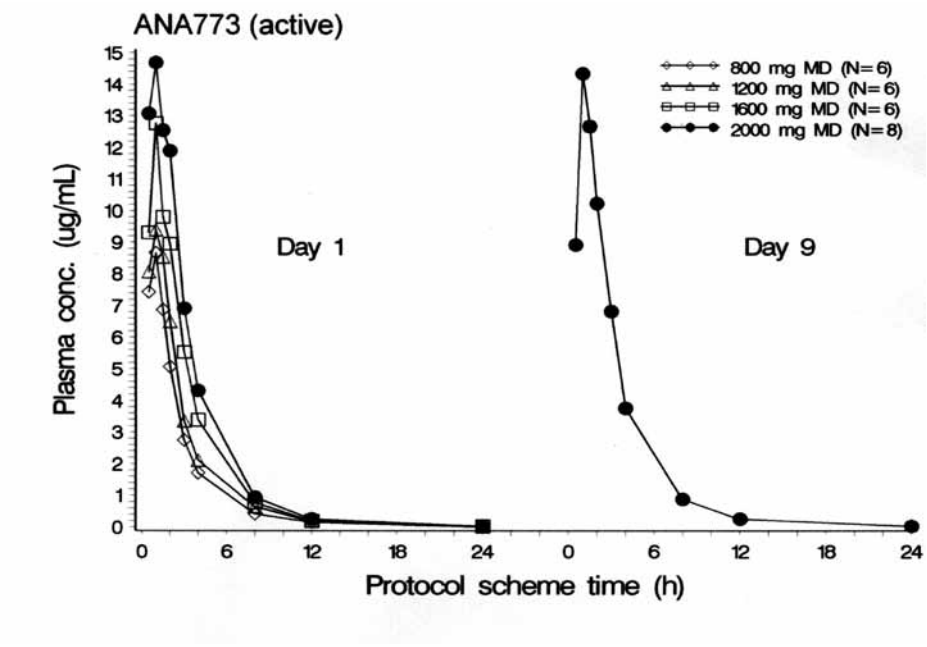
Subject	HCV genotype	Sex	Baseline viral load[1]	Nadir	Day (N)	Day 1 (N)	Day 5 (N)	Day 28 (N) / Day 10 (N)[2]	EOT / EOT9 / 10[3]	Final follow-up
1600 mg										
Patient 1	3	M	7.04	-1.15	4	-0.74	-0.70	-0.49	6.52	6.86
Patient 2	1A	F	6.59	-2.52	6	-1.64	-2.52	-2.24	4.41	6.02
2000 mg										
Patient 3	3A	F	6.77	-1.63	10	-1.62	-1.30	-1.63	5.35	6.10
Patient 4	4D	M	6.23	-1.35	6	-1.01	-1.35	-0.55	6.10	5.56
Patient 5	4A	M	5.11	-1.53	4	-0.65	-1.12	-0.28	4.72	4.67
Patient 6	3A	M	6.99	-3.10	4	-2.16	-3.07	-1.68	5.25	5.91
Patient 7	2	M	6.75	-1.78	8	-0.55	-1.03	-1.07	5.75	6.95

[1] Mean of Day -1 and pre-dose Day 1.

[2] Maximum negative change from baseline value at Day 28 or at Day 10.

[3] Average of the Day 27 and Day 28 or Day 9 and Day 10 (2000 mg only) HCV RNA concentrations.

Figure 1. Mean plasma concentration of the active metabolite of ANA773 on Day 1 (all groups) and Day 9 (2000 mg only)



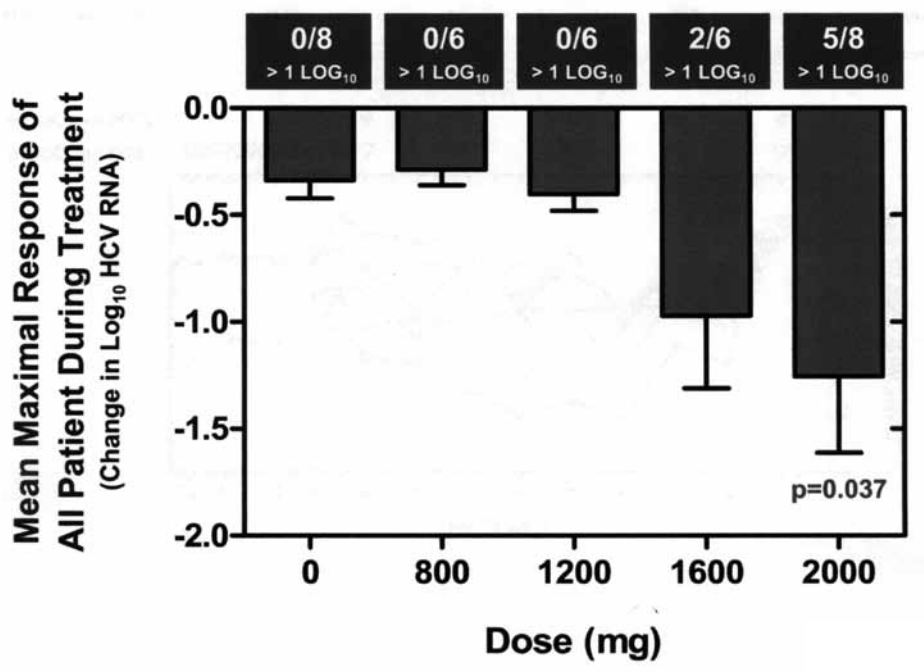
SD: single dose; MD: multiple dose.

a PK profile was also obtained on Day 9. Plasma concentrations of the active metabolite of ANA773 were below LLQ at pre-dose on Day 9. The PK profile on Day 9 after multiple doses of ANA773 was similar to the PK profile on Day 1 after a single dose of ANA773 (Figure 1). This indicates that no accumulation during multiple dosing was observed. Two other metabolites of ANA773 (metabolite A and metabolite B) also appeared in plasma within 0.5 h after dosing. Metabolite A is an intermediate in the conversion of ANA773 to the active metabolite and metabolite B is a minor degradation product. The maximum mean concentrations were observed for metabolite A at 0.5 to 1 h post-dose and for metabolite B at 1.5 to 2.5 h post-dose. The plasma concentrations of metabolite A were on average approximately 2 to 3-fold lower than of the active metabolite, whereas for metabolite B the plasma concentrations were more than 100-fold lower. Evaluation of the dose-normalized individual values and geometric mean PK parameters and evaluation of dose proportionality with an exploratory ANOVA analysis showed that Cmax of the active metabolite of ANA773 increased with dose in a less than dose proportional manner (estimate of slope of 0.44 [95% CI 0.12-0.75]). The AUC for the active metabolite of ANA773 increased at slightly less than dose proportionally (estimate of slope 0.79 [95% CI 0.60-0.97]).

HCV RNA levels

At the end of treatment 1 patient in the 1600 mg dose group and 3 patients in the 2000 mg dose group, achieved a viral load decline > 1.0 $\log_{10}$ IU/ml, which was considered a clinically relevant decline. Another 3 patients (1 patient in the 1600 mg dose group and 2 patients in the 2000 mg dose group) showed transient declines of > 1.0 $\log_{10}$ IU/ml during treatment. In the 800 and 1200 mg

Figure 2. Mean maximum HCV RNA response of all patients during treatment with ANA773

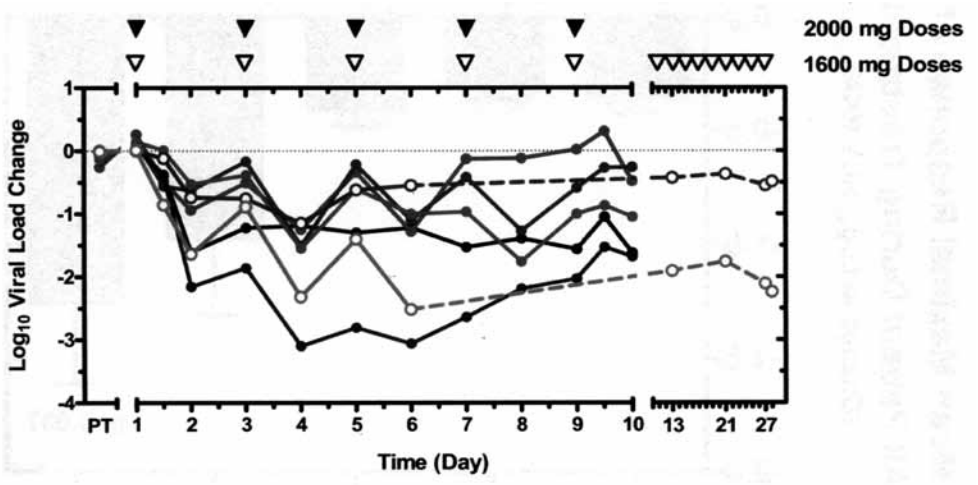


Mean (+ SEM) viral load nadir values are plotted for each dose group. The maximum viral load decline at 2000 mg is statistically significant relative to the placebo group. The number of patients in each group with a maximum decline in viral load of >1.0 $\log_{10}$ IU/ml is displayed above the plot.

Note: patients in the 800, 1200 and 1600 mg group were dosed for 28 days. Patients in the 2000 mg group were dosed for 10 days.

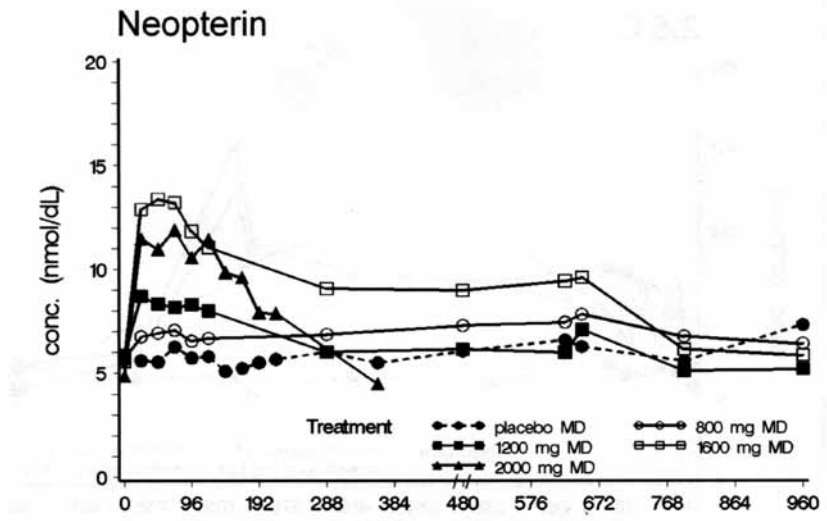
dose groups there were no patients with a > 1.0 $\log_{10}$ IU/ml reduction in viral load at any timepoint. The mean maximum decline of serum HCV RNA per dose group is shown in Figure 2. Individual viral load data of the 7 patients with > 1.0 $\log_{10}$ IU/ml decline during treatment are shown in Table 4 and in Figure 3. The time of nadir for these patients varied between 3 to 9 days after start of treatment and was always 24 h after ANA773 dosing. After reaching nadir, HCV viral load started to increase in several of these patients (Figure 3). Serum viral load levels generally reverted to baseline after cessation of treatment. Viral load reductions of > 1.0 $\log_{10}$ IU/ml during treatment were observed in all genotypes. The pre-treatment viral load ranged from 5.11 to 7.04 $\log_{10}$ IU/ml in the 7 patients who showed a maximum virological response of > 1.0 $\log_{10}$ IU/ml. A response to ANA773 treatment was seen in both treatment-naive and relapse patients. The 2 patients with a clinically relevant virological response in the 1600 mg dose group had relapsed from previous treatment, whereas the 5 patients with a clinically relevant virological response in the 2000 mg dose group were all treatment-naive.

Figure 3. Viral load time course in patients with > 1.0log$_{10}$ decline during treatment



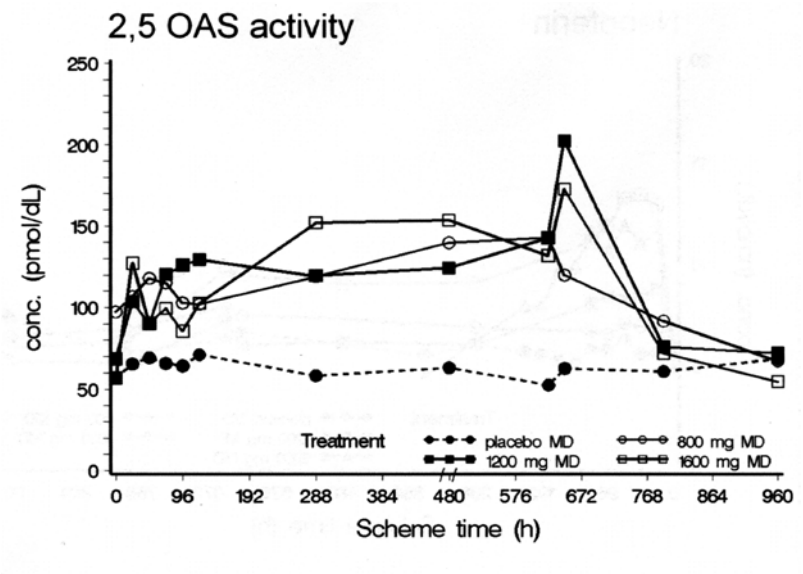
Five 2000 mg patients (closed circles) and two 1600 mg patients (open circles) had a maximum decline in viral load of > 1 log$_{10}$ during treatment. Each line represents the viral load change for one of these patients during the treatment period (10 days for the 2000 mg group, 28 days for the 1600 mg group). Patients in the 2000 mg group had samples taken pre-treatment (PT) and on days 1 through 10 (24 hours after the last dose). Patients in the 1600 mg group had samples taken pre-treatment, on days 1-6, and then on days 13, 21, 27 and 28 (24 hours after the last dose). For these patients, the period between days 6-28 is represented by a dashed line.

Figure 4A. Mean neopterin concentration throughout the study



Note: patients in the 800, 1200 and 1600 mg group were dosed for 28 days. Patients in the 2000 mg group were dosed for 10 days.

Figure 4B. Mean 2,5-OAS activity throughout the study

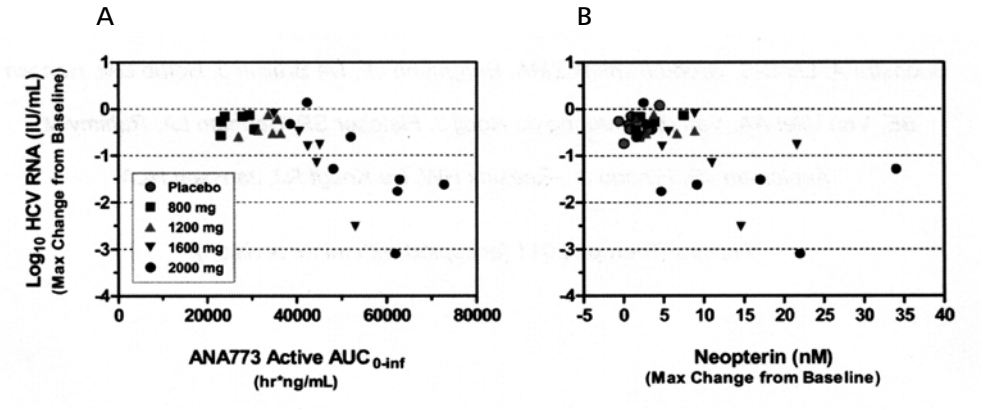


Immunological parameters

In total 10 patients had a measurable interferon alfa response, defined as having one or more interferon concentrations above LLQ that increased after ANA773 dosing. For the patients treated with placebo no interferon concentrations above LLQ were observed. The incidence of the interferon responses appeared to increase with increasing dose of ANA773. The response was rapid: interferon concentrations above LLQ were observed at 3 h post-dose in several patients. In most cases, the peak interferon concentrations were observed at 6 to 9 h post-dose. The interferon concentrations rapidly decreased thereafter and were below LLQ prior to the administration of the next ANA773 dose. Due to the small number of patients that showed a quantifiable response, it was not clear whether the magnitude of the response increased with dose. Of the 7 subjects who showed a decline in viral load of > 1.0 $\log_{10}$ IU/ml, 4 patients also had a measurable interferon response (one patient in the 1600 mg group and 3 patients in the 2000 mg group). However, for 3 of the subjects with a virological response of > 1.0 $\log_{10}$ IU/ml no interferon concentrations above LLQ were observed (one patient) or at only one time point (2 patients). In one patient (800 mg group) measurable interferon concentrations were present throughout the study period. Since these substantial levels of circulating interferon in this subject were present even at baseline, this is considered to be unrelated to the study treatment. Nonetheless, the interferon concentrations increased following ANA773 dosing on 4 of the 5 days with post dose interferon assessments; this subject is therefore considered as having a measurable interferon response.

The expression of two different interferon-response genes, OAS1 and ISG15, was evaluated as markers of interferon-response. The extent of the increase (mean fold change in mRNA levels) was higher for ISG15 than for OAS1, but other than that the profiles were very similar with regard to timing and dose-response relationship. As previously reported in healthy volunteers dosed with ANA773, the maximum increases in mRNA levels were observed approximately 6 to 12 h post dose and mean mRNA levels were near baseline by 48 h post dose. The extent of the induction of these genes appeared to be approximately constant for the first 3-5 dosing occasions. All 7 patients who showed a decline in viral load of > 1.0 $\log_{10}$ IU/ml, also had clearly increased expression of OAS1 and ISG15.

Figure 5. Correlation of antiviral effect of ANA773 with (A) exposure to ANA773 and (B) induction of interferon-dependent response



Each point represents an individual patient. The plots display the correlation between maximum change in viral load with (A) exposure to ANA773 after the first dose; r = -0.699, p<0.0001, and (B) maximum change in neopterin levels during treatment; r = -0.457, p = 0.0066. Placebo values are not included in (a).

Mean neopterin concentrations increased for all ANA773 dose levels tested (Figure 4A). There was a dose-response relationship up to the 1600 mg ANA773 dose. Following the 2000 mg ANA773 dose, the maximum increase in mean neopterin concentrations was slightly lower than following the 1600 mg dose. The highest increases in neopterin concentrations were observed at the beginning of the treatment period. After the first 3 to 4 doses, the neopterin concentrations wained over time such that in most patients neopterin concentrations had returned to values near baseline by the end of the treatment period. In the patients with a viral decline > 1.0 $\log_{10}$ IU/ml a clear increase in neopterin concentrations was observed. At the 2000 mg ANA773 dose level, the 5 patients with a virological response > 1.0 $\log_{10}$ IU/ml had a neopterin mean maximum change (Rmax, FC) ranging from 2.02- to 6.48-fold. These patients also had the highest maximum absolute neopterin concentrations. The 3 patients in this dose group with no virological response had a neopterin Rmax, FC similar to values measured for placebo, indicating no clear increase in neopterin.

An increased 2,5-OAS activity was observed for the 1200 and 1600 mg dose level (Figure 4B). For the 2000 mg dose group no 2,5-OAS activity was determined. Although the mean plots do not indicate a clear dose-response relationship, the mean values for Rmax, FC did seem to indicate the presence of a dose-response relationship. Since the samples of the 2000 mg dose group were not analyzed, only a limited evaluation of a possible relation between increased 2,5-OAS activity and virological response could be done. The 2 patients who had a decrease in viral load of > 1.0 $\log_{10}$ IU/ml, did not appear to have the highest increases or absolute values of 2,5-OAS activity in their dose groups. The increase in mean 2,5-OAS activity was observed starting from 24 h after the first dose. A further increase was observed after the second dose; thereafter the 2,5-OAS activity appeared to remain constant throughout the treatment period.

Discussion

The present study is the first clinical trial to evaluate ANA773, an oral inducer of endogenous interferons that acts via TLR7, in chronic hepatitis C patients. This multiple dose escalation study was designed to explore the safety, tolerability, pharmacokinetics, pharmacodynamics and antiviral activity of ANA773.

Our primary objective was to investigate safety and tolerability of ANA773. There were no serious AEs observed and there were no premature discontinuations. The most frequently reported AEs, which were considered to be related to the study medication, were flu-like symptoms, which were only observed early following the start of treatment. Since these symptoms are known interferon-like side effects, the observed AE profile is consistent with the expected mechanism of action of the compound. With increasing dose of ANA773, a trend was seen towards a higher frequency and stronger intensity of flu-like symptoms. Overall, treatment with multiple doses of ANA773 at dose levels up to 2000 mg were well tolerated by the 26 patients chronically infected with HCV. The same mild profile of side effects was seen with an intravenous TLR7 agonist (isatoribine), although only few flu-like symptoms were seen in this study (12). In contrast, oral administration of resiquimod (a TLR7 and TLR8 agonist) was associated with typical interferon-related adverse events which limited dose escalation (16).

ANA773 was rapidly absorbed following oral administration, and all three metabolites were detected in plasma at the first sampling time point 30 minutes after start of treatment. Systemic exposure to the active metabolite of ANA773 was dose proportional, although the Cmax increased with dose in a less than dose proportional manner. It is possible that the rate of conversion of ANA773 into the active metabolite may become more limiting at the higher doses. An additional factor contributing to the less than dose proportional enhancement of the Cmax of the active metabolite of ANA773 could be the high number of capsules administered which may limit the efficiency of the dissolution and absorption process. For the Cmax of metabolite A no deviations from dose proportionality were observed, which would also be expected if dissolution and absorption was a major factor. The pharmacokinetic profile of ANA773 in chronic HCV infected patients was similar to healthy volunteers (data not shown). For the 2000 mg group it was shown that all three metabolites were below LLQ at pre-dose on Day 9, indicating that no accumulation during multiple dosing occurred.

Repeated administration with 1600 mg or 2000 mg of ANA773 resulted in a mean maximum viral load decrease of –0.97 and –1.26 $\log_{10}$ IU/ml, respectively, compared to -0.34 $\log_{10}$ IU/ml in placebo-treated HCV patients (p=0.037 at 2000 mg relative to the placebo group). At the 1600 mg dose level 2 of 6 HCV patients and at the 2000 mg dose level 5 of 8 HCV patients had a decline in viral load of > 1.0 $\log_{10}$ IU/ml. The decline of serum HCV RNA levels was generally observed within the first several days, and HCV RNA levels remained reduced until the end of therapy in most patients. Interestingly, HCV viral load decline did not resemble the typical biphasic pattern as seen with interferon-based regimens, which may be due to a different antiviral mechanism, suboptimal drug dosing, or due to the specific dosing regimen.

Stimulation with TLR7 agonists induces the production of type I interferons, pro-inflammatory cytokines and the expression of co-stimulatory molecules on various cell populations (11, 17, 18). In all patients with a decline of viral load of more than 1.0 $\log_{10}$ these factors were induced or activated, demonstrating the immunomodulatory activity of ANA773 leading to antiviral effects. Our findings demonstrated that the decline in serum HCV RNA levels correlated with exposure to the active metabolite of ANA773 as well as neopterin levels, respectively (Figure 5). Further studies are needed to examine why some patients respond to ANA773 as demonstrated by activation of innate immune

responses, whereas no decline of viral load is observed in these patients. We observed that HCV genotype and the baseline HCV RNA levels were not predictive of the responsiveness to administration of the TLR7 agonist.

Whereas current peginterferon administration consists of a single interferon subtype (2a or 2b), TLR activation induces several interferon subtypes which may potentially improve antiviral efficacy, but may also lead to more cytokine mediated adverse events; however, both improved antiviral efficacy as deterioration of the AE profile were not seen (with respect to peginterferon), suggesting that the optimal dosage of ANA773 remains to be determined.

In this proof of concept study conclusions are limited by the number of subjects and the heterogeneity of the study population. But due to its heterogenous population it is possible to conclude that immune activation and viral decline can be achieved by ANA773 in HCV infected patients, irrespective of genotype, viral load, sex or previous response to interferon-based therapies.

In conclusion, oral administration of ANA773 was safe and modest anti-HCV activity was seen in combination with marked therapy induced activation of the immune system. Potential advantages of ANA773 relative to weekly subcutaneous injections with peginterferon include the route of administration (oral versus injection) and a good safety profile. For the near future it is likely that peginterferon will remain the cornerstone of antiviral combination therapies. However, with the advent of direct antiviral compounds targeting the HCV protease and polymerase proteins and leading to strong viral suppression, the clinical development of immune activators, such as TLR-agonists, may be advantageous. In this study, we show that forced viral decline combined with activation of the immune system by the TLR7 agonist ANA773 is safe and exhibits modest anti-HCV activity. In addition, our findings pave the way for future studies to explore whether higher doses of ANA773 administered via the oral route will further enhance the antiviral efficacy of this TLR7 agonist in lowering the serum HCV RNA levels in chronic HCV patients. It will be of particular interest to investigate this drug in combination with direct antivirals or with ribavirin, as currently is being planned.

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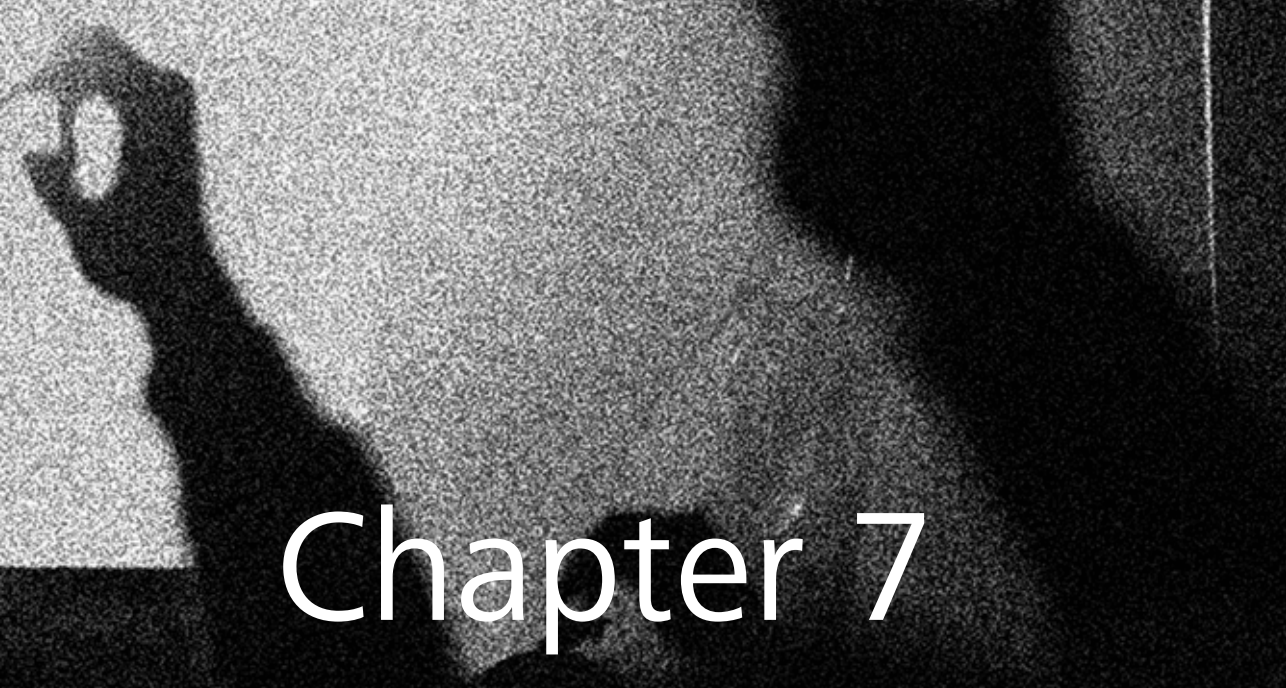


Chapter 7

Potent immune activation in chronic hepatitis C patients upon administration of an oral inducer of endogenous interferons that acts via TLR7

Boonstra A., Liu B-S., Groothuismink Z.M.A., Bergmann J.F., De Bruijne J., Hotho D.M., Hansen B.E., Van Vliet A.A., Van de Wetering de Rooij J., Fletcher S.P., Bauman L.A., Rahimy M., Appleman J.R., Freddo J.L., Reesink H.W., De Knegt R.J., Janssen H.L.A.

Antiviral Therapy (in press)

Abstract

Background: ANA773, an oral prodrug of a small-molecule TLR7 agonist, induces a dose-related decrease in serum HCV RNA levels in chronic hepatitis C patients.

Methods: The prodrug ANA773 was administered to healthy individuals and chronic hepatitis C patients. At different time-points during the course of treatment, modulation of the phenotype and function of peripheral leukocytes were evaluated to determine the role of distinct immune cells on the clinical outcome of therapy.

Results: Early after administration of the TLR7 agonist, a mild, transient reduction of the number of lymphocytes was observed in both healthy individuals and chronic hepatitis C patients. Moreover, repeated administration of ANA773 resulted in transiently reduced numbers of myeloid and plasmacytoid dendritic cells (DC) in blood. Interestingly, reduced plasmacytoid DC numbers as well as increased serum interferon alfa and IP-10 levels were observed only in virological responders (≥ 1 $\log_{10}$ IU/ml reduction of HCV RNA levels upon ANA773 treatment), but were absent in virological non-responders. In vitro stimulation of peripheral blood mononuclear cells from virologic responders showed a high frequency of interferon alfa producing plasmacytoid DC upon stimulation in vitro with ANA773, whereas no interferon alfa was induced in non-responders.

Conclusions: These findings indicate that the viral load decline in chronic hepatitis C patients treated with the TLR7 agonist ANA773 is likely due to intrinsic differences in the induction of endogenous interferons and interferon-stimulated gene products (interferon alfa and IP-10) upon TLR7 ligation.

Introduction

The hepatitis C virus (HCV) is a major cause of chronic liver disease, affecting more than 170 million individuals globally. In about 80% of individuals infected with HCV, the infection does not resolve spontaneously, resulting in persistent infection. Chronic HCV infected patients are at increased risk for developing liver fibrosis, cirrhosis and/or hepatocellular carcinoma, which may take decades to become apparent. The long-term complications of liver failure, as a result of chronic HCV infection, are worldwide the most common causes for liver transplantation (1, 2). At present, no vaccine to prevent persistent HCV infection is available. The standard treatment for chronic HCV infection is pegylated interferon alfa plus ribavirin. This combination therapy has many adverse effects, and a sustained viral response is only observed in about 50% of HCV genotype 1 infected patients. Thus, improved therapies are urgently needed.

Patients who eventually develop chronic hepatitis C, initially have a strong T cell response, but this response is not sustained. In fact, during chronic infections HCV-specific CD4+ and CD8+ T cell responses are difficult to detect in blood and liver, and are functionally impaired, which may be a direct consequence of high viral load, viral escape mutations, or due to active suppression mediated by immunoregulatory mechanisms (3-6).

Stimulation of the immune system in order to boost antiviral immunity is the basis for research in search of effective T cell vaccines against HCV (7). However, an alternative approach is to activate the innate immune system making use of its ability to respond to pathogen-derived products. Activation of DC and macrophages by pathogens can be achieved by the specific interaction between pattern recognition receptors, such as the members of the TLR family, and pathogen-derived products (8, 9). Distinct leukocyte populations in both mice and humans have been shown to express different TLRs, and consequently to respond to distinct microbial products (10, 11). For example, human plasmacytoid DC express TLR7 mRNA, and respond to specific TLR7 agonists, such as single-stranded RNA and R848, to produce type I interferons (12).

Activation of the innate immune system by intravenous administration of a TLR7 agonist isatoribine (13) and oral administration of the TLR7/8 agonist resiquimod (14) have been previously described for the treatment of chronic hepatitis C patients. However, the latter compound interacts with TLR7 and TLR8 and therefore activates not only plasmacytoid DC but also other leukocytes such as monocytes (15), leading to more severe adverse effects. We recently reported the first results of the clinical study in which the TLR7 agonist ANA773 was administered to chronic HCV infected patients via oral administration (16). In this trial, we observed a significant treatment-induced viral decline of serum HCV RNA levels (range 0.14 to -3.10 log at the highest dosing group receiving 2000 mg), which was observed in some, but not all patients. In the current study, we examined the immunological effects following oral administration of the TLR7 agonist ANA773 in patients, and evaluated the immunological differences between responders and non-responders.

Methods

Study design

The characteristics of the chronic hepatitis C patients and healthy individuals who participated in this study have been described in detail before (16). This study was a phase I study, which was conducted at the Erasmus Medical Center (Rotterdam), Academic Medical Center (Amsterdam) and PRA International (Zuidlaren), the Netherlands, in accordance with Good Clinical Practice and the World Medical Association Declaration of Helsinki, after approval by the institutional review board. All patients and

healthy individuals provided written informed consent before participating in any study-related activity. For the ancillary study the cohorts of chronic HCV infected patients receiving a dose of 1600 mg or 2000 mg ANA773 were evaluated for immune status, as well as a cohort of healthy controls receiving 1600 mg ANA773. The highest dose cohorts were examined since considerable reductions of serum HCV RNA load were observed in these cohorts. In the 1600 mg group, 6 chronic HCV infected patients received oral ANA773 and 2 received placebo. In the 2000 mg group, 8 patients received ANA773 and 2 received placebo. Blood samples of the 1600 mg group were drawn on day 0, 5, 13, 27, and 41; the blood samples of the 2000 mg group were drawn on day 0, 5, 9 and 18. No blood was collected from one patient in each dosing group, and therefore immunological assays were performed on PBMC from 5 patients in the 1600 mg group and from 7 patients in the 2000 mg group. The patient details are described before (16). Patients were dosed with oral ANA773 every-other-day for either 28 days (1600 mg group) or 10 days (2000 mg group). Study medication (100 mg capsules) and placebo capsules were supplied by Anadys Pharmaceuticals, Inc., San Diego, USA.

Patients

Key inclusion criteria included male and female chronic HCV patients between 18 to 65 years, with body mass indexes of 18 to 35 kg/m^2, treatment-naive or relapse from prior interferon-based therapies (defined as recurrence of HCV RNA following a full course of treatment and having achieved an undetectable HCV RNA during treatment), and an HCV RNA level $\geq 75 \times 10^3$ IU/mL. Key exclusion criteria included decompensated liver disease (consistent with Child Pugh B/C liver cirrhosis), and co-infection with HIV or HBV. Patients receiving antiviral therapy or immunomodulatory therapy within 90 days prior to administration of the first dose of ANA773 were excluded.

Enumeration of monocytes and leukocytes in whole blood, and quantitation of lymphocyte subpopulations

Absolute numbers of leukocytes, lymphocytes, monocytes and granulocytes in whole blood were measured by an automated impedance hematology analyzer (ABX Micros-60, Horiba Medical). To determine the frequency of distinct leukocyte subpopulations, whole blood was lysed using ammoniumchloride, stained with antibodies against CD4 (SK3, BD), CD8 (RPA-T8, BD), CD56 (MY31, BD), CD19 (SJ25C1, BD), CD14 (61D3, eBioscience), BDCA1 and BDCA4 (both from Miltenyi Biotech). NK cells were defined as CD3-negative lymphocytes that expressed CD56. This population included both CD56dim and CD56bright NK cells. In addition, the expression of CD69 on NK cells was assessed using CD56-PE (MY31, BD) and CD69-APC (L78, BD). All events were evaluated by flow cytometry (Canto-II, BD), and the data was analyzed using BD FACS Diva software. All assays were performed on the day of blood collection.

Intracellular cytokine staining

PBMC were isolated from peripheral blood of patients prior to treatment with ANA773 (2000 mg-group only). Cells were isolated from peripheral blood by density centrifugation on Ficoll-Hypaque (GE healthcare). Fresh PBMC were stimulated on the day of blood collection with medium, ANA773 (300 M) or R848 (1 g/ml; Alexis) in RPMI-1640 medium (BioWhittaker) supplemented with 10% human serum for 5 h, with brefeldin-A (10 g/ml; Sigma) present for the last 4 h. Samples were then fixed with 2% formaldehyde, permeabilized with 0.5% saponin and stained with antibodies against CD14-Pacific Blue (M5E2, BD Pharmingen), BDCA4-APC (AD5-17F6, Miltenyi Biotech), TNF-PE-Cy7 (MAb11, eBioscience), and interferon alfa FITC (MMHA-1, PBL). Cytokine-producing plasmacytoid DC and monocytes were detected by flow cytometry (Canto-II, BD).

Immunoassay for detection of cytokines

The levels in serum of interferon alfa and IP-10 during the course of treatment with ANA773 were detected by enzyme-linked immunosorbent assays by Alta Analytical Laboratory, San Diego, USA. 2,5-OAS was analyzed by radio-immunoassay at PRA International, Assen, The Netherlands.

Statistics

Values are expressed as mean values, unless indicated otherwise. Data were analyzed with Prism 5.0 (Graphpad software) using the Mann-Whitney t-test to compare variables between two independent groups. In all analyses, a two-tailed p-value of less than 0.05 (confidence internal 95%) was considered statistically significant.

Results

Administration of TLR7 agonist ANA773 leads to a transient reduction of the absolute number of lymphocytes in blood of healthy individuals and HCV infected patients

To examine the consequence of administration of the TLR7 agonist ANA773 on immune parameters, we first assessed the effect of treatment on the absolute numbers of various leukocyte subpopulations prior to treatment and 6 hours after the first administration by comparing paired blood samples. As shown in Figure 1, treatment of healthy individuals with a dose of 1600 mg ANA773 every other day did not affect the absolute numbers of peripheral leukocytes, monocytes or neutrophils. Comparable findings were observed when chronic HCV infected patients were treated with a dose of 1600 mg or 2000 mg ANA773 every other day, except for the number of monocytes, which declined within 6 hours following administration of 2000 mg TLR7 agonist. The absolute numbers of lymphocytes was significantly reduced 6 hours after start of treatment in both healthy individuals (dose 1600 mg) and chronic HCV patients (dose 1600 and 2000 mg).

The reduction in the number of lymphocytes 6 hours after administration of ANA773 was transient, since the number of leukocytes, lymphocytes and monocytes was similar as their pre-treatment numbers after day 5 (Figure 2A). Further phenotyping of the lymphocytes in CD4+ T cells, CD8+ T cells, CD3-CD56+ NK cells and CD19+ B cells did not demonstrate any significant shifts in cell numbers during the treatment period.

Administration of ANA773 leads to a transient reduction of the number of plasmacytoid DC only in virologic responders

Since ANA773 interacts with the TLR7, which is expressed at high levels by plasmacytoid DC, we determined the numbers of plasmacytoid DC and myeloid DC, in blood of chronic hepatitis C patients during treatment. As shown in Figure 2B, repeated administration of 1600 mg ANA773 showed a reduction of plasmacytoid DC numbers in blood and myeloid DC which was most prominent on day 13 (1.5×10^6 to 0.8×10^6 cells/l and 9.4×10^6 to 3×10^6 cells/l, respectively), and returned to baseline levels thereafter. Similar to the 1600 mg group, multiple dosing of 2000 mg ANA773 showed the same trend with respect to the decline of the numbers of DC, which was not significant.

Administration with 2000 mg ANA773 resulted in a viral load reduction of more than one log in 5 out of 7 patients. We determined whether the differential clinical responsiveness was reflected by a differential effect on the numbers of plasmacytoid DC. Indeed, as shown in Figure 3, all patients who were considered responders to treatment with TLR7 agonists showed a significant reduction of circulating plasmacytoid DC and myeloid DC numbers at day 9, which was not observed in patients who did not respond to TLR7 ligation. Shortly after ending treatment at day 10, plasmacytoid DC numbers

recovered in responders, whereas the number of myeloid DC were still reduced in some, but not all, patients. It is interesting to note that the baseline plasmacytoid DC frequency is lower in the 2 non-responder patients as compared to the responder patients, which was also observed when examining the non-responder patients of the 1600 mg group.

Differential effects of TLR7-induced responses in virologic responders versus non-responders

To explore the differences between the observed effects of TLR7 ligation in chronic hepatitis C patients who responded and patients who were non-responders, we examined the serum levels of interferon stimulated genes interferon alfa, interferon-induced protein IP-10 and mRNA levels for 2,5-OAS. As presented in Figure 4A, interferon alfa and IP-10 were detectable in serum from most responders, but undetectable in patients who did not respond to ANA773 as defined by no reduction of serum HCV RNA levels. However, in both responder and non-responders to TLR7 ligation, the levels of 2,5-OAS mRNA in serum were induced 6 hours after start of treatment.

In addition, we examined the activation status of NK cells in treated patients. By performing flow-cytometry, we observed that 6h after the first administration, the expression of the early activation marker CD69 was increased on the majority of CD3-CD56dim NK cells in responding patients, but not non-responding patients (Figure 4B). We did not observe TLR7-induced changes of activation markers expressed on plasmacytoid DC or myeloid DC, such as CD80, CD86 or CD40, at different time-points following ANA773 administration (data not shown).

Finally, we compared the in vitro response of PBMC to ANA773 and R848 (a TLR7/8 agonist) with the patient's subsequent virologic response to ANA773 treatment. As shown in Figure 5, a high frequency of interferon alfa producing plasmacytoid DC upon stimulation in vitro was detected in PBMC from patients who were subsequently virologic responders, whereas no interferon alfa was induced in cells from non-responders. As a control experiment, we observed that monocytes were unresponsive to ANA773, whereas activation by R848 induced a high frequency of TNF-producing monocytes. These findings suggest that the in vitro assay may be used as a screening tool for the expected efficacy of antiviral activity of TLR agonists such as ANA773, and that intrinsic properties of plasmacytoid DC may determine the efficacy of treatment with TLR7 agonist of patients with chronic HCV infections.

Discussion

At present, TLR7 agonists to treat HCV infection are not used in clinical practice. These compounds act by specifically inducing antiviral activity initiated by the induction of endogenous interferon as well as by specific TLR7-induced activation of various leukocyte populations, such as plasmacytoid DC. Direct stimulation of the immune system may be an important advantage over the use of exogenous interferon-based antiviral therapy, which does not lead to activation of leukocyte populations. Previously, intravenous administration of a TLR7 agonist isatoribine (13) and oral administration of the TLR7/8 agonist resiquimod (14) has been described in the treatment of chronic hepatitis C patients. The disadvantage of the combined TLR7/8 agonist resiquimod over specific TLR7 agonists is that TLR8 is also expressed on monocytes, and will thus induce pro-inflammatory cytokines other than interferon alfa (Figure 5). The consequence of this is a higher chance of adverse effects (17), and this was indeed observed in the clinical study with resiquimod (14).

The present study demonstrates that treatment of chronic hepatitis C patients with the TLR7 agonist ANA773 activates the immune system by the release of interferon alfa and interferon alfa induced molecules as well as the NK cell compartment. We demonstrate that oral administration of TLR7 agonists

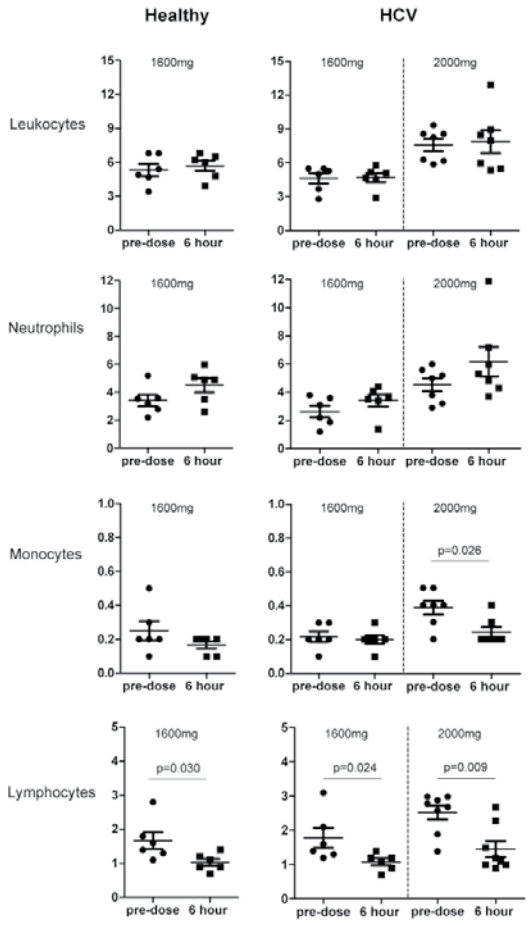
leads to a mild and transient reduction of circulating lymphocytes, plasmacytoid DC and myeloid DC in viral responders to ANA773 treatment. As a direct consequence of TLR7 ligation, or indirectly as a result of enhanced interferon alfa activity, viral responders exhibited increased IP-10 and 2',5'-OAS. Together with activated NK cell activity, this illustrated that important components of the antiviral immune responses were activated upon ANA773 administration. In addition, elevated levels of circulating interferon alfa and IP-10, as well as TLR7-induced activation of NK cells, were only demonstrated in patients with a significant drop in HCV RNA levels upon treatment with TLR7 agonists. Differential responsiveness to TLR7 ligation upon treatment could be reproduced in vitro, suggesting that intrinsic differences between patients accounted for the different efficacy of ANA773. Interestingly, also evaluation of the effect of ANA773 on PBMC from healthy individuals showed induction of interferon alfa by plasmacytoid DC in the majority of individuals (8 out of 10 individuals; data not shown).

Despite activation of various components of the innate antiviral immune response, the decline of serum HCV RNA levels was mild. To explain this, we can not exclude that the highest dose of ANA773 administered in this study was still suboptimal with respect to viral decline. As an alternative explanation, it has been described that the TLR7 signaling pathway is selectively impaired in plasmacytoid DC (18) and monocyte-derived DC (19) from chronic HCV infected patients, as well as in hepatoma cell lines (20). However, we show that upon oral administration of ANA773, no differences were observed between healthy individuals and chronic hepatitis C patients in the immune parameters examined, which were mainly focused on shifts in leukocyte populations and the expression of activation markers. Moreover, functionally, plasmacytoid DC from chronic HCV infected patients were still capable of responding to TLR7 ligation using either ANA773 or R848, indicating that plasmacytoid DC were not completely inert to stimulation via TLR7. Another possible explanation for the modest viral decline observed after ANA773 administration is the reduction of the number of circulating plasmacytoid DC, which may affect the interferon levels that are induced during therapy. TLR ligation as well as exogenous administration of interferon alfa in mice also showed a transient lymphopenia which was the result of redistribution rather than deletion of lymphocytes (21). At present, the transient nature of the response is not clear. However, tight regulation of TLR7 expression may lead to lower responsiveness of cells to TLR ligation upon repeated exposure to the ANA773.

We observed that not all patients responded to ANA773 administration with regard to a decline in viral load. Interestingly, we showed that the responsiveness to ANA773 during the course of treatment was determined by intrinsic characteristics of the individual's leukocytes, since the ability to respond in vivo was paralleled by the in vitro stimulation of the cells with ANA773 prior to treatment. These differences in responsiveness may be influenced by TLR7 polymorphisms which were found to correlate with the response to interferon-based therapy in chronic HCV infected patients (22), and also gender differences are known to influence the levels of interferon alfa produced upon TLR7 ligation (23). Another mechanism that may limit the efficacy of treatment with TLR agonists is elicitation of compensatory mechanisms that regulate and prevent excessive inflammation (24, 25). In mice, it was shown that CD4+CD25+FoxP3+ regulatory T cells were induced upon topical administration of imiquimod in a model of human breast cancer, and also serum levels of the immunosuppressive cytokine IL-10 were elevated following treatment with imiquimod (26). In our study, we did not find any shifts in the number of CD4+CD25+FoxP3+ regulatory T cells during the course of treatment with ANA773 (data not shown), thereby limiting the possibility that the induction of FoxP3+ regulatory T cells underlies the weak antiviral activity.

The effect of TLR7 agonist therapy on the immune system of patients with chronic HCV infections was evaluated in this phase I study. The conclusions drawn from this study have to be considered in

Figure 1.

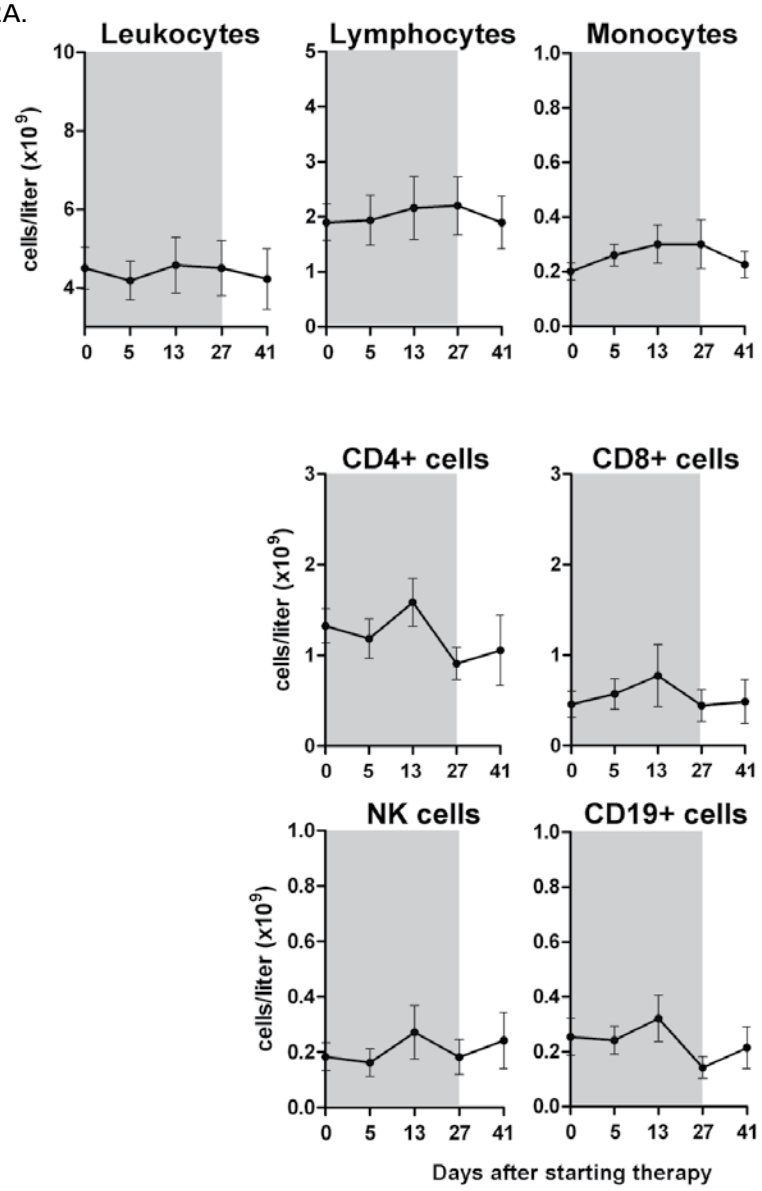


The effect of ANA773 on the numbers of blood leukocytes early after administration. Healthy individuals and chronic hepatitis C patients were administered a single dose of 1600 mg or 2000 mg ANA773. Blood was collected before and 6 h after administration. The absolute numbers of cells were expressed as 10^9 cells/liter, and shown for individual patients.

light of the limited number of patients per dosing group. Despite the small group size, our findings demonstrate that the treatment of chronic hepatitis C patients with the TLR7 agonist ANA773 resulted in a decrease of serum HCV RNA levels, and that this treatment strategy activates parts of the innate immune system. Importantly, those patients that display potent induction of endogenous interferons and interferon-stimulated gene products, most likely via an effect on plasmacytoid DC, also show a therapy-induced decline of viral load. To further improve strategies to develop ANA773 as an approach for HCV treatment, it will be important to examine the mechanism underlying the observation that certain patients are responsive and others are unresponsive to treatment.

In conclusion, the fact that administration of TLR7 agonists lead to a significant viral load reduction in chronic hepatitis C patients, combined with clearly detectable activation of the components of the

Figure 2A.

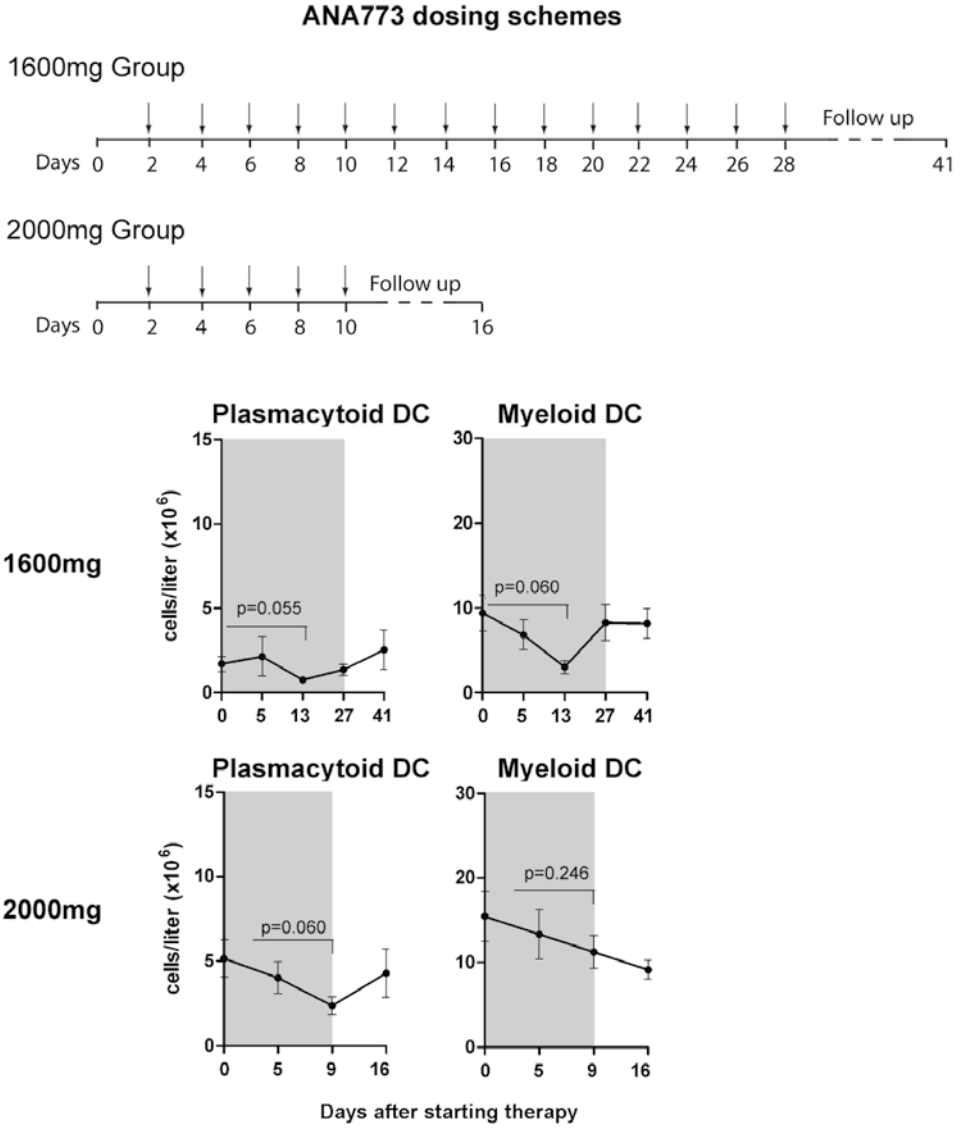


anti-viral immune response, make these novel immunomodulatory compounds promising for further development. Combined or sequential treatment regimens of direct antiviral agents or standard of care to reduce the viral load with the use of TLR7 agonists, as immunomodulators to stimulate the immune system, may well be efficient to eradicate the virus, and simultaneously allow the development of effective HCV-specific T cell memory responses to prevent relapses and re-infection.

Acknowledgements

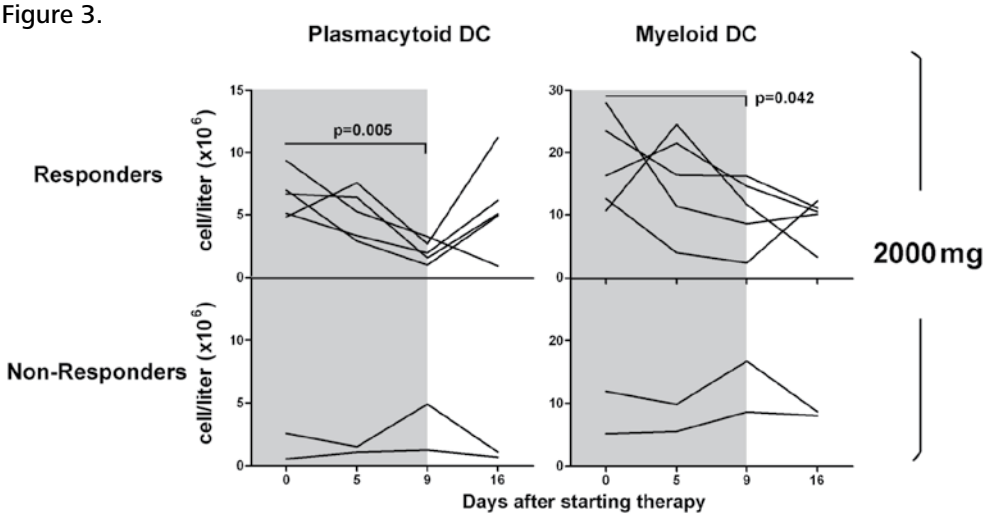
We would like to thank the patients who agreed to participate in the clinical study. We also acknowledge the contribution of Cokki van der Ent and Irene Brings (Clinical Research Bureau, Erasmus MC) and Martine Peters (AMC, Amsterdam). Furthermore, we would like to thank B. Eam, M.V. Sergeeva, and T.W. Harding with their help during various stages of this project.

Figure 2B.



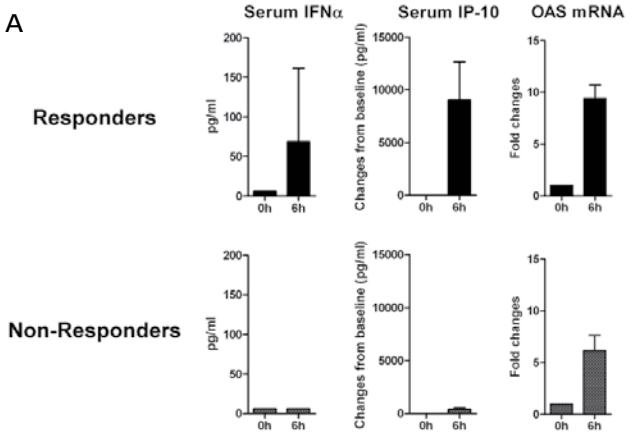
Repeated administration of ANA773 does not influence the number of leukocyte subpopulations over a period of 4 weeks. (A) Chronic hepatitis C patients were treated with ANA773 every 48h for a period of 28 days (1600 mg), and blood was collected at the indicated time-points. Leukocyte subpopulations were determined in whole blood by automated analyses and flowcytometry as described in the material and methods. (B) The effect of ANA773 on DC populations was determined in whole blood of patients treated with 1600 mg ANA773 (as described above) or 2000 mg, which was administered every 48h for 10 days.

Figure 3.



Reduced plasmacytoid DC numbers in chronic HCV infected patients with a decline of HCV RNA levels upon treatment with ANA773, but not in non-responders. Chronic HCV patients were treated with ANA773 at a dose of 2000 mg. The absolute numbers of plasmacytoid DC and myeloid DC are presented at different time points after start of treatment, and displayed separately for patients with a viral decline of more than one log (responders) or less than one log (non-responders).

Figure 4.



Ex vivo analysis demonstrates stronger activation of immunity in virologic responders to ANA773 as compared to non-responders. (A) The serum levels of interferon alfa and IP-10 were determined by ELISA, and the 2,5-OAS levels in serum by RIA before and 6 h after start of treatment. (B) The expression of CD69 on CD3-CD56+ NK cells is determined in whole blood before and 6 h after start of treatment.

Figure 4.

B

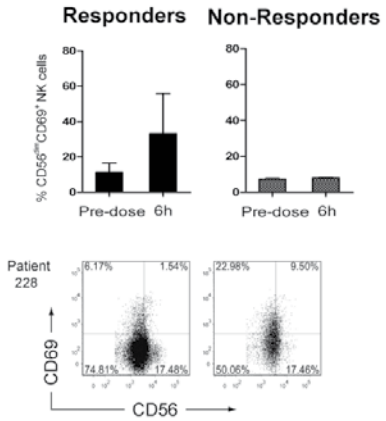
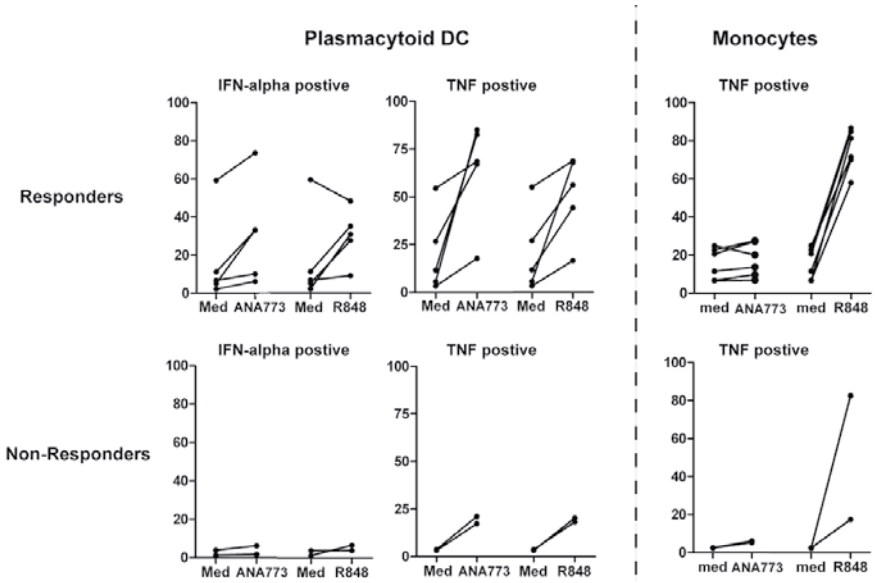


Figure 5.



The frequency of interferon alfa producing plasmacytoid DC in vitro was higher in PBMC from patients that were subsequently virologic responders, whereas no interferon alfa was induced in non-responders. PBMC, collected prior to treatment with 2000 mg ANA773, were stimulated in vitro with medium, ANA773 or R848. The percentage of cytokine producing plasmacytoid DC and monocytes was determined by intracellular cytokine staining for interferon alfa and TNF.

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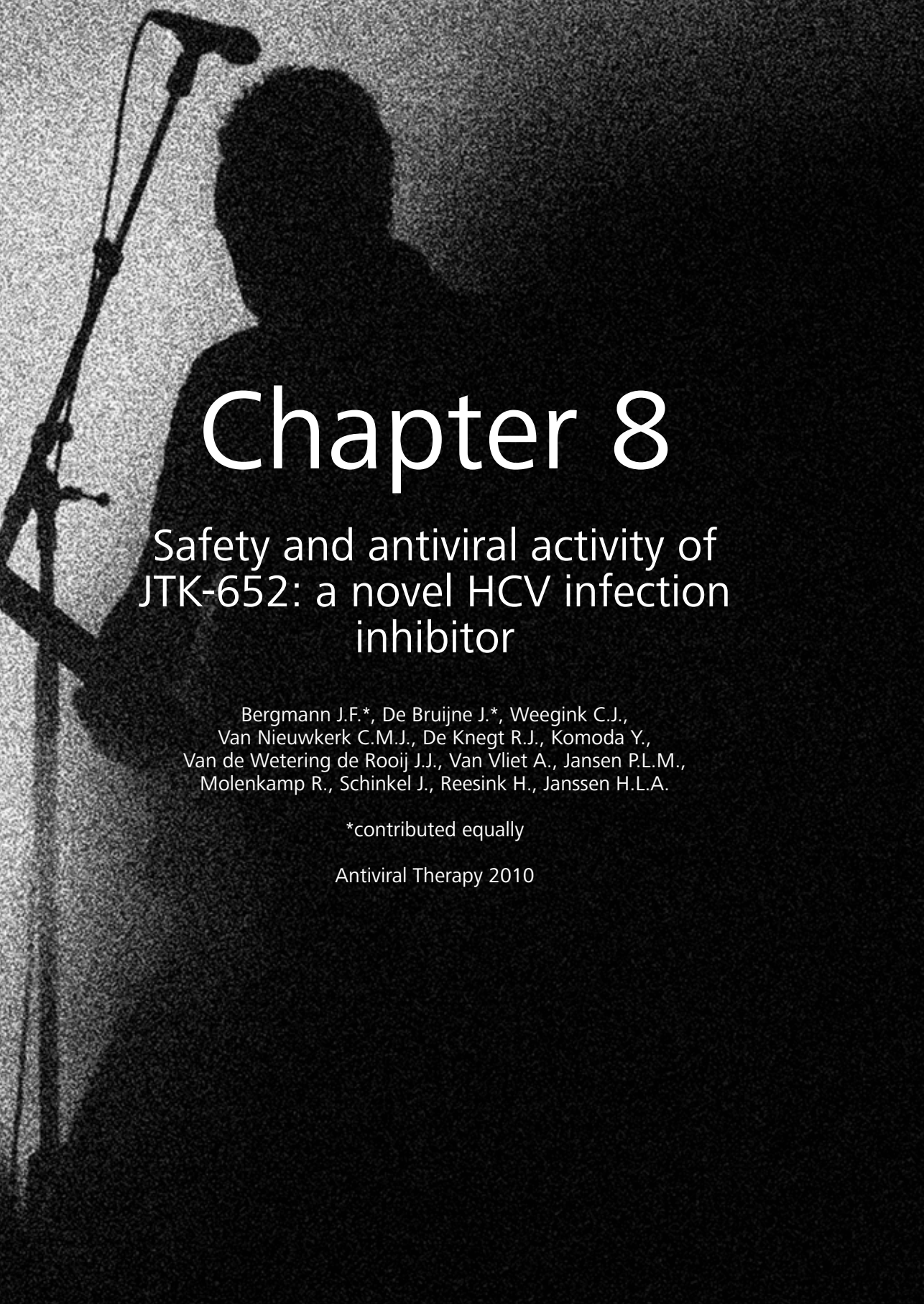

Chapter 8

Safety and antiviral activity of JTK-652: a novel HCV infection inhibitor

Bergmann J.F.*, De Bruijne J.*, Weegink C.J.,
Van Nieuwkerk C.M.J., De Knegt R.J., Komoda Y.,
Van de Wetering de Rooij J.J., Van Vliet A., Jansen P.L.M.,
Molenkamp R., Schinkel J., Reesink H., Janssen H.L.A.

*contributed equally

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Abstract

Background: Standard treatment of chronic hepatitis C with pegylated interferon and ribavirin is associated with suboptimal virological response rates and substantial side effects. This study describes the in vitro and in vivo development of JTK- 652, a novel pyrrolopyridazin-derived HCV infection inhibitor.

Methods: JTK-652 was evaluated in multiple cell lines using an in vitro HCV infection model consisting of HCV pseudotype vesicular stomatitis virus bearing HCV E1/E2 envelope proteins. Safety, tolerability, pharmacokinetics and efficacy of JTK-652 were tested in a randomized double-blind and placebo-controlled study in healthy male volunteers (n=36) and chronic hepatitis C patients. A total of 10 HCV genotype 1 infected patients (treatment-naïve [n=2] and treatment-experienced [n=8]) with HCV RNA> 1×10^5 IU/ml received an oral dose of 100 mg JTK-652 three times daily or placebo (8:2 ratio) for 4 weeks.

Results: JTK-652 showed potent inhibitory activity against HCV genotype 1a and 1b pseudotype viruses bearing HCV E1/E2 envelope proteins in HepG2 cells and in human primary hepatocytes. No significant clinical laboratory, vital sign, ECG or physical examination abnormalities were observed during the phase I trial. JTK-652 was found to be well tolerated. No significant changes in HCV RNA levels compared with baseline were observed at the end of treatment.

Conclusions: Although results from the preclinical studies indicated that JTK-652 has well-established anti-viral properties and a phase I clinical trial has showed that JTK-652 was safe and well tolerated at a 100 mg three times daily dose level, plasma HCV RNA levels in chronically HCV-infected patients did not decrease during 28 days of dosing at a 100 mg three times daily dose level.

Introduction

Hepatitis C results from infection with HCV through exposure to infected blood. Approximately 170 million people worldwide, 3% of the world's population, are infected with HCV. Globally, it is estimated that 3-4 million people are newly infected each year (1), with 50–80% of HCV-infected patients developing chronic hepatitis (2). The most important sequelae of chronic HCV infection are progressive liver fibrosis leading to cirrhosis and hepatocellular carcinoma (3). Standard hepatitis C treatment consists of 24-48 weeks of combination therapy with pegylated interferon alfa and ribavirin, which is dependent on HCV genotype, baseline HCV RNA titre and virological response (4-6). Antiviral treatment should be considered in all chronically HCV-infected individuals; however, suboptimal response rates, abundant side effects and high treatment costs are major disadvantages of the current standard of care.

Development of new specifically targeted antiviral therapy against hepatitis C is eagerly anticipated. The target cells of HCV are hepatocytes and the natural hosts are humans and chimpanzees. Because of the lack of a cell culture system that supports efficient production of infectious particles, studying HCV entry has been difficult; for this reason, several surrogate in vitro systems have been developed to study the process of HCV entry into host cells (7-9). Recombinant HCV envelope glycoproteins have been successfully used as surrogate models to study virus-host interaction. Development of these functional models (HCV-like particles, recombinant HCV envelope glycoproteins, HCV pseudotype particles and vesicular stomatitis virus [VSV]/HCV pseudotypes) to analyse HCV entry has led to the identification of several cell surface molecules (including CD81, scavenger receptor B type 1, claudin-1, occludin, heparin sulphate, glucosaminoglycans, DC-SIGN, L-SIGN and low-density lipoprotein receptor) that are involved in HCV entry (10-16). Recent data suggest that HCV entry requires a complex, tightly regulated interaction between the host and HCV.

VSV can be efficiently propagated in animal cells and readily forms pseudotypes with the envelope proteins of different viruses, including HCV. VSV pseudotype viruses expressing HCV E1/E2 chimeric proteins, containing transmembrane and cytoplasmic domains of the VSV-G glycoprotein, have been developed as HCV pseudotype models to study HCV binding and entry (17-18).
JTK-652, a novel pyrrolopyridazin-derived HCV infection inhibitor, was identified by high-throughput screening of a large number of potential compounds (Central Pharmaceutical Research Institute, Japan Tobacco Inc., Osaka, Japan). Using the in vitro HCV infection model consisting of HCV pseudotype VSV bearing HCV E1/E2 envelope proteins, JTK-652 was evaluated in multiple cell lines.

This study describes the preclinical development of this first-in-class infection inhibitor, followed by clinical studies testing safety, tolerability, pharmacokinetics and efficacy of JTK-652 in healthy volunteers and chronic hepatitis C patients.

Methods

Preclinical development of JTK-652

Pharmacology

To evaluate the inhibitory effect of JTK-652 on HCV infection, HepG2 cells, human primary hepatocytes and Huh7 cells were infected with HCV genotype 1a and/or 1b pseudotype VSV (19). The secreted alkaline phosphatase gene was inserted into the VSV genome, and its secretion from the infected cells into the culture supernatant was used to measure the viral infectivity. JTK-652, at various concentrations, was added at the same time as inoculation of target cells with HCV pseudotype virus. Subsequently, the secreted alkaline phosphatase activity in the culture supernatant was measured after

cultivation for 24 h. Recombinant VSV bearing the G protein, the original envelope protein of VSV, was used as a control.

HepG2 cells were infected with both HCV genotype 1a and 1b pseudotype virus and human primary hepatocytes were infected with HCV genotype 1b pseudotype virus. The effect of JTK-652 on infection of Huh7 cells with HCV genotype 1a pseudotype virus was evaluated using HCV pseudotype virus produced in HCV E1/E2 protein-expressing 293T cells.
To determine the influence of human serum proteins on the inhibitory effect of JTK-652, human serum was added to the inoculum of HepG2 cells infected with HCV genotype 1b pseudotype virus at a final concentration of 50%.

To evaluate the cytotoxicity of JTK-652 in the different target cells, living-cell-derived ATP-dependent luciferase activity was measured after cultivation for 24 h. Using the assay system for infection of HepG2 cells with HCV genotype 1b pseudotype virus, the effect of the timing of adding JTK-652 on its inhibitory activity was investigated. JTK-652 was added to HepG2 cells at a final concentration of 30 nmol/l at 0, 0.5, 1, 2, 3, 4 and 5 h after inoculation with HCV pseudotype virus.

The effect of JTK-652 on replication of HCV replicon RNA was investigated using HCV replicon-containing cells (8). JTK-652 was added at various concentrations and the total RNA from the cells was extracted after cultivation for 48 h. The amount of HCV replicon RNA was measured by real-time reverse transcriptase PCR to evaluate the effect of JTK-652. As a positive control, JTK-109, an HCV-RNA-dependent RNA polymerase inhibitor, was used (20).

Clinical evaluation of JTK-652

Safety, tolerability and pharmacokinetics of JTK-652 in healthy volunteers

Safety, tolerability and pharmacokinetics of JTK-652 in healthy male volunteers was evaluated by two phase I, randomized double-blind placebo-controlled ascending dose studies. A single ascending dose study in 18 healthy male volunteers was conducted. In an alternating panel design, two panels of nine volunteers were administered single oral doses of 100, 200, 400, 800 (fed and fasted) and 1200 mg JTK-652 (six participants) or placebo (three participants), over three study periods. Subsequently, two dose cohorts (400 and 800 mg) each consisting of nine volunteers were randomized to receive JTK-652 (n=6) or placebo (n=3) three times daily for 14 days. The main inclusion criteria for both studies were being a healthy male between 18 and 55 years of age and having a body mass index (BMI) of 19–28 kg/m^2.

Safety, tolerability, pharmacokinetics and antiviral activity of JTK-652 in chronic hepatitis C patients

The primary objective in this phase Ib trial was to determine the safety, tolerability and antiviral activity of JTK-652 in patients with genotype 1 chronic hepatitis C. Our secondary objective was to assess the pharmacokinetics of multiple oral doses of JTK-652.

This randomized double-blind placebo-controlled study was conducted at two sites: Academic Medical Center (University of Amsterdam, Amsterdam, the Netherlands) and PRA International (Zuidlaren, the Netherlands). The Erasmus Medical Center (Erasmus University Rotterdam, Rotterdam, the Netherlands) and the VU Medical Center (VU University, Amsterdam, the Netherlands) referred patients for the study. The JTK-652 starting dose of 100 mg three times daily was based on the safety, pharmacokinetic and tolerability results of the 14-day multiple increase dose study in healthy volunteers and was considered to be a minimum dose level to reduce viral load in chronic hepatitis C patients. In the first planned cohort, 10 patients (8 active and 2 placebo) were randomized to receive JTK-652 100 mg

or placebo every 8 h for 4 weeks. Placebo was included to ensure a controlled assessment of safety during the study. The 4-week treatment period included a 2-week in-house admission period and a 2-week ambulatory period with hospital visits on days 22 and 29. Follow-up included two visits for medical examinations on days 36 and 43. JTK-652 was supplied as 100 mg tablets for oral administration. Placebo formulation and dosing was identical to JTK-652 except for the active compound. The main inclusion criteria were being a male or a post-menopausal female (follicle-stimulating hormone level > 40mIU/ml or lack of menses for > 12months) between 18 and 65 years of age with chronic genotype 1a, 1b or mixed 1a/1b HCV infection, having HCV RNA $\geq 1 \times 10^5$ IU/ml, BMI of 18.5-32.0 kg/m^2, alanine aminotransferase concentrations ≤ 5 the upper limit of normal and no clinically significant deviations from the normal range for haematology or clinical chemistry values. Haemophilia patients were allowed to participate in this study. Plasma samples for JTK-652 pharmacokinetics were collected on days 1 and 14 before the morning dose and 0.5, 1, 1.5, 2, 3, 4, 6, 8 and 16 (only day 1) h after the morning dose. Pre-morning dose samples were also collected on days 2–13, 22 and 29. Safety assessments were performed at regular intervals during the treatment and follow-up period. Safety assessments included ECG, vital sign examination, laboratory testing (chemistry, haematology and urinalysis), physical examination and adverse events registration. HCV RNA concentrations were determined using the COBAS® AmpliPrep/Cobas® TaqMan® (Roche, Basel, Switzerland) assay. The dynamic range of the assay was 43-6.9 $\times 10^7$ IU/ml. The limit of quantification of the assay was 43 IU/ml and the limit of detection was 15 IU/ml.

For HCV RNA measurements, serum samples were collected before the morning dose of study drug on days 1-4, 7, 14 and 22 of the dosing period and on days 29, 36 and 43 after the end of study.

Ethical committees of the participating centres (Academic Medical Center and PRA International) approved the study and all participants provided written informed consent before participating in any study-related activity. All studies were conducted in full compliance with the guidelines of Good Clinical Practice and the Declaration of Helsinki and were in accordance with the principles of Good Laboratory Practice.

Results

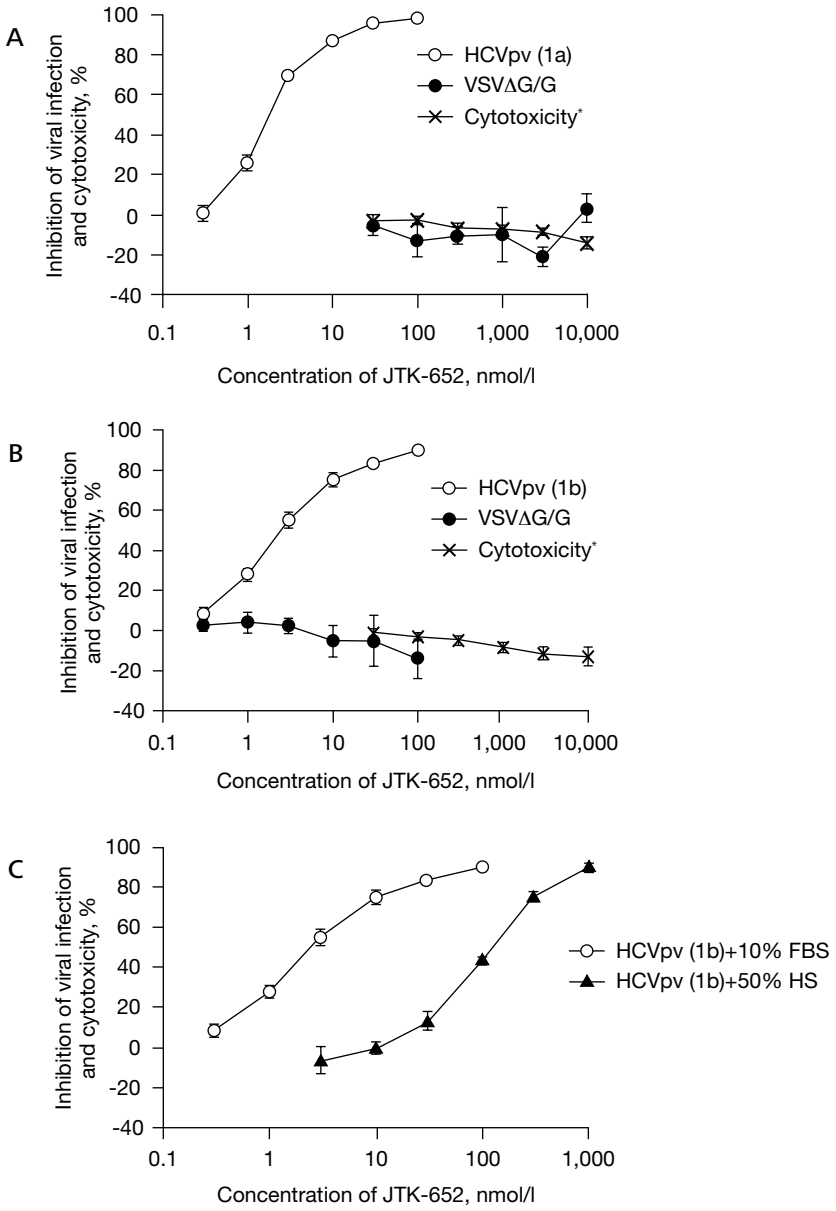
Preclinical development of JTK-652

Pharmacology

In vitro pharmacology studies demonstrated that JTK-652 inhibits infection of HepG2 cells with HCV genotype 1a and 1b pseudotype virus in a dose-dependent manner. The half maximal inhibitory concentrations (IC50) ±SE were 1.9 ±0.07 nmol/l and 3.1 ±0.49 nmol/l, respectively (Figure 1A and 1B). In the presence of 50% human serum, inhibition of infection with HCV genotype 1b pseudotype virus decreased approximately 40-fold to an IC50 ±SE value of 127 ±12.4 nmol/l (Figure 1C). No inhibitory effect on infection with recombinant VSV bearing G protein of original envelope protein of VSV was seen. JTK-652 did not show cytotoxicity in HepG2 cells up to a concentration of 10 µmol/l (Figure 1).

JTK-652 inhibits infection of human primary hepatocytes with HCV genotype 1b pseudotype virus with an IC50 value of 5.8 nmol/l (Figure 2A). This inhibition was comparable to that observed in HepG2 cells. JTK-652 did not show cytotoxicity in human primary hepatocytes up to a concentration of 10 µmol/l (Figure 2A). JTK-652, up to a concentration of 10 µmol/l did not inhibit infection of Huh7 cells with HCV pseudotype 1a virus (Figure 2B).

Figure 1. Inhibitory effect of JTK-652 on infection of HepG2 cells and the effect of human serum on JTK-652



Values shown are mean ± SE (n = 3). (A) Inhibitory effect of JTK-652 on infection of HepG2 cells with vesicular stomatitis virus (VSV)-based pseudotype virus bearing HCV genotype 1a E1/E2 envelope proteins (HCVpv [1a]) or with recombinant VSV (VSVΔG/G). (B) Inhibitory effect of JTK-652 on infection of HepG2 cells with genotype 1b HCVpv (HCVpv [1b]) or with VSV (VSVΔG/G). (C) Effect of 10% bovine serum (FBS) and 50% human serum (HS) on the inhibitory effect of JTK-652 on infection of HepG2 cells infected with HCVpv (1b). *Cytotoxicity in HepG2 cells.

When JTK-652 was added to the cell culture at the same time as inoculation with HCV genotype 1b pseudotype virus, JTK-652 showed a potent inhibitory activity of approximately 90%. The inhibitory effects were reduced and almost disappeared when JTK-652 was added ≥ 3 h after inoculation (Figure 3).

JTK-652 showed no inhibition of replication of HCV replicon up to a concentration of 10 µmol/l whereas JTK-109, an HCV-RNA-dependent RNA polymerase inhibitor, inhibited the replication of HCV replicon RNA with an IC50 ±SE value of 0.43 ±0.01 µmol/l (YK, data not shown). Safety pharmacology studies indicated that JTK-652 had no effect on the central nervous, cardiovascular, gastrointestinal, renal/urinary or respiratory systems in rats and dogs. In the phototoxicity studies, a positive reaction was observed dose-dependently at 300 mg/kg with single oral administration in mice (dose levels were 30, 100, 300 or 1000 mg/kg).

Clinical evaluation of JTK-652

Safety, tolerability and pharmacokinetics of JTK-652 in healthy volunteers

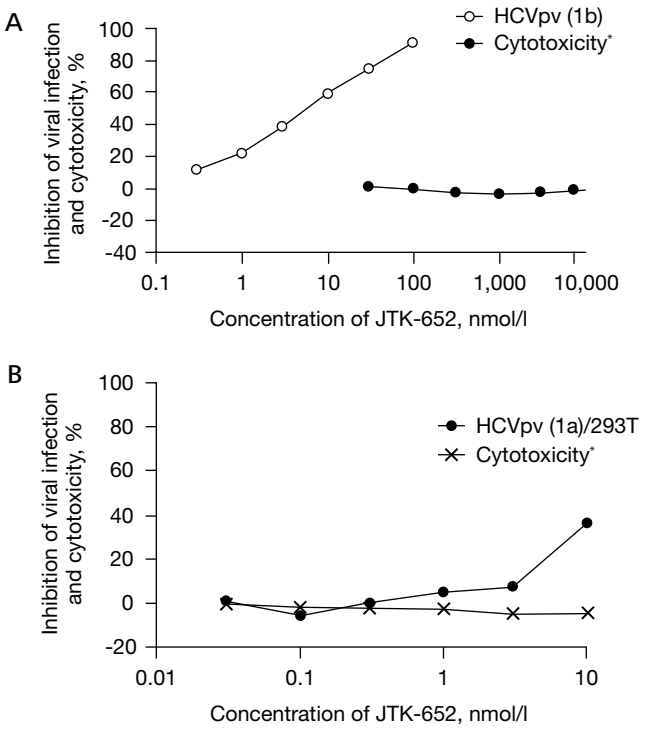
Treatment with single oral doses of JTK-652 at doses ranging from 100 to 1200 mg was safe and well-tolerated in all healthy male volunteers. Multiple oral doses of 400 and 800 mg JTK-652 three times daily during 14 days were safe and well-tolerated by five out of six and three out of six healthy male volunteers, respectively. Four participants, one after treatment with 400 mg JTK-652 three times daily and three after treatment with 800 mg JTK-652 three times daily, developed a rash of mild intensity at approximately day 9 or day 10 and were withdrawn. Except for the transient rash, there were no other clinically significant findings considered to be related to study drug administration with regard to clinical laboratory, vital sign, body weight, ECG or physical examination. After multiple dosing with 400 and 800 mg JTK-652 three times daily, maximum JTK-652 plasma concentrations were reached between 1.50 and 1.75 h post-dose (median time to reach the maximum plasma concentration [tmax]). For area under the curve (AUC)0-T, maximum concentration (Cmax) and trough concentration (Ctrough), a less than dose-proportional increase was observed for the dose range of 400-800 mg JTK-652. The mean half-life (t1/2) (ty=t1/2) for JTK-652 was approximately 21 h for both doses studied, with a steady state reached by day 5. The accumulation ratio (day 14:day 1) with respect to AUC0-T was, on average, 1.53 after treatment with 400 mg JTK-652 three times daily and 1.19 after treatment with 800 mg JTK-652 three times daily.

Safety, tolerability, pharmacokinetics and antiviral activity of JTK-652 in chronic hepatitis C patients

A total of 10 patients were enrolled and randomized into the study and 8 patients completed the study (7 active and 1 placebo). Demographic characteristics and baseline HCV RNA levels were similar in all dosed patients (Table 1). Of the 10 patients in the study, all were male and Caucasian. Premature discontinuation occurred in two patients (one active and one placebo) on day 13 and day 19, respectively, because of a rash of mild intensity. Most patients, 8 out of 10 (80%), had been treated previously for HCV infection. All 10 dosed patients were fully compliant with study drug dosing.

With regard to safety and tolerability, administration of multiple oral doses of 100 mg JTK-652 or placebo three times daily for 28 days was safe and well-tolerated by 8 out of 10 male participants with chronic hepatitis C. There were no serious adverse events reported during this study. All adverse events (AEs) were of mild intensity. Treatment was not tolerated by two participants and they were withdrawn during the course of the study because of an AE. During treatment with 100 mg JTK-652 three times daily, one participant developed a rash on day 17. The other participant developed a rash on day 12 during treatment with placebo three times daily. Both rashes were mild in intensity for these two participants and dosing was discontinued on day 19 and day 13, respectively. The rash disap-

Figure 2. Inhibitory effect of JTK-652 on viral infection of human primary hepatocytes and Huh7 cells



Values shown are means (n = 2). (A) Inhibitory effect of JTK-652 on infection of human primary hepatocytes with vesicular stomatitis virus-based pseudotype virus bearing HCV genotype 1b E1/E2 envelope proteins (HCVpv [1b]). (B) Inhibitory effect of JTK-652 on infection of Huh7 cells with genotype 1a HCVpv (HCVpv [1a]) produced in HCV E1/E2 protein-expressing 293T cells exhibiting different cell tropism. *Cytotoxicity in human primary hepatocytes. #Cytotoxicity in Huh7 cells.

peared within days after stopping JTK-652 administration and additional treatment was not necessary. In total, 16 of the 46 AEs were considered as possibly related to the study medication. These related AEs were mainly skin and to a lesser extent gastrointestinal disorders. Except for the rash, there were no other clinically significant findings with regard to clinical laboratory, vital sign, body weight, ECG or physical examinations.

The pharmacokinetics of JTK-652 were studied and its appearance in plasma shortly after oral intake led to the median tmax being reached at approximately 1.25-1.75 h post-dose. After the final dose, a gradual decrease in plasma JTK-652 concentrations was observed. The mean accumulation ratio (day 14:day 1) with respect to AUC0-8 h was 1.64 after treatment with 100 mg JTK-652 three times daily. The mean accumulation ratio (day 14:day 1) with respect to Cmax and Ctrough was 1.47 and 1.81, respectively (Table 2). JTK-652 Ctrough remained almost steady after day 2 (Figure 4) and the range

Figure 3. Effect of the timing of adding JTK-652 on HCV genotype 1b pseudotype virus infection

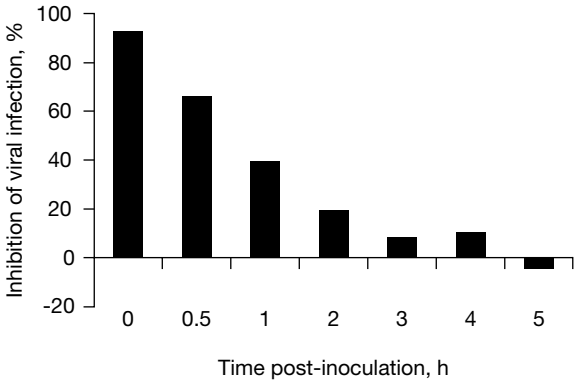


Table 1. Patient baseline characteristics

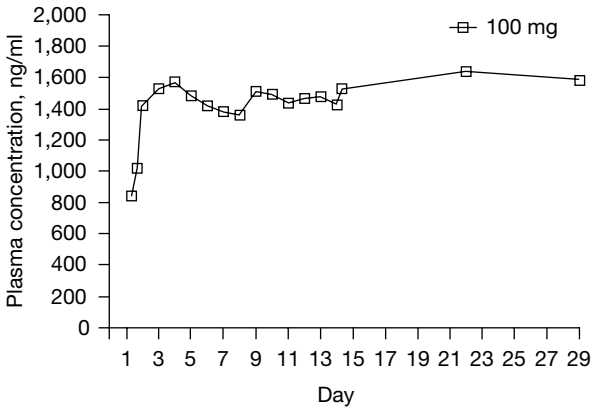
Characteristic	JTK-652 100 mg* (n = 8)	Placebo (n = 2)	All participants (n = 10)
Male, n (%)	8 (80.0)	2 (20.0)	10 (100.0)
White, n (%)	8 (80.0)	2 (20.0)	10 (100.0)
Mean age, years (range)	51 (43-61)	45 (40-49)	49 (40-61)
BMI			
Mean, kg/m^2 (± sd)	24.8 (3.6)	24.7 (2.4)	24.8 (3.3)
Range, kg/m^2	20.8-31.8	23.0-26.4	20.8-31.8
Median ALT level, U/l	113.0	85.5	101.0
HCV genotype			
1a, n (%)	5 (50.0)	2 (20.0)	7 (70.0)
1b, n (%)	2 (20.0)	-	2 (20.0)
Mixed type 1a/1b, n (%)	1 (10.0)	-	1 (10.0)
Mean HCV RNA load, $\log_{10}$ IU/ml (± sd)	6.51 (0.49)	6.06 (0.04)	6.42 (0.47)
Prior HCV treatment, n (%)	6 (60.0)	2 (20.0)	8 (80.0)

*Three times daily. ALT, alanine aminotransferase; BMI, body mass index.

of Ctrough values on day 14 in patients was 780–2,369 ng/ml. The Ctrough values in all patients, therefore, exceeded the IC50 value by > 10-fold (127 nmol/l in the presence of 50% human serum; approximately 70 ng/ml).

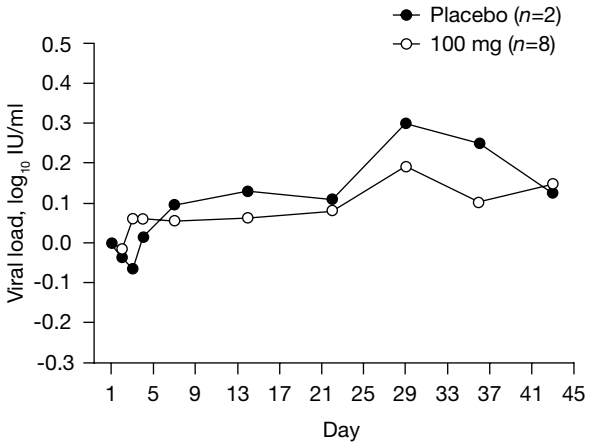
An investigation into the efficacy of JTK-652 found that after multiple dosing with 100 mg three times daily, no decrease in viral load was observed at the expected therapeutic dose level (Figure 5). Individual maximum viral load change from baseline in all participants was within 0.5 $\log_{10}$ IU/ml. Alanine aminotransferase levels were unaffected by treatment with multiple doses of 100 mg JTK-652 three times daily.

Figure 4. Mean* plasma JTK-652 100 mg three times daily trough concentrations in patients



*Geometric mean (n = 8). For days 22 and 29, n = 7.

Figure 5. Mean* plasma HCV RNA values throughout the study



*Arithmetic mean (placebo: n = 1 on days 14, 22, 29 and 36; and 100 mg: n = 7 on days 22, 29 and 36).

Discussion

This study is the first to describe the in vitro development of an HCV infection inhibitor and its clinical evaluation in chronic hepatitis C patients. Firstly, we described the in vitro antiviral properties of JTK-652 against HCV pseudotype virus, using multiple cell lines as target cells. Secondly, we evaluated JTK-652 in chronic hepatitis C patients to assess safety, tolerability, pharmacokinetics and antiviral activity.

Although we are still far from understanding the details of HCV entry, recent data show that viral entry into target cells is a complex multistep process requiring the presence of several entry factors (21).

Table 2. Summary statistics of JTK-652 plasma PK parameters in patients

Treatment	Day	n	C_{max}, ng/ml	t_{max}, h	C_{trough}, ng/ml*	AUC_{0-8}, ng•h/ml	Accumulation ratio AUC_{0-8}	Accumulation ratio C_{max}	Accumulation ratio C_{trough}
100 mg JTK-652 three times daily	1	8	2,061 (968-3,363)	1.25 (1.00-2.00)	842 (532-1,409)	10,228 (5,531-17,471)	-	-	-
	14	8	3,025 (1,981-4,770)	1.75 (1.00-2.00)	1,524 (780-2,369)	16,751 (11,349-25,582)	1.64 (1.20-2.44)	1.47 (1.04-2.64)	1.81 (1.47-2.58)

For the maximum concentration (C_{max}), trough concentration (C_{trough}), AUC0-8 (area under the curve for 0-8 h) and the accumulation ratio, the geometric mean (range) is presented; for time to reach the maximum plasma concentration (tmax) the median (range) is presented. *C_{trough} of day 1 and day 14 is the concentration at 8 h after dosing. PK, pharmacokinetic.

Initial attachment of the virion involves the tetraspanin CD81, glucosaminoglycans, heparan sulphate and the low-density lipoprotein receptor, followed by the sequential interaction with the scavenger receptor class B type I and the tight junction proteins claudin-1 and occludin. Furthermore, unidentified factors might be involved in the HCV entry process. Previous studies described the crucial role of CD81 in the process of HCV infectivity (14,22,23). Binding of CD81 to E2 is crucial for an HCV viral particle to penetrate into the host cell; however, recent studies showed that cell lines with little or no CD81 were capable of direct cell-to-cell viral transmission, suggesting a CD81-independent HCV entry process (24-26). This indicates that there are at least two modes by which a virus can transmit from an infected cell to an uninfected cell: cell-free transmission and cell-to-cell transmission.

In this study, three different cell lines were used to evaluate the inhibitory effect of JTK-652 on infection with HCV genotype 1a and 1b pseudotype viruses: HepG2 cells, human primary hepatocytes and Huh7 cells. JTK-652 showed inhibition of infection of HepG2 cells with HCV genotype 1a and 1b pseudotype viruses bearing HCV E1/E2 envelope proteins. HepG2 cells do not express CD81 on their cell surface; however, human fibroblast growth factor receptor 5 has been reported as a possible candidate for CD81-independent HCV entry in HepG2 cells (27). We then demonstrated a similar inhibition when human primary hepatocytes were used as target cells. These results suggest that JTK-652 possesses a potent inhibitory activity against infection of HepG2 cells and human primary hepatocytes with HCV genotype 1a or 1b pseudotype virus. JTK-652 showed no inhibition of infection of Huh7 cells with HCV genotype 1a pseudotype virus. These Huh7 cells were infected with HCV pseudotype virus produced in HCV E1/E2 protein-expressing 293T cells. A possible explanation for the lack of inhibitory effect in these cells could be because of the amount of E1 and E2 expressed on the cell surface or because of differences of glycosylation as described before (19).

The timing of adding JTK-652 on the viral inhibitory effect on HepG2 cells infected with pseudotype 1b virus was investigated. Maximum inhibition of viral infection was achieved when JTK-652 was added during inoculation with HCV pseudotype virus. The inhibitory effect was reduced upon delayed addition of JTK-652 to the assay system and little inhibitory effect was observed when JTK-652 was added $\geq$ 3 h after the inoculation. In addition, because JTK-652 showed no inhibition of the replication of HCV replicon RNA, JTK-652 had apparently no effect on the replication process of HCV.

Safety and tolerability experiments in animals showed a positive, dose-dependent skin reaction in the phototoxicity studies. Phototoxicity was observed in mice after drug exposure at a dose level of 1000 mg/kg; therefore, when JTK-652 was administered to humans in clinical studies, safety measures were taken, such as avoiding sunlight exposure. Despite such measures being taken, 4 out of 12 healthy volunteers were withdrawn during the course of the study because of a rash of mild intensity. These events of rash were considered to be related to the study drug and as a consequence the study dose in the phase Ib study in chronic hepatitis C patients was lowered. Because JTK-652 plasma pharmacokinetic parameters in healthy volunteers had revealed that 100 mg dosing appeared to be sufficient as far as Cmax, tmax, AUC0–∞ and t1/2 were concerned, it was decided to reduce the dose for the first cohort of chronic hepatitis C patients from 400 to 100 mg three times daily.

Data of the phase Ib study revealed that JTK-652 at 100 mg three times daily was well tolerated in the patients; however, two patients (one placebo and one active) had to stop treatment prematurely because of a mild rash. As one of these patients had received placebo, the rash might have been caused by an excipient that was present in the placebo as well as in the active medication. JTK-652 could not be excluded as the causal factor in the rash developed by the participant on the active treatment.

JTK-652 did not demonstrate HCV RNA decreases in eight chronic hepatitis C patients. It was decided to waive a higher dosing cohort because of the lack of HCV RNA decrease in the first dosing cohort (100 mg three times daily) and the observed rash in healthy volunteers and chronic HCV patients. Despite inhibition of infection of HepG2 cells and human primary hepatocytes with HCV genotype 1a and/or 1b pseudotype virus by JTK-652, no viral inhibitory effect of JTK-652 was seen during the Phase Ib trial in chronic hepatitis C patients. therefore, it can be concluded that the in vitro viral inhibition of JTK-652 against HCV pseudotype virus infection might not represent the actual in vivo process of HCV infection. Other approaches to study HCV infection inhibition, such as lentiviral particles bearing HCV glycoproteins or HCV produced in cell culture or eventually small animal models, could potentially help to bridge the gap from in vitro models to effective HCV entry inhibitors.

In summary, JTK-652 monotherapy did not decrease HCV RNA in chronic hepatitis C patients. The majority of adverse events were mild and there were two premature discontinuations in the Phase Ib study because of rash. Further development of JTK-652 was discontinued. Nevertheless, HCV infection inhibition remains a potential target for antiviral therapy within the HCV life cycle.

Acknowledgements

The authors thank the patients who generously agreed to participate in this clinical study. We also acknowledge the contribution of the following individuals: M.W. Peters (study nurse at Academic Medical Center), S. Menting, S.P.H. Rebers (laboratory staff, Section of Virology, Academic Medical Center), and C. van der Ent and I. Brings (Clinical Research Bureau, Erasmus Medical Center).

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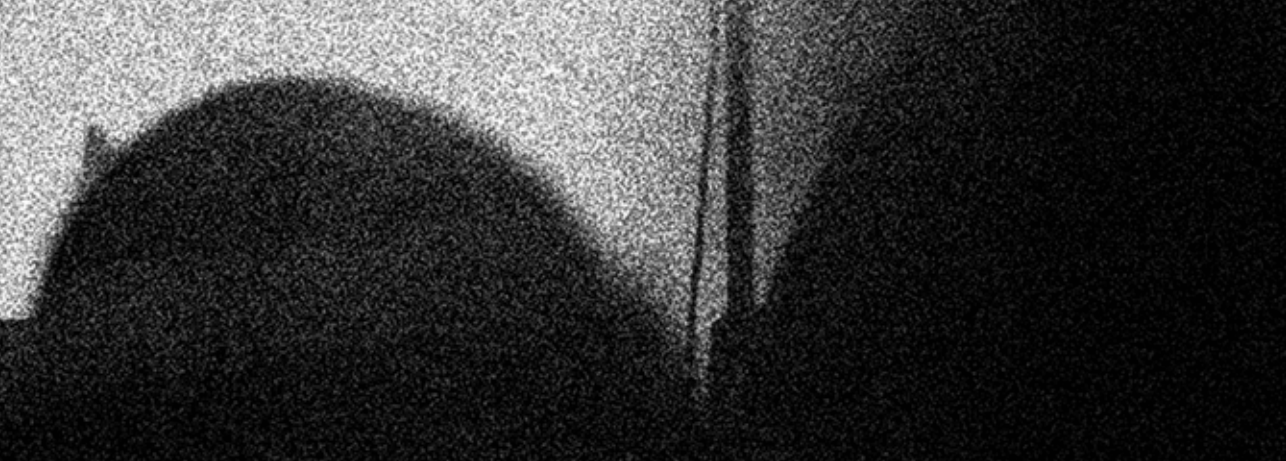

Chapter 9

Antiviral activity of narlaprevir combined with ritonavir and pegylated interferon in chronic hepatitis C patients

Bergmann J.F.*, De Bruijne J.*, Reesink H.W., Weegink C.J., Molenkamp R., Schinkel J., Tong X, Li J., Treitel M.A., Hughes E.A., Van Lier J.J., Van Vliet A.A., Janssen H.L.A., De Knegt R.J.

*contributed equally

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Abstract

Narlaprevir (SCH 900518) is a potent inhibitor of the hepatitis C virus (HCV) non-structural protein 3 serine protease that is primarily metabolized by the cytochrome P450-3A4 system. In order to explore the use of ritonavir-based pharmacokinetic enhancement of an HCV protease inhibitor, this study investigated the safety, tolerability, pharmacokinetics, and antiviral activity of narlaprevir (with or without ritonavir) administered as monotherapy and as combination therapy with pegylated interferon alfa-2b to HCV genotype 1 infected patients. This was a randomized, placebo-controlled, two-period, blinded study in 40 HCV genotype 1 infected patients (naïve and treatment-experienced). In period 1, narlaprevir was administered for 7 days as 800 mg three times daily without ritonavir or 400 mg twice daily with 200 mg ritonavir twice daily. In period 2, after a 4-week washout, the same dose and regimen of narlaprevir was administered in combination with peginterferon alfa-2b for 14 days. Upon completion of period 2, all patients initiated peginterferon alfa-2b and ribavirin treatment. A rapid and persistent decline in plasma HCV RNA was observed in both treatment-experienced and treatment-naïve patients during period 1, with a mean viral load decline of at least 4 $\log_{10}$ in all treatment groups. A high percentage of both treatment-experienced (50%) and treatment-naïve (≥ 60%) patients had undetectable HCV RNA (< 25 IU/ml) after period 2. Standard of care resulted in sustained virological response (SVR) rates of 38% and 81% in treatment-experienced and treatment-naïve patients, respectively. Narlaprevir (with or without ritonavir) alone or in combination with peginterferon alfa-2b was safe and well tolerated. Conclusion: Narlaprevir administration resulted in a robust HCV RNA decline and high SVR rates when followed by standard of care in both treatment-experienced and treatment-naïve HCV genotype 1 infected patients.

Introduction

Chronic hepatitis C virus (HCV) infection is a major cause of liver cirrhosis and hepatocellular carcinoma. HCV-related end-stage liver disease is now the main indication for liver transplantation in North America and western Europe (1). Estimates suggest that there are 170 million HCV-infected patients worldwide and that 3 to 4 million people are newly infected each year (2). Approximately 80% of patients who become infected with HCV develop chronic hepatitis C (3). The current standard of care (SOC), combination therapy of pegylated interferon alfa and ribavirin (RBV), achieves a sustained virological response (SVR) in only approximately 40% of patients infected with HCV genotype 1 (4,5).

The HCV nonstructural protein 3 (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 interferon response genes (6,7). Direct-acting antiviral agen ts such as NS3 protease inhibitors are currently being evaluated in phase 3 clinical trials in combination with peginterfeon and ribavirin. The addition of these first-generation protease inhibitors (VX-950, telaprevir; SCH 503034, boceprevir) (8,9) to the backbone therapy of peginterfeonand ribavirin has improved the treatment outcomes significantly for HCV genotype 1 infected patients (10,11).

For many years, human immunodeficiency virus (HIV)-specific protease inhibitors have been widely used as part of highly active antiretroviral therapy (12). Ritonavir is frequently prescribed with highly active antiretroviral therapy, not necessarily for its antiviral activity but for its ability to inhibit cytochrome P450-3A4 (CYP3A4). Inhibition of CYP3A4 by ritonavir leads to higher plasma concentrations of the coadministered HIV protease inhibitors, allowing a lower dose and a less frequent dosing schedule of HIV protease inhibitors (13). This discovery has significantly improved dosing convenience for patients and has resulted in increased efficacy of protease inhibitors for HIV treatment (14,15).

Narlaprevir (SCH 900518) is a novel potent oral direct-acting antiviral agent that prevents viral replication in infected host cells by inhibiting the HCV NS3 protease. The mechanism of inhibition involves the covalent, yet reversible, binding of narlaprevir to the NS3 protease active site serine through a ketoamide functional group. In the replicon system, the 50% and 90% maximal effective concentration for suppression of the HCV genotype 1b is approximately 20 ± 6 nM and 40 ± 10 nM (~28 ng/mL), respectively (16). These data indicate that narlaprevir is approximately 10-fold more potent in vitro than other protease inhibitors currently in phase 3 trials (telaprevir and boceprevir) (17,18). The replicon data also suggest that combination therapy with interferon alfa may enhance HCV RNA reduction and may suppress the selection of resistant HCV mutations in a clinical setting (16).

Additional in vitro studies have identified that narlaprevir is primarily metabolized by CYP3A4, and phase I clinical studies have confirmed that significantly increased plasma exposures of narlaprevir in healthy volunteers can be achieved when coadministered with ritonavir (data on file at Schering-Plough Research Institute). Effective telaprevir minimum were plasma concentrations (Cmin) approximately five-fold higher than the 90% maximal effective concentration (EC90) as determined by the replicon system (8). Therefore, in order to achieve narlaprevir exposures that would demonstrate potent antiviral activity, the doses administered in this study (800 mg narlaprevir three times daily and 400 mg narlaprevir with 200 mg ritonavir twice daily) were targeted to attain a therapeutic exposure and a mean Cmin at least five- to 10-fold above the replicon assay EC90 value of 40 nM (~28 ng/mL).

We report the safety and tolerability of narlaprevir administered at two dose levels as monotherapy

and in combination with peginterferon alfa-2b in 40 treatment-naïve and treatment-experienced patients infected with HCV genotype 1. We also present the antiviral activity and pharmacokinetic profile of narlaprevir and the response to SOC (peginterferon alfa-2b/ribavirin) following the completion of narlaprevir administration.

Patients and methods

Study design

This randomized, placebo-controlled, double-blind, two-period phase Ib study was conducted at three sites in The Netherlands. Narlaprevir dosing was conducted at a single site as an inpatient study; SOC was administered on an outpatient basis. Study medication (PegIntron, Rebetol, and narlaprevir) was supplied by Schering-Plough Research Institute. Ritonavir (Norvir; Abbott Laboratories) was also used in this study. Narlaprevir and matched-placebo were administered as an amorphous suspension.

The study was conducted as a two-period, fixed-sequence study in 40 HCV genotype 1 infected patients enrolled in four cohorts (Figure 1). Cohorts 1 and 3 each included 10 patients naïve to HCV treatment; cohorts 2 and 4 each included 10 HCV treatment-experienced patients. In each cohort, patients were randomized in a 4:1 ratio to either narlaprevir (n = 8) or placebo (n = 2) according to a computer-generated random code. Treatment was prepared and dispensed in a blinded fashion by a third party for administration to the patients. The third party was not involved with the study procedures, assessments, or data recording and did not reveal the randomization during the study according to the Consolidated Standards of Reporting Trials guidelines (19).

During period 1, patients received either 800 mg narlaprevir (or placebo) three times daily (cohorts 1 and 2) as monotherapy or 400 mg narlaprevir (or placebo) in combination with 200 mg ritonavir twice daily (cohorts 3 and 4) for 7 consecutive days. There was a washout period of approximately 4 weeks between the final treatment administration in period 1 and the first treatment in period 2. During the washout period, patients returned to the study site for assessments on days 14 and 21. Period 2 consisted of 14 consecutive days of dosing with the same dosing regimen as in period 1 in combination with 1.5 µg/kg/week peginterferon alfa-2b (days 1 and 8). Upon completion of the second treatment period, patients were offered SOC with 1.5 µg/kg/week peginterferon alfa-2b and daily weight-based ribavirin (800-1400 mg) for 24 or 48 weeks. Initiation of SOC began immediately after confinement at the clinical site. Patients were treated for 24 (only if rapid viral response [RVR] was achieved) or 48 weeks at the discretion of the patients, provided standard stopping rules did not require premature discontinuation. Rapid viral response (RVR) was defined as HCV RNA undetectable after 4 weeks of SOC. This study was conducted in accordance with Good Clinical Practice and with the Declaration of Helsinki after approval by each center's institutional review board. All patients provided written informed consent before participating in the study.

Patients

Key inclusion criteria included men and women between 18 and 65 years with body mass indexes of 18-40 kg/m^2, HCV genotype 1 (any subtype), and HCV RNA level > 1 x 10^5 copies/ml (or equivalent international units). Chronic hepatitis C patients were naïve, nonresponders or relapsers to previous interferon-based treatment. Relapse was defined as undetectable HCV RNA upon completion of a previous interferon-based treatment, but positive HCV RNA during follow-up. Nonresponse was defined as positive HCV RNA at the end of a previous interferon-based treatment or < 2 log decline in HCV RNA levels at 12 weeks and discontinued treatment. Key exclusion criteria included decompensated liver disease, findings consistent with Child-Pugh class B or C liver cirrhosis, and coinfection with HIV

or hepatitis B virus. Patients with chronic stable hemophilia or on stable methadone substitution treatment were eligible for the study.

Viral assessments

The Truegene assay was used to determine the genotype and subtype of all patients. Multiple samples for determination of plasma HCV RNA levels and viral sequencing were obtained in both periods on day 1, followed by daily sample collection. HCV-RNA was measured during the SOC treatment at the start or treatment; at treatment weeks 4, 12, and 24; at end of treatment; and 24 weeks after treatment cessation. HCV RNA levels during the narlaprevir treatment phase of the study were measured using the Roche Cobas TaqMan HCV/HPS assay version 2.0 (Covance, Switzerland) with a lower limit of quantification of 25 IU/ml and a lower limit of detection of 9.3 IU/ml. Plasma HCV RNA levels during SOC were assessed at the Academic Medical Center (Amsterdam, The Netherlands) using the Roche Cobas Ampliprep/Cobas TaqMan assay version 1.0 with a lower limit of detection of 15 IU/ml. Viral population sequencing of the NS3 protease domain (amino acids 1-181) was performed for all patients at all time points collected if sufficient RNA was available. Viral RNA was extracted from human plasma samples using a commercially available silica-gel membrane based kit (Qiagen, Valencia, CA) and processed on an automated BioRobot 9604 system (Qiagen). Reverse-transcription of RNA was performed using a SuperScript III First Strand Synthesis Supermix kit (Invitrogen, Carlsbad, CA) with random hexamers according to the manufacturer's instructions. Polymerase chain reaction was conducted with a Platinum PCR SuperMix kit (Invitrogen). Reaction products were purified on a Biomek FX system (Beckman Coulter, Fullerton, CA) using a magnetic bead kit (Agencourt Bioscience Corporation, Beverly, MA). DNA sequencing of purified material was conducted on a 3730xl DNA Analyzer (Applied Biosystems).

Pharmacokinetic assessments

To investigate a potential correlation between exposure to narlaprevir and antiviral activity, plasma samples were collected over the course of the study for pharmacokinetic profiling of narlaprevir with or without ritonavir. In period 1, serial blood narlaprevir pharmacokinetic samples were taken on days 1 and 7. Additional narlaprevir pharmacokinetic sampling was performed on days 2, 5, 6, and 7 for trough level (Cmin) determination. In period 2, serial blood narlaprevir pharmacokinetic samples were collected on days 1, 7, and 14, and additional pharmacokinetic samples for Cmin determination were obtained on days 2, 8, 10, 12, and 14. Plasma concentrations of narlaprevir were determined using a validated liquid chromatographic–tandem mass spectrometric method with a limit of quantification of 6.08 ng/ml.

Statistical analysis

The determination of the sample size was based on empirical considerations rather than statistical justification. The sample size of 8 subjects in each cohort could detect a difference of 0.92 in $\log_{10}$ change in HCV RNA levels from baseline between the treatment and placebo group with 80% power. Patients who received placebo were pooled. Adverse events, ECGs, vital signs, and laboratory values were listed for each patient and adverse events were tabulated by cohort and summarized using descriptive statistics. Plasma narlaprevir concentrations and pharmacokinetic parameters were summarized using descriptive statistics. Log_{10} HCV RNA values were summarized and graphically displayed by treatment period and cohort.

Safety assessments

Patients were monitored for safety and tolerability at regular intervals from the start of dosing through a follow-up visit 24 weeks after completion of SOC. Safety assessments included physical examination, vital signs, clinical laboratory tests, electrocardiograms, and the recording of all adverse events.

Table 1. Baseline Characteristics

	800 mg narlaprevir TID		400 mg narlaprevir BID + ritonavir	
	Cohort 1: Tx-Naïve (n = 10)	Cohort 2: Tx-Experienced (n = 10)	Cohort 3: Cohort 3: Tx-Naïve (n = 10)	Cohort 4: Tx-Experienced (n = 11)
Male	6 (60)	7 (70)	8 (80)	10 (91)
Race				
Caucasian	9 (90)	8 (80)	8 (80)	7 (64)
Black	1 (10)	1 (10)	1 (10)	0
Other	0	1 (10)	1 (10)	4 (36)
Age, years	51.1 (3.9)	47.9 (7.4)	43.6 (9.2)	51.1 (7.2)
Weight, kg	73.5 (11.5)	82.2 (12.5)	79.4 (14.8)	84.4 (13.2)
BMI, kg/m^2	24.1 (2.1)	27.8 (4.4)	25.3 (4.1)	26.3 (5.2)
Patients receiving methadone	1 (10)	0	1 (10)	1 (9)
Patients with hemophilia	0	0	1 (10)	1 (9)
Genotype				
1	1	4	2	2
1a	4	1	4	3
1b	5	5	4	6
Baseline HCV RNA	4.8×10^6 (3.2×10^6)	6.3×10^6 (4.4×10^6)	3.8×10^6 (2.9×10^6)	4.3×10^6 (4.6×10^6)

Data are presented as n(%) or mean (SD).
BID, two times daily; BMI, body mass index; TID, three times daily; Tx, treatment.

Results

Demographic and baseline characteristics

Fortyone patients (10 patients each in cohorts 1-3 and 11 patients in cohort 4) were enrolled in the study. One patient in cohort 4 discontinued immediately after the first dose on day 1 because of intolerance to the drug suspension; study medication was stopped at the discretion of the investigator, and this patient was replaced. A total of 40 patients completed the narlaprevir treatment phase of the study and initiated SOC immediately after period 2. Treatment-experienced patients consisted of 12 relapse patients and eight nonresponders. Demographic and other baseline characteristics of the randomized patients are shown in Table 1.

Pharmacokinetics

The pharmacokinetic profile of narlaprevir 800 mg three times daily or 400 mg with ritonavir two times daily was characterized (Table 2). Exposure to narlaprevir with and without coadministration of peginterferon alfa-2b for both treatment-naïve and treatment-experienced patients was comparable. Narlaprevir was eliminated more slowly when coadministered with ritonavir than when administered alone. Dose-normalized daily exposures (area under the curve) on day 14 in the presence of peginterferon alfa-2b and ritonavir increased 7.6- and 7.1-fold in treatment-naïve and treatment-experienced patients, respectively, compared with narlaprevir monotherapy. Based on the pharmacokinetic methodology employed in this trial, the narlaprevir terminal T1/2 could not be determined for all treatment groups.

Table 2. Pharmacokinetic parameters of narlaprevir after 14 days of narlaprevir with peginterferon alfa-2b with or without ritonavir (period 2) in chronic hepatitis C patients

Period 2, Day 14	800 mg narlaprevir TID		400 mg narlaprevir BID + ritonavir	
	Cohort 1: Tx-Naïve (n = 8)	Cohort 2: Tx-Experienced (n = 7)*	Cohort 3: Tx-Naïve (n = 8)	Cohort 4: Tx-Experienced (n = 8)
Tmax, hours (range)	2.0 (2.0-4.0)	2.0 (0.5-4.0)	2.0 (2.0-4.0)	2.0 (2.0-12.0)
Cmax, ng/ml (CV %)	1,630 (60)	1,480 (35)	3,640 (32)	2,640 (25)
AUC 0- τ, ng/hour/ml (CV %)	7,890 (61)	6,400 (41)	29,900 (30)	22,600 (24)

AUC, area under the plasma concentration time curve over the dosing interval; BID, two times dailys; Cmax, maximum plasma concentration; CV, coefficient of variance; TID, three times daily; Tmax, time of maximum plasma concentration; Tx, treatment. *One patient was excluded because no pharmacokinetic samples were available.

Table 3. Mean changes of HCV RNA levels (log_{10} IU/mL) from baseline after each treatment period in narlaprevir-treated patients

	800 mg narlaprevir TID		400 mg narlaprevir BID + ritonavir	
	Cohort 1: Tx-Naïve	Cohort 2: Tx-Experienced (n=8)	Cohort 3: Tx-Naïve (n=8)	Cohort 4: Tx-Experienced (n=8)
End of Period 1	-4.49	-4.42	-4.18	-4.01
End of Period 2	-4.90	-4.10	-5.01	-3.50
Patients with HCV RNA levels < 25 IU/ml at end of period 2, n (%)	6 (75%)	5 (63%)	4 (50%)	4 (50%)

BID, two times daily; TID, three times daily; Tx, treatment.

Table 4. Therapy outcome after treatment with peginterferon alfa-2b and ribavirin in patients treated with narlaprevir or placebo

	Treatment-Naïve patients (n = 20)		Treatment-Experienced patients (n = 20)	
	Narlaprevir (n = 16)	Placebo (n = 4)	Narlaprevir (n = 16)	Placebo (n = 4)
SVR[1]	13 (81)	3 (75)	6 (38)	0
Non-response[2]	0	0	2 (12)	3 (75)
Relapse[3]	0	0	1 (6)	1 (25)
Breakthrough[4]	3 (19)	1 (25)	7 (44)	0

Data are presented as n(%).

[1] Undetectable HCV RNA 24 weeks after completion of treatment.

[2] Positive HCV RNA at end of treatment.

[3] Undetectable HCV RNA at completion of treatment, but relapsed during follow-up.

[4] 1 $\log_{10}$ increase of HCV RNA from nadir during SOC.

RBV, ribavirin.

Table 5. Treatment-emergent mutations at the NS3 domain (defined as changes from reference sequence) observed throughout the narlaprevir treatment phase and SOC based on population sequencing

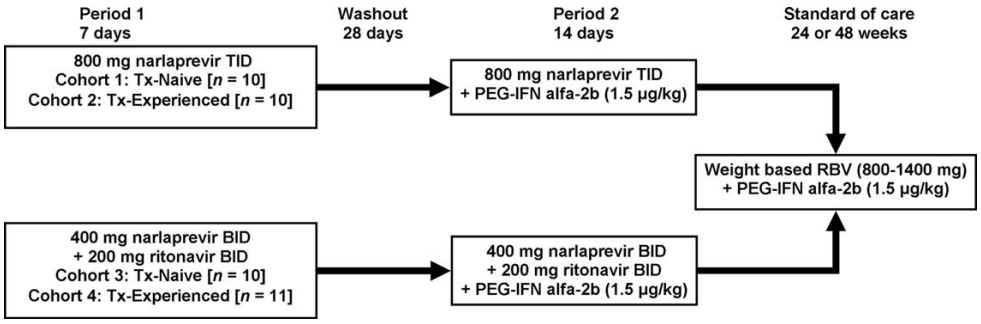
	NS3 mutations				
	101	202	309	409	411
Genotype	1a	1a	1a	1a	1a
Pretreatment	-	-	-	-	-
Period 1	-	-	-	V36M, R155T, A156T	-
Period 2	V36M, R155K	-	R155K	A156T	V36M, R155K, A156S
SOC	V36M, R155K	V36M, R155K	R155K	V36M, R155K, A156T	V36M, R155K, A156S
Treatment outcome	Breakthrough	Breakthrough	Breakthrough	Breakthrough	Nonresponse

Table 6. Summary of most frequently reported AEs (≥ 10% of patients) during 7 days of narlaprevir monotherapy with or without ritonavir (period 1) and after 14 days of narlaprevir with peginterferon alfa-2b with or without ritonavir (period 2)

	800 mg narlaprevir TID (n = 16)	400 mg narlaprevir BID + ritonavir (n = 16)	Placebo (n = 4)	Placebo + Ritonavir (n = 4)
Period 1 (7 days)				
Patients reporting any AE	15*	15*	3*	3*
Headache	3	8*	0	0
Diarrhea	3	7	0	0
Anorectal discomfort	6	3	0	0
Nausea	6	2	1	0
Dizziness	4	2	0	1
Somnolence	6	0	1	0
Abdominal discomfort	2	1	0	3*
Influenza-like illness	1	3	0	0
Abdominal distension	1	2	0	1
Period 2 (14 days), + peginterferon				
Patients reporting any AE	16*	16*	3*	4*
Influenza-like illness	16*	14*	3*	3*
Diarrhea	3	5	0	0
Headache	3	3	0	1
Dyspepsia	2	2	0	0
Injection site erythema	3	1	0	0
Nausea	1	3	0	0

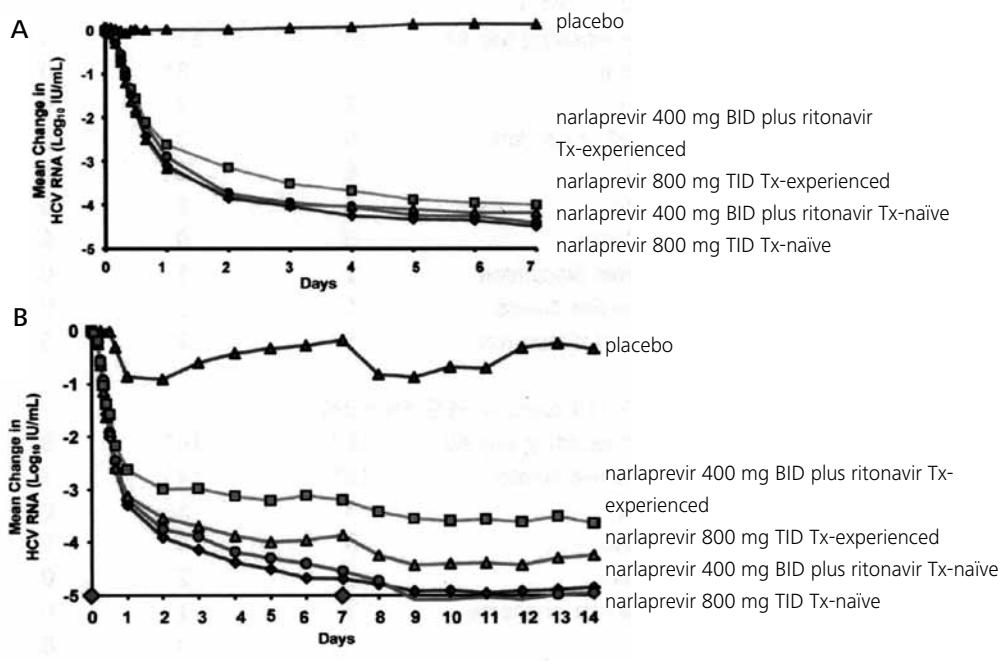
AEs are summarized by body system and preferred term regardless of severity and drug relatedness. No AEs were considered probably or very likely related to study medication. All AEs reported were grade 1.
BID, two times daily; TID, three times daily.
*Reported by ≥ 50% of patients.

Figure 1. Study flow diagram



BID, twice daily; TID, three times daily; Tx, treatment.

Figure 2.



Narlaprevir treatment resulted in robust reductions of HCV RNA. The day 0 baseline value is the mean of all predose values per cohort. Mean changes of HCV RNA levels from baseline are shown for all 40 patients who received the following treatment regimens: (A) period 1 with narlaprevir monotherapy with or without ritonavir for 7 days and (B) period 2 with narlaprevir with or without ritonavir plus peginterferon alfa-2b for 14 days. Peginterferon alfa-2b was administered at baseline and at day 7.

HCV RNA levels

A rapid and persistent decline in plasma HCV RNA levels was observed that was strikingly similar in all cohorts. Therapy with narlaprevir with or without ritonavir (period 1) resulted in a mean > 4 $\log_{10}$ IU/ml decline in plasma HCV RNA levels in all treatment groups (Figure 2A). The mean HCV RNA changes from baseline in all narlaprevir treated patients are listed in Table 3. All groups demonstrated a similar return of viral load to baseline during the 4-week washout period after period 1. No significant changes in HCV RNA levels were observed in patients who received placebo.

When narlaprevir 6 ritonavir was coadministered with peginterferon alfa-2b, similar declines in HCV viral load were achieved across all treatment groups (Figure 2B). All patients achieved a > 3 $\log_{10}$ IU/ml decline in HCV RNA levels, and the majority of patients had a maximal HCV RNA decline of 4-5 $\log_{10}$ IU/ml. The mean HCV RNA changes from baseline for each treatment cohort are listed in Table 3. Patients randomized to the placebo group demonstrated a mean HCV decline of 0.45 $\log_{10}$ IU/ml in response to peginterferon alfa-2b treatment (Figure 2B).

All 40 patients completed period 2 and initiated SOC within 1 day after the last narlaprevir dose. Patients were treated with SOC for 24 weeks at the discretion of the patient if HCV RNA was undetectable after 4 weeks of SOC. The treatment outcomes (SVR, relapse, nonresponse, or breakthrough) according to prior treatment history of all patients are listed in Table 4. Treatment-naïve patients

treated with narlaprevir had an overall SVR rate of 81% (13/16) compared with 38% (6/16) in the treatment-experienced group. In the placebo group, 38% (3/8) of patients achieved SVR after at least 48 weeks of treatment; all responders were treatment naïve. Of the six treatment-experienced patients who achieved SVR, five patients were previous relapsers, and one was a previous nonresponder. Nine treatment-naïve patients treated with narlaprevir had an RVR and subsequently achieved SVR (100%) with 24 weeks (six patients) or 48 weeks (three patients) of SOC. Seven treatment-experienced patients treated with narlaprevir had an RVR, of whom six achieved SVR (86%) after 48 weeks of SOC.

Mutational analysis

In this study, viral variants were detected in all cohorts and in both treatment-naïve and treatment-experienced patients. Treatment-emergent variants known to be associated with resistance to narlaprevir, characterized in biochemical and cell-based assays, were observed in five patients (Table 5). These were observed at loci V36, R155, and A156. Susceptibility to narlaprevir has not been characterized for the treatment-emergent mutation R155T (16). Of the 40 patients enrolled, 24 had an isoleucine-170 (I170) polymorphism detected in pretreatment samples. This variant is not known to confer reduced susceptibility to narlaprevir. All patients with treatment-emergent resistance variants failed to achieve undetectable viral HCV RNA levels. Virological breakthrough was observed in four patients; one previous non-responder appeared to be a non-responder again during SOC. One treatment-experienced patient with a serine-54 polymorphism at baseline associated with reduced susceptibility to narlaprevir achieved undetectable viral load levels in period 2 (cohort 2). This patient remained HCV RNA undetectable during SOC but relapsed after 24 weeks of treatment.

Safety

No severe or serious adverse events (AEs) and no dosing interruptions or discontinuations were reported during narlaprevir dosing. A complete listing of the most frequently reported AEs recorded for both period 1 and period 2 is provided in Table 6. During period 1, the most commonly reported AEs were gastrointestinal symptoms (diarrhea, anorectal discomfort, abdominal discomfort, abdominal distension). Gastrointestinal symptoms were reported in 25 (76%) patients who received narlaprevir and 4 (50%) patients who received placebo. During period 2, when peginterferon alfa-2b was added to the treatment regimen, the most commonly reported AE was influenza-like illness, which was observed in 30 (94%) patients who received narlaprevir and 6 (75%) patients who received placebo. Also during period 2, there was an elevated rate of gastrointestinal symptoms. Gastrointestinal-related AEs were reported by 24 (75%) patients who received narlaprevir, compared with no patients in the placebo group. No significant difference in AEs was noted between patients that were treatment-naïve versus treatment-experienced. Ritonavir coadministration did not significantly affect the AE profile. Three serious AEs (one instance of elevated CRP and two instances of pyrexia) occurred during SOC administration. All three events occurred in the same patient and required hospital admission, but they were not considered related to narlaprevir treatment. No clinically significant changes in blood chemistry or hematological parameters, vital signs, or electrocardiograms occurred in any treatment group.

Discussion

The present study was the first clinical trial to evaluate narlaprevir in chronic hepatitis C patients and to evaluate a treatment regimen that used a pharmacokinetic enhancer (ritonavir) in combination with an HCV NS3 protease inhibitor for the treatment of hepatitis C. In addition, this was one of the first phase Ib studies to offer treatment with peginterferon alfa-2b and ribavirin to all patients following treatment with narlaprevir in order to explore the potential of increasing the RVR and, consequently, the SVR rates. Finally, the first clinical mutational analysis of narlaprevir was performed to investigate the development of NS3/4 genome sequence changes during and after narlaprevir treatment.

The primary objective of this study was to assess the safety and tolerability of narlaprevir with or without ritonavir and peginterferon alfa-2b in chronic hepatitis C patients. During narlaprevir dosing, there were no treatment discontinuations and no serious AEs. The most frequently reported AEs were gastrointestinal symptoms and influenza-like illness. Addition of peginterferon alfa-2b to the treatment regimen increased the frequency of AEs, however, these AEs (flu-like symptoms) were consistent with those expected for pegylated interferon. Combination with ritonavir did not significantly affect the AE profile. Most AEs reported in patients receiving narlaprevir were mild or moderate in severity. None of these moderate events was considered to be related to the study drug. Consistent with the results in healthy volunteers, narlaprevir appeared to be safe and well tolerated in all patients.

The secondary objectives were to investigate the antiviral activity and pharmacokinetic profile of narlaprevir. Narlaprevir demonstrated a profound antiviral activity in both treatment-naïve and treatment-experienced patients. A rapid and persistent mean HCV RNA decline of at least 4 $\log_{10}$ IU/ml was achieved in all patients whether narlaprevir was administered for 7 days alone or with ritonavir. HCV RNA levels returned to baseline at the end of a 4-week washout period. During 14 days of treatment with narlaprevir with or without ritonavir in combination with peginterferon alfa-2b, plasma HCV RNA levels declined in two phases: a rapid decline within the first day followed by a more gradual viral decline thereafter. Four patients who received narlaprevir achieved undetectable HCV RNA (< 15 IU/ml) after 14 days. Follow-up treatment with peginterferon alfa-2b and ribavirin resulted in high SVR rates of 81% (13/16) in treatment-naïve patients and 38% (6/16) in treatment-experienced patients treated with narlaprevir (with or without ritonavir). A rapid viral response was a strong positive predictor for SVR in treatment-naïve (9/9) and treatment-experienced patients (6/7). These results demonstrate that the rapid and profound decline in HCV RNA that was observed after a short initial period (14 days) of narlaprevir dosing could result in an increased RVR rate and subsequently an increased SVR rate in both treatment-naïve and treatment-experienced patients compared with regular SOC (4,5,20). This finding suggests that SVR rates may be further enhanced when the dosing period of narlaprevir is extended to a 12-week regimen, which is currently being assessed in a phase IIa trial (21).

The pharmacokinetic objective of this study was to generate a mean Cmin at least five- to 10-fold above the replicon assay EC90 value of 40 nM (~28 ng/ml). Analysis of the pharmacokinetic profile of narlaprevir monotherapy revealed plasma concentrations at least six times the EC90 at trough in all treatment groups after a 7-day dosing period. A quartile distribution of median Cmin of 170, 296, 1150, and 1725 ng/ml represented a median Cmin six- to 62-fold higher than the EC90 for narlaprevir. Although these doses generated an extraordinary range of narlaprevir plasma trough exposures, the antiviral activity observed was similar for all of the quartiles. The enhanced trough levels observed when narlaprevir was administered with ritonavir and the associated robust antiviral activity observed in this study provided a proof of principle for the use of pharmacokinetic enhancement in HCV therapy. This study justified and guided the further clinical investigation of a once daily dosing regimen of narlaprevir (200 mg and 400 mg) in combination with low-dose ritonavir (100 mg) in a phase IIa study (21).

Although the results of this phase Ib study demonstrate the great potential of narlaprevir to improve therapy for HCV-infected patients, several limitations should be considered. Clearly, the short duration of narlaprevir dosing influenced its potential impact on SVR rates following SOC. However, despite this limitation, administration of narlaprevir before initiation of SOC still appeared to benefit the patients significantly. In addition to the short duration of narlaprevir dosing, the study was limited by a heterogenous and small patient population. A further complication was secondary to the sequential dosing periods interrupted by a 1-month washout period. To address these study limitations, several modifications to future study designs could be employed. First, the small size (10 patients per cohort) and

heterogeneity (differences in treatment history, baseline HCV RNA, wide range of body mass index, different ethnic groups, and patients with hemophilia) of the study population could have biased the treatment effect estimate. A larger and more restricted study population could remove this potential bias. Such changes were implemented in a subsequent phase IIa study of narlaprevir (21). Second, the approach of two sequential dosing periods separated by a washout period was chosen to investigate narlaprevir monotherapy and viral rebound after removal of drug pressure, as well as to attempt to demonstrate the additional antiviral effect of narlaprevir when used in combination with peginterferon alfa-2b. However, as shown in other studies with protease inhibitor monotherapy (22,23) 7 days of narlaprevir monotherapy most likely induced resistant variants with reduced susceptibility and complicated the interpretation of combination therapy during period 2 of the study. Detection of single variants (A156T), double variants (V36M together with R155K), and in one case a triple variant (V36M and R155K together with A156S) showed that the treatment regimens in this study selected for virus variants with a high level of resistance to narlaprevir. Based on population sequencing during the washout period, one patient had a viral population consisting of V36M, R155T, and A156T associated with high levels of resistance to narlaprevir (Table 5). This patient had a less profound HCV RNA decline during period 2, and HCV RNA even increased after day 8.

In total, sequence analysis of the NS3/4A protease domain showed that viral variants (R155K, V36L/M, A156T/S) associated with reduced susceptibility to narlaprevir were present in five patients. Treatment outcome of these five genotype 1a patients included a viral breakthrough in four patients, and one patient appeared to be a nonresponder. Longer duration of narlaprevir treatment in combination with peginterferon alfa-2b and ribavirin may increase the durability of antiviral response to this treatment regimen and add protection against potential viral breakthrough and emergence of viral variants (10). Longer follow-up and clonal analysis is needed to fully understand the kinetics of these resistance variants.

Combination of protease inhibitor–based regimens with SOC (peginterferon alfa-2b and ribavirin) has dramatically improved chronic hepatitis C treatment outcomes (10,11). Telaprevir and boceprevir, both of which are HCV specific NS3 protease inhibitors, are currently being evaluated in phase III clinical trials with a three times daily dosing regimen. The requirement of these compounds for high frequency dosing may lead to a lack of adherence and consequently lowered protease inhibitor exposure that could potentially lead to the development of resistant virus and a failure to achieve SVR (24). Since the mid-1990s, combining a pharmacokinetic enhancer with protease inhibitors in antiretroviral drug regimens has provided HIV patients with potent therapies that durably suppress HIV replication to undetectable levels and reduce the likelihood of generating drug resistance (25). Inhibition of the CYP-450 (3A4) metabolic pathway by ritonavir provides the basis for pharmacokinetic enhancement of concomitantly administered HIV protease inhibitors. CYP3A4 is present in the intestinal tract and liver, where it plays a key role in protease inhibitor first-pass metabolism (26). A once daily dosing regimen of narlaprevir and ritonavir could be a major advantage, because the pill burden will likely increase with the addition of future direct-acting antiviral agents to the current SOC.

The potential of undesired effects of ritonavir during HCV treatment is low due to a possibility for a shorter treatment duration (compared with HIV treatment), administration of a low dose, and reduced dosing frequency (once daily). However, coadministration of a metabolic enhancer will require attention to possible interactions with other medications metabolized by CYP3A4 (such as statins and benzodiazepines) (26). Other protease inhibitors such as TMC435 have demonstrated potent antiviral activity with once daily dosing without ritonavir boosting (27). It is therefore uncertain if ritonavir boosting will be useful in future treatment regimens that potentially include three or four drug combi-

nations. Nevertheless, knowledge about the coadministration of HCV protease inhibitors with ritonavir will be important in the large HIV-coinfected subpopulation of patients.

In conclusion, narlaprevir demonstrated potent antiviral activity when administered as monotherapy (with and without ritonavir) and in combination with peginterferon alfa-2b. For the first time during the treatment of HCV infection, pharmacokinetic and pharmacodynamic modeling supports once daily dosing of an HCV NS3 protease inhibitor (narlaprevir) with low-dose ritonavir in chronic hepatitis C patients (21). An ongoing study is currently assessing the efficacy of once daily dosing (narlaprevir and ritonavir), the impact on viral resistance, and the possibility of a shorter SOC treatment duration due to the potency of the compound.

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Summary and discussion

After two decades of interferon-based therapy for chronic hepatitis C we have seen sustained virological response (SVR) rates climb from less than 20% with interferon alfa monotherapy up to 80% in selected patients with the current standard of care (SOC): peginterferon and ribavirin combination therapy (1, 2). This remarkable increase in SVR can be regarded as a tremendous success, whereas achievement of SVR can be considered a comlete recovery from chronic hepatitis C (3, 4). However, the current SOC coincides with substantial adverse events like flu-like symptoms, cytopenia, dermatitis and depression, which affect drug compliance and which limit successful treatment outcome. Moreover, the recommended treatment duration can be long (up to 72 weeks) and peginterferon/ribavirin combination therapy has a major impact on health-related quality of life. In addition, there are currently hardly treatment options for previous non-responders. Retreatment with peginterferon and ribavirin of non-responders to a previous course of peginterferon and ribavirin results in approximately 5-15% SVR (5, 6). In previous relapsers, SVR rates after retreatment are moderately higher (30-40%). This means that in the vast majority of these patients continued HCV replication will keep them at risk for developing liver cirrhosis and hepatocellular carcinoma. Especially for patients with end stage liver disease or with known factors that accelerate liver disease progression (alcohol consumption, diabetes mellitus, HIV or HBV co-infection) there is an obvious need for HCV eradication. It has been calculated that HCV-related mortality will peak within the next two decades and that the current SOC will only modestly impact the mortality rate (7, 8).

During the past 10 years huge efforts have been made to optimize the current SOC and to develop specific compounds with direct acting antiviral activity (DAA) against HCV infection, in order to give patients a better chance of HCV eradication. The ultimate treatment goals include high SVR rates, shorter therapy duration and less drug induced side effects. However, we are still far from that. In this thesis we describe our studies that evaluated adjusted (peg)interferon-based regimens and several new antiviral drugs for the treatment of chronic hepatitis C patients.

Optimizing current interferon-based therapy

As described in *chapter 2*, treatment duration of SOC should be tailored to the on-treatment virological response (response-guided therapy). Key time points for assessing HCV RNA include start of treatment, week 4, week 12 and week 24. The likelihood of SVR is directly proportional to the time of HCV RNA negativity. Based on virological responses at week 4 or 12, treatment duration can be reduced or extended conform the most recent international guidelines (9). Patients with genotype 1 with low baseline viral load who achieve a rapid virological response (RVR; HCV RNA negativity at week 4) can be treated for 24 week (10-13). Patients with genotype 1 achieving early virological response (EVR; more than 2 log decline at week 12) should be treated for 48 weeks. Patients with genotype 1 with delayed virological response (DVR; HCV RNA negativity between week 12- 24) might be treated for 72 weeks to reduce the relapse rate (14-20). The above mentioned recommendations can also be applied to genotype 4 patients. Patients with genotypes 2 and 3 with an RVR and low baseline viral load might be treated for 16 weeks, although a slightly higher relapse rate is seen compared to 24 weeks of therapy (21-25). Shortening of therapy duration is not recommended in patients with advanced fibrosis, cirrhosis or other cofactors associated with poor response. Patients with genotypes 2 and 3 and EVR or DVR might be treated for 48 or 72 weeks (10). There are insufficient data available for genotypes 5 and 6 patients, although in general similar response rates are seen as compared to genotype 3 patients (26-28).

With regard to the importance of achieving an RVR or EVR, higher than standard dosing of (peg) interferon has been evaluated extensively in order to obtain higher RVR and EVR rates and to improve clinical outcome (29-31). Especially for previous non-responders an intensified peginterferon regimen

might be of interest as alternative treatment-options are still limited. However, the benefit of peginterferon induction for 4 or 12 weeks remains debatable and concerns about increased toxicity have risen. In *chapter 3* we evaluated a peginterferon induction regimen combined with prolongation of therapy duration (72 weeks) versus SOC in patients unresponsive to a previous course of standard interferon with or without ribavirin. We could not detect any safety issues, but response rates were comparable between both groups (approximately 40% SVR). Importantly, achievement of RVR was highly predictive for SVR. Of interest was our finding that baseline gamma-glutamyltransferase levels less than 2 x upper limit of normal were significantly associated with SVR.

In *chapter 4* we evaluated high-dose continuous interferon infusion (6, 9 or 12 MU interferon per day) combined with ribavirin in previous peginterferon/ribavirin non-responders. We hypothesized that stable serum levels of standard interferon might improve viral suppression and avoid adverse events associated with serum interferon peaks during once weekly peginterferon injections. In this difficult-to-treat population, an overall SVR rate of 20% was seen. During treatment 6 serious adverse events occurred and side effects appeared dose-dependent. We concluded that treatment with continuous subcutaneous inteferon can be successful, but side effect management should improve, especially for the highest and most effective dose group (12 MU interferon per day).

Further in depth viral kinetic analyses of continuous interferon administration are described in *chapter 5*. We showed that median HCV RNA decline at week 4 was significantly stronger in patients receiving 12 MU interferon per day compared to 9 MU, respectively (2.58 log versus 0.84, p=0.017). Interestingly, stronger HCV RNA decline was associated with higher neopterin levels (as marker of immune activation) but not with 2,5-oligoadenylate synthetase activity or beta2-microglobulin levels. Also in this study it was found that a profound HCV RNA decline at week 4 of therapy is essential for achieving SVR.

These studies show that adjusted (peg)interferon-based regimens in combination with ribavirin can lead to SVR in selected patients with difficult-to-treat characteristics. Although SVR rates are modest, it will be of utmost interest to combine these regimens with new direct acting antivirals, wich are expected to become available soon.

New anti-HCV drugs

Improved understanding of the HCV life cyclus has led to several classes of new drugs that are being developed for chronic hepatitis C patients. Since our review in 2008 (*chapter 2*), some developments were halted due to lack of efficacy, safety concerns or for economic reasons. Other drugs went on to phase III clinical trials and a variety of new compounds has entered phase I and phase II studies.

In *chapter 6* we evaluated ANA773, a toll-like receptor (TLR)7 agonist. The TLR family is of particular interest due to its ability to activate the innate and adaptive immune response (32). ANA773, an oral prodrug of a small-molecule TLR7 agonist, has shown induction of endogenous interferon alfa of multiple subtypes in preclinical studies and in healthy volunteers. In this phase I dose-finding study we showed that ANA773 was safe and well tolerated, and can lead to a significant HCV RNA decline up to 3.10 log, compared to placebo. However, this decline was transient and was only seen in the two highest dose groups. A subsequent analysis of HCV infected patients and healthy volunteers showed that reduced plasmacytoid dendritic cell numbers as well as increased serum interferon alfa and interferon gamma inducible protein (IP)-10 levels were only observed in patients with a ≥ 1 log decline of HCV RNA upon ANA773 administration (*chapter 7*). These findings show that important components of the antiviral immune response were activated in responding patients. Future studies should evaluate

higher dosages of ANA773 and combination regimens with ribavirin and new direct antivirals.

The first-in-class compound JTK-652, a novel pyrrolopyridazin-derived HCV infection inhibitor, was studied in *chapter 8*. In vitro studies in three different cell lines identified the inhibitory effect of JTK-652 on infection with HCV genotype 1a and 1b pseudotype viruses. Although details of HCV entry in the host cell are still poorly understood, we hypothesized that JTK-652 might also prevent HCV infection in vivo. Oral administration of JTK-652 in multiple doses showed mild side effects in healthy volunteers and patients. However, JTK-652 monotherapy did not decrease HCV RNA in chronic HCV infected patients. We concluded that viral inhibition of JTK-652 against HCV pseudotype virus infection in the replicon system might not represent in vivo HCV infection.

The most promising groups of drugs include protease inhibitors and polymerase inhibitors, which are also referred to as direct acting antiviral drugs (DAAs). Two protease inhibitors (telaprevir and boceprevir) are expected to enter clinical practice in 2011 or 2012. Landmark studies have shown that telaprevir or boceprevir administration can result in 27-31% higher SVR rates compared to SOC, when combined with peginterferon and ribavirin in treatment-naïve genotype 1 patients (9, 33-35). Pivotal trials in previous genotype 1 treatment-experienced patients showed SVR rates up to 80% with triple therapy, although substantial lower SVR rates (approximately 30%) were seen in true non-responders (36, 37).

With regard to the optimal treatment duration of triple therapy in previously untreated patients, a response-guided approach based on viral decline at week 4 is recommended. Treatment duration can be shortened in approximately 57-65% of patients treated with telaprevir and in 44% of patients treated with boceprevir, respectively. Previous non-responders, however, should be treated for 48 weeks with triple therapy containing telaprevir or boceprevir (38). For boceprevir, a 4-week lead-in approach with peginterferon and ribavirin is used based on better SVR results and lower relapse rates compared with triple therapy without a lead-in phase (39). The lead-in approach identifies patients that are susceptible to SOC, which assists further individualization of therapy duration.

The impact of introducing triple therapy into clinical practice is unquestionable, both for patients as for doctors. However, there are several important factors that should be considered before starting triple therapy.

Besides the substantial side effect profile of peginterferon and ribavirin, there will be more side effects when adding one of the new drugs. The most frequent telaprevir related adverse events included rash, anemia, pruritus, nausea and diarrhea. Discontinuation of all study drugs due to adverse events was seen in 17% of patients (35). The most frequent boceprevir related adverse events included anemia and dysgeusia (metal taste). Complete discontinuation of study drugs because of any adverse event was seen in 12-16% of patients (34). Adequate side effect management will be a key element in optimizing compliance and treatment outcome.

A major issue will be the occurrence of viral resistance upon administration of an HCV protease inhibitor. Experiences with hepatitis B and HIV have indicated the risk of selecting viral variants that have a decreased susceptibility to DAAs (40). Numerous drug-related mutations have been identified at several positions in the NS3/4A gene that induce resistance to telaprevir and boceprevir. Importantly, peginterferon and ribavirin might prevent emergence of drug-resistant mutations and most drug-resistant HCV strains remain sensitive to peginterferon and ribavirin (41).

Although HCV genotype 1 is the most prevalent in Western countries, the current registration trials have not evaluated non-genotype 1 patients. Preliminary data show that protease inhibitors are less effective for treatment of HCV genotype 2, 3 and 4 patients, but further studies are required (42).

As expected, patients who are most in need of HCV eradication (advanced liver fibrosis, cirrhosis, transplant recipients, hepatitis B or HIV co-infection) are not included or underrepresented in the current registration trials. Proper studies with DAAs are needed in these patients.

Another potential pitfall will be the thrice daily dosing of telaprevir and boceprevir. Suboptimal drug compliance might induce viral resistance and subsequent viral non-response or breakthrough. Second generation protease inhibitors are currently being developed in order to allow less frequent daily dosing (once or twice) and to improve the side effect profile.

In *chapter 9* we evaluated narlaprevir, a second generation direct inhibitor of the HCV NS3 protease, in a phase I placebo-controlled randomized study in naïve and treatment-experienced genotype 1 patients. In vitro studies indicated that narlaprevir is approximately 10-fold more potent than telaprevir and boceprevir (43, 44). Co-administration with ritonavir was also evaluated in order to enhance the pharmacokinetic profile of narlaprevir by inhibition of cytochrome P450-3A4, allowing twice daily dosing of narlaprevir (instead of thrice daily dosing with narlaprevir only). During narlaprevir dosing only mild adverse events were seen and combination with ritonavir did not significantly affect the side effect profile. A more than 4 log HCV RNA decline was seen in all patients during 7 days of narlaprevir administration, with or without ritonavir. After a second period of 14 days with narlaprevir and peginterferon (with or without ritonavir), SOC was started and resulted in 38% SVR in treatment-experienced patients and 81% SVR in treatment-naïve patients, respectively. As with other HCV protease inhibitors, drug-induced viral variants were detected during narlaprevir monotherapy and showed a high level of resistance to narlaprevir. Nonetheless, the great potential of this drug is currently being evaluated in a phase II trial, in which once daily dosing will be studied as well.

Despite the very potent antiviral activity of most DAAs that are currently being developed, peginterferon and ribavirin remain the backbone of anti-HCV combination therapy. Immunomodulatory stimulation with peginterferon seems inevitable in order to definitely eradicate chronic HCV. Nevertheless, there are promising data becoming available evaluating combinations of DAAs without peginterferon (45). Several phase I and II studies are currently being performed, in which protease inhibitors are combined with polymerase inhibitors. If these studies will result in a true peginterferon-free regimen leading to SVR remains to be determined.

Finally, in a time of global financial crisis and exploding national health care budgets, it seems reasonable to evaluate cost-effectiveness of future triple therapies. Especially when supportive side-effect measures are taken into account (like erythropoietin administration for anemia), the price of triple therapy might increase significantly compared to SOC. These data, however, are not available yet.

Conclusion

We are now on the eve of a new era of antiviral therapy for chronic hepatitis C patients. After a decade of peginterferon and ribavirin combination therapy, the cocktail will be expanded with a direct antiviral agent. In this thesis we showed that there is still room for optimizing the immunomodulatory part of anti-HCV therapy. In addition, several new drugs were studied with different mechanisms of interrupting the HCV life cycle, including a second generation protease inhibitor. Although major challenges lie ahead including viral resistance and side effect management, the therapeutic success story of chronic hepatitis C is definitely to be continued.

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Samenvatting en bespreking

Na twee decennia van antivirale behandeling voor chronische hepatitis C met interferon is de kans op een blijvende virologische respons (SVR; sustained virological response) geklommen van 20% met interferon alfa monotherapie tot 80% in geselecteerde patiënten met de huidige standaard behandeling (SOC; standard of care): gepegyleerd interferon alfa in combinatie met ribavirine (1, 2). Deze opmerkelijke verbetering van SVR kan gezien worden als een enorm succes, aangezien het bereiken van SVR beschouwd wordt als genezing van chronische hepatitis C (3, 4). Maar de huidige standaard behandeling gaat helaas gepaard met aanzienlijke bijwerkingen waaronder griepachtige verschijnselen, bloedbeeld afwijkingen (anemie, leukopenie en trombopenie), dermatitis en depressie. Deze bijwerkingen beperken de kans op een succesvolle behandeling. Daarnaast is de aanbevolen behandelduur lang (soms tot 72 weken) en is er een significante vermindering van de kwaliteit van leven tijdens behandeling. Verder zijn er op dit moment nauwelijks behandelmogelijkheden voor patiënten die eerder niet succesvol zijn behandeld met SOC. Herbehandeling met peginterferon en ribavirine van eerdere non-responders leidt tot ongeveer 5-15% SVR (5, 6). In patiënten die wel respons lieten zien maar bij wie het virus terugkwam na het staken van de behandeling (relapsers) wordt een iets hogere SVR gezien als zij worden herbehandeld met peginterferon en ribavirine (30-40%). Dit betekent dat in de meerderheid van deze patiënten virusreplicatie zal continueren waardoor zij het risico lopen op de ontwikkeling van levercirrose en hepatocellulair carcinoom. Met name in patiënten met eindstadium leverziekte of met bekende risicofactoren die de progressie van leverziekte versnellen (alcohol gebruik, diabetes mellitus, HIV of HBV co-infectie) is het belang van HCV eradicatie eminent. Het is berekend dat de huidige HCV-gerelateerde mortaliteit de komende decennia zal pieken en dat de huidige SOC hierop slechts een bescheiden invloed heeft (7, 8). De afgelopen 10 jaar heeft er zeer veel onderzoek plaatsgevonden om de huidige behandeling te verbeteren en om nieuwe medicijnen te ontwikkelen met antivirale activiteit tegen HCV. De ultieme behandeldoelen bestaan uit 100% SVR, een kortere behandelduur en minder bijwerkingen. Deze doelen liggen echter nog niet binnen handbereik. In dit promotieonderzoek beschrijven wij onze studies waarin we hebben gekeken naar aangepaste (peg) interferon behandelingen en verschillende nieuwe medicijnen voor chronische hepatitis C patiënten.

Verbetering van de huidige behandeling gebaseerd op interferon

Zoals beschreven in *hoofdstuk 2* kan de behandelduur met SOC worden aangepast aan de virologische respons tijdens behandeling. Essentiële tijdspunten om HCV RNA te bepalen zijn start van de behandeling, week 4, week 12 en week 24. De kans om SVR te behalen is direct gerelateerd aan het moment dat HCV RNA niet meer aantoonbaar is. Afhankelijk van respons op week 4 en 12 kan de behandelduur worden verkort of verlengd volgens de meest recente internationale richtlijn (9). Patiënten met genotype 1 en lage virale load bij start van de behandeling kunnen 24 weken worden behandeld indien zij HCV RNA negatief zijn op week 4 (RVR; rapid virological response) (10-13). Patiënten met genotype 1 die een meer dan 2 log daling hebben op week 12 (EVR; early virological response) moeten 48 weken worden behandeld. Patiënten met genotype 1 met een late virologische respons tussen week 12 en 24 (DVR; delayed virological respons) zouden 72 weken kunnen worden behandeld om relaps te voorkomen (14-20). De hiervoor genoemde aanbevelingen kunnen ook worden toegepast op genotype 4 patiënten. Patiënten met genotype 2 en 3 die RVR halen en een lage virale load hebben bij start van de behandeling zouden 16 weken kunnen worden behandeld, alhoewel er iets meer relaps optreedt in vergelijking met 24 weken behandeling (21-25). Het inkorten van de behandeling is niet aanbevolen voor patiënten met ernstige leverfibrose of cirrose of met andere cofactoren die geassocieerd zijn met een slechte respons. Patiënten met genotype 2 en 3 die EVR of DVR halen zouden 48 of 72 weken kunnen worden behandeld (10). Er zijn onvoldoende gegevens over genotype 5 en 6 patiënten, alhoewel de kans op respons vergelijkbaar is met genotype 3 patiënten (26-28).

Om de kans op RVR of EVR te verhogen is er uitgebreid onderzoek gedaan naar hogere doseringen (peg)interferon (29-31). Met name voor eerdere non-responders kan een behandeling met een hogere dosering peginterferon een optie zijn, aangezien er momenteel nog geen alternatieven voorhanden zijn. Het voordeel van hoge dosis peginterferon inductieschema's gedurende 4 of 12 weken is echter discutabel en er is bezorgdheid over toxiciteit tijdens de behandeling. In *hoofdstuk 3* evalueerden wij een peginterferon inductie schema gecombineerd met een langere therapieduur (72 weken) ten opzichte van SOC in patiënten die eerder niet reageerden op een behandeling met standaard interferon met of zonder ribavirine. Wij konden geen verschil vinden met betrekking tot de veiligheid, maar de respons percentages waren vergelijkbaar tussen beide groepen (ongeveer 40% SVR). Het behalen van RVR was een belangrijke voorspeller voor het behalen van SVR. Daarnaast bleek een laag gamma-glutamyltransferase niveau bij start van de behandeling (minder dan 2 x de bovenlimiet van normaal) significant geassocieerd met SVR.

In *hoofdstuk 4* evalueerden wij een hoge dosis continue interferon infusie (6, 9 of 12 MU interferon per dag) gecombineerd met ribavirine in eerdere peginterferon/ribavirine non-responders. Hierbij werd de hypothese aangenomen dat stabiele serum concentraties van standaard interferon de virale suppressie zouden kunnen verbeteren en dat er minder bijwerkingen zouden optreden zoals die gezien worden bij wekelijkse subcutane peginterferon toediening. In deze moeilijk te behandelen patiëntengroep werd 20% SVR gezien. Er werden 6 serieuze bijwerkingen gezien en de bijwerkingen leken dosisafhankelijk. Wij concludeerden dat continue subcutane interferon toediening succesvol kan zijn, maar dat de aanpak van bijwerkingen zou moeten verbeteren, met name voor de hoogste en meeste effectieve dosering (12 MU).

Een verdere verdieping in de virale kinetiek van continue interferon toediening wordt beschreven in *hoofdstuk 5*. Wij lieten zien dat de mediane HCV RNA daling op week 4 significant sterker was in patiënten die 12 MU interferon per dag kregen in vergelijking met 9 MU, respectievelijk (2.58 log versus 0.84, $p = 0.017$). Een sterkere daling van HCV RNA was geassocieerd met hogere neopterine concentraties (als marker van immuunactivatie) maar niet met 2,5-oligoadenylaat synthetase activiteit of beta2-microglobuline concentraties. Daarnaast werd gezien dat een sterke HCV RNA daling op week 4 essentieel is om SVR te behalen.

Deze studies laten zien dat aangepaste (peg)interferon schema's in combinatie met ribavirine tot SVR kan leiden in geselecteerde patiënten met karakteristieken die zijn geassocieerd met een lage virale respons. De gevonden SVR percentages zijn bescheiden, maar het zal bijzonder interessant zijn om deze schema's te combineren met de nieuwe directe antivirale middelen, die op korte termijn verwacht worden.

Nieuwe anti-HCV medicijnen

Door een toegenomen begrip van de HCV levenscyclus zijn er verschillende klassen nieuwe medicijnen ontwikkeld voor patiënten met chronische hepatitis C. Sinds onze review in 2008 (*hoofdstuk 2*) zijn sommige ontwikkelingen gestaakt door gebrek aan effectiviteit, toxiciteit of economische motieven. Daarnaast zijn er verschillende medicijnen doorontwikkeld in fase III klinische onderzoeken. Een hele reeks medicijnen wordt momenteel in fase I en fase II onderzocht.

In *hoofdstuk 6* evalueerden wij ANA773, een toll-like receptor (TLR)7 agonist. De TLR familie is interessant gebleken aangezien na stimulatie activatie van zowel het specifieke als het niet-specifieke immuunsysteem wordt gezien (32). ANA773 heeft endogene inductie laten zien van verschillende interferon alfa subtypen in preklinische studies en in gezonde vrijwilligers. In deze fase I studie lieten

wij zien dat ANA773 veilig is en goed wordt verdragen. Daarnaast werd een significante HCV RNA daling gezien tot 3.10 log in vergelijking met placebo. Deze daling was echter tijdelijk en werd alleen gezien in de twee hoogste doseringsgroepen. Een vervolg analyse van HCV geïnfecteerde patiënten en gezonde vrijwilligers liet zien dat verminderde plasmacytoïd dendritische celpopulaties en verhoogde serum interferon alfa en IP-10 (interferon-gamma inducible protein) concentraties alleen werden gezien in patiënten die ≥ 1 log HCV RNA daling hadden tijdens behandeling met ANA773 (*hoofdstuk 7*). Deze bevindingen laten zien dat belangrijke onderdelen van de antivirale immuun respons werden geactiveerd in patiënten die een virale daling lieten zien. Vervolgstudies zouden hogere doseringen van ANA773 moeten onderzoeken en behandelschema's gecombineerd met ribavirine en nieuwe directe antivirale middelen.

Het middel JTK-652 is een nieuwe HCV infectieremmer die werd bestudeerd in *hoofdstuk 8*. In vitro studies in drie verschillende cellijnen lieten het remmende effect van JTK-652 zien op de entree van HCV genotype 1a and 1b pseudotype virussen. In deze studie werd de hypothese aangenomen dat JTK-652 mogelijk de entree van HCV in de gastheercel in vivo zou kunnen voorkomen, alhoewel het exacte mechanisme hiervan nog niet goed begrepen is. Verschillende doseringen van JTK-652 lieten milde bijwerkingen zien in gezonde vrijwilligers en patiënten. JTK-652 liet echter geen HCV RNA daling zien in patiënten met chronische hepatitis C. Wij concludeerden dat virale remming van JTK-652 tegen HCV pseudotype virus in het replicon system waarschijnlijk niet overeenkomt met in vivo HCV infectie.

Protease- en polymeraseremmers behoren tot de meest veelbelovende groep nieuwe medicijen, ook wel DAAs genoemd (direct acting antiviral drugs). Introductie in de klinische praktijk van twee proteaseremmers (telaprevir en boceprevir) wordt verwacht in 2011 of 2012. Belangrijke studies in genotype 1 patiënten die niet eerder werden behandeld hebben laten zien dat telaprevir of boceprevir in combinatie met peginterferon en ribavirine het percentage SVR met 27-31% kan verhogen in vergelijking met SOC (9, 33-35). Vergelijkbare studies in genotype 1 patiënten die wel eerder werden behandeld lieten SVR percentages tot 80% zien met triple therapie, alhoewel beduidend lagere SVR percentages (ongeveer 30%) werden gezien in echte non-responders (36, 37).

De optimale behandelduur met deze middelen is afhankelijk van virale respons op week 4. De behandelduur met telaprevir kan in 57-65% van de onbehandelde patënten worden verkort en met boceprevir in 44% van de patiënten, respectievelijk. Eerdere non-responders zouden echter 48 weken moeten worden behandeld (38). Met boceprevir werden betere SVR resultaten gezien na een zogenaamde lead-in fase van 4 weken waarin alleen peginterferon en ribavirine werden gebruikt (39). Deze lead-in fase kan patiënten identificeren die gevoelig zijn voor SOC, waardoor verdere individualisatie van de behandeling mogelijk is.

De impact van deze triple therapie in de klinische praktijk zal enorm zijn, zowel voor patiënten als voor dokters. Er zijn echter een aantal belangrijke factoren waarmee rekening gehouden moet worden alvorens te starten met triple therapie.

Naast de bekende bijwerkingen van peginterferon en ribavirine zullen er nog meer bijwerkingen optreden zodra één van de nieuwe middelen wordt toegevoegd. De belangrijkste bijwerkingen van telaprevir zijn anemie, jeuk, rash, misselijkheid en diarree. Het stoppen van de behandeling door bijwerkingen van telaprevir was noodzakelijk in 17% van de patiënten (35). De belangrijkste bijwerkingen van boceprevir zijn anemie en smaakveranderingen. Het stoppen van de behandeling door bijwerkingen van boceprevir werd gezien in 12-16% van de patiënten (34). Een adequate aanpak van deze bijwerkingen zal essentieel zijn om goede behandelresultaten te halen.

Een belangrijk punt betreft het optreden van virale resistentie na toediening van een proteaseremmer. Ervaringen met hepatitis B en HIV hebben laten zien dat selectie van virale varianten kan leiden tot een verminderde gevoeligheid voor DAAs (40). Meerdere mutaties zijn geïdentificeerd op verschillende posities in het NS3/4A gen die virale resistentie tegen telaprevir en boceprevir kunnen induceren. De meeste resistente mutaties blijven echter gevoelig voor peginterferon en ribavirine (41).

De huidige registratie-studies hebben alleen de werking van triple therapie in genotype 1 patiënten geëvalueerd. Er zijn gegevens bekend dat proteaseremmers minder effectief zijn bij HCV genotype 2, 3 en 4, maar verder onderzoek is nodig (42).

Zoals verwacht zijn patiënten waarbij de noodzaak van HCV eradicatie het hoogst is (ernstige lever fibrose, cirrose, donorontvangers, hepatitis B of HIV co-infectie) niet geïncludeerd in de huidige registratie-studies. Goede studies in deze patiëntenpopulaties met DAAs zijn gewenst.

Een ander belangrijk punt betreft de driemaal daagse dosering van telaprevir en boceprevir, hetgeen de therapie-trouw kan bemoeilijken. Therapie-ontrouw kan virale resistentie in de hand werken met als gevolg virale breakthrough of non-respons.

In *hoofdstuk 9* evalueerden wij narlaprevir, een tweede generatie HCV NS3 proteaseremmer, in onbehandelde en behandelde genotype 1 patiënten. In vitro studies lieten zien dat narlaprevir ongeveer 10 x sterker is dan telaprevir en boceprevir (43, 44). Gecombineerde toediening met ritonavir werd ook geëvalueerd, aangezien hierdoor het farmacokinetische profiel van narlaprevir werd verbeterd door remming van cytochroom P450-3A4, waardoor tweemaal daags doseren van narlaprevir mogelijk werd (in plaats van driemaal daags doseren met alleen narlaprevir). Tijdens narlaprevir behandeling werden alleen milde bijwerkingen gezien en in combinatie met ritonavir werd het bijwerkingen profiel niet significant anders. Meer dan 4 log HCV RNA daling werd gezien in alle patiënten gedurende 7 dagen behandeling met of zonder ritonavir. Na een tweede periode van 14 dagen behandeling met narlaprevir en peginterferon (met of zonder ritonavir), werd gestart met SOC waarbij 38% SVR werd gezien in eerder behandelde patiënten en 81% SVR in onbehandelde patiënten, respectievelijk. Net als met andere proteaseremmers werden resistente virale varianten gedetecteerd gedurende narlaprevir behandeling. Echter, gezien de veelbelovende resultaten is gestart met fase II onderzoek, waarbij ook een eenmaal daags doseringsschema zal worden geëvalueerd.

Ondanks de zeer sterke antivirale activiteit van de meeste DAAs die momenteel in ontwikkeling zijn zullen peginterferon en ribavirine voorlopig de ruggegraad zijn van anti-HCV combinatie therapie. Immuuncontrole door peginterferon lijkt noodzakelijk voor definitieve HCV eradicatie. Er zijn echter interessante nieuwe gegevens beschikbaar waarbij verschillende DAAs gecombineerd worden zonder toevoeging van peginterferon (45). Momenteel worden fase I en II studies uitgevoerd waarbij proteaseremmers gecombineerd worden met polymeraseremmers. Of dergelijke schema's een peginterferon-vrij tijdperk zullen inluiden zal de toekomst moeten uitwijzen.

Een belangrijk punt van aandacht betreft de kosteneffectiviteit van toekomstige triple therapieën. De prijs van triple therapie zal waarschijnlijk significant toenemen in vergelijking met SOC, zeker als extra kosten voor de aanpak van bijwerkingen (bijvoorbeeld erytropoëtine toediening bij anemie) hierin worden meegenomen. Deze gegevens zijn op dit moment echter nog niet beschikbaar.

Conclusie

We staan aan de vooravond van een nieuw tijdperk van antivirale behandeling voor chronische hepatitis C. Na een decennium van peginteferon en ribavirine combinatie therapie zal de cocktail zich uitbreiden met een direct antiviraal middel (telaprevir of boceprevir). In dit proefschrift lieten we zien dat er nog ruimte is om het immunomodulatoire gedeelte van de anti-HCV behandeling te verbeteren. Daarnaast werden drie nieuwe middelen geëvalueerd met verschillende werkingsmechanismen, inclusief een tweede generatie proteaseremmer. Er liggen nog grote uitdagingen voor ons, waaronder de aanpak van bijwerkingen en virale resistentie, maar het therapeutische succes verhaal van chronische hepatitis C krijgt een passend vervolg.

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List of Publications

1. Bergmann JF, Vrolijk JM, van der Schaar P, Vroom B, van Hoek B, van der Sluys Veer A, de Vries RA, Verhey E, Hansen BE, Brouwer JT, Janssen HLA, Schalm SW, de Knegt RJ. Gamma-glutamyltransferase and rapid virological response as predictors of successful treatment with experimental or standard peginterferon-alpha-2b in chronic hepatitis C non-responders. Liver Int 2007;27:1217-1225.

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PhD portfolio summary

Oral and poster presentations

2006 Double-dose induction with prolongation of treatment duration vs standard dose peginterferon alfa-2b and ribavirin in patients with chronic hepatitis C unresponsive to previous therapy: a pilot study (PIT-study). NVGE, Veldhoven, The Netherlands (oral).

2007 FibroScan superior to APRI in detecting significant liver fibrosis in chronic hepatitis B and C patients. NVGE, Veldhoven, The Netherlands (oral).

2008 FibroScan superior to APRI in detecting significant liver fibrosis in chronic hepatitis B and C patients. EASL, Milan, Italy (poster).

2009 Safety and antiviral activity of SCH900518 administered as monotherapy and in combination with peginterferon alfa-2b to naïve and treatment-experienced HCV-1 infected patients. NVGE, Veldhoven, The Netherlands (oral).

JTK-652 is a novel HCV entry inhibitor: results of a phase 1 study evaluating safety, tolerability and antiviral activity in chronic hepatitis C patients. EASL, Copenhagen, Denmark (poster).

ANA773, an oral inducer of endogenous interferons that acts via TLR7, reduced serum viral load in patients chronically infected with HCV. AASLD, Boston, USA (poster).

SVR results in chronic hepatitis C genotype 1 patients dosed with SCH900518 and peginterferon alfa-2b for 2 weeks, followed by peginterferon alfa-2b and ribavirin for 24/48 weeks: an interim analysis. AASLD, Boston, USA (poster).

2010 Viral kinetics and immunological response with continuous subcutaneous administration of high-dose interferon alfa-2b in treatment-experienced chronic hepatitis C patients. NVGE, Veldhoven, The Netherlands (oral) & AASLD, Boston, USA (poster).

Continuous subcutaneous administration of high-dose interferon alfa-2b combined with ribavirin in chronic hepatitis C patients: a dose-finding and safety study in treatment-experienced patients. AASLD, Boston, USA (poster).

International conferences

2005 Annual meeting of the American Association for the Study of Liver Diseases (AASLD), San Francisco, USA.

2006 Annual meeting of the European Association for the Study of the Liver (EASL), Vienna, Austria.

2008 Annual meeting of the American Association for the Study of Liver Diseases (AASLD), San Francisco, USA.

Courses

2006 Erasmus Summer Programme: introduction to data-analysis. (Erasmus University, Rotterdam, The Netherlands).

Memberships

2005 Dutch Society of Gastroenterology (NVGE).

Dutch Society of Hepatology (NVH).

2009 Dutch Society of Internal Medicine (NIV).

2011 Dutch Society of Gastroenterologists (NVMDL).

Dankwoord

Zonder informed consent geen research… Dus ten eerste wil ik alle HCV patiënten die hebben geparticipeerd in de verschillende trials bedanken voor hun medewerking (lees: geduld, heel veel formulieren en liters bloed). Alhoewel elke patiënt maar één doel voor ogen had (namelijk een blijvende virologische respons), deelname gaf geen enkele garantie op genezing. Gelukkig zagen vele patiënten in dat wetenschappelijk onderzoek de enige manier is om de huidige behandeling te verbeteren. Maar hoe was ik hierin eigenlijk verzeild geraakt?

Na mijn oudste co-schap MDL in Rotterdam kwam ik tot de conclusie dat dit inderdaad mijn vak moest worden. De meerwaarde van wetenschappelijk onderzoek was toen nog niet tot mij doorgedrongen (ik blijf een laatbloeier), dus het leek mij een goed plan om bij Rob (dr. de Man) en Ernst (prof.dr. Kuipers) aan te geven dat ik wel meteen met de opleiding kon beginnen… Een half uur later zat ik bij Solko (prof.dr. Schalm). Hij had nog wel een mooi project voor me. Althans, een idee. Na ampele overwegingen bedacht ik me dat het toch wel een voorrecht is om in de MDL hoofdstad van Nederland een opleiding te genieten, en stemde dus in met het onderzoeks-voorstel.

En toen begon het. In een duistere vleugel van het Erasmus MC (bereikbaar per brandtrap) werd ik geplaatst in kamertje 1 van de zogenaamde dakpoli, alwaar zich computers bevonden van vlak na het Atari tijdperk. Zucht. Hoe te beginnen? Het werd een veelbelovend investigator-initiated studieprotocol waarvan talloze versies werden geschreven. Vol overgave werkte ik eraan en na maanden van werk mocht het voorstel gepresenteerd worden aan een aantal internationale toppers en een potentiële financier… De studie heeft helaas nooit plaatsgevonden. Goed, les 1 was geleerd. Hoe is het dan toch zo ver gekomen? Zoals een uitgeputte renner op de Alpe d'Huez naar de top wordt geschreeuwd, zo hebben vele personen mij in meer of mindere mate bijgestaan.

Best Rob (dr. de Knegt), het is prachtig dat we dit bereikt hebben! Als HCV expert werd jij mijn copromotor en moest jij je over mij ontfermen en zorgen dat mijn promotietraject tot een succesvol einde zou worden gebracht. Geen makkelijke taak, maar ik dank je voor alle energie die je erin hebt gestoken. Jij bent echt uniek. Uren kun jij vullen met relevante en minder relevante zaken. Ik zou graag nog een avond brieven met je schrijven naar de KLM over slechte service aan boord of verontwaardigd het lidmaatschap opzeggen van één of andere (niet nader te noemen) politieke partij… Maar mogelijk dat je daar geen tijd meer voor hebt aangezien je nu meerdere promovendi hebt die de laatste hand aan hun boekje leggen. Gefeliciteerd, mede dankzij jou staat hepatitis C weer op de kaart!

Beste Solko, jij hebt diepe indruk op mij gemaakt als wetenschapper en medicus pur sang. De wisseling van de wacht was aanstaande en je hebt mij uiteindelijk een klein jaar begeleid. Je bent de eminence grise uit vervlogen tijden terwijl je je tijd tot op de dag van vandaag ver vooruit bent. Je huidige activiteiten als directeur van ExpertDoc en NHGDoc voor online physician to physician consulting getuigen daarvan. Veel dank voor je inspiratie.

Beste Harry (prof.dr. Janssen), ik mag mijzelf gelukkig prijzen dat jij mijn promotor werd toen je het overnam van Solko. Jij was de stuwende kracht achter dit proefschrift en jouw finishing touch zorgde ervoor dat de manuscripten het vereiste niveau haalden om voor wetenschappelijke publicatie in aanmerking te komen. Zoals je de hepatologie unit momenteel door de inter-nationale Champions League loodst is indrukwekkend!

Beste Henk (dr. Reesink) en Joep (collega promovendus), veel dank voor jullie AMC bijdrage aan dit proefschrift. De Ajax-Feyenoord rivaliteit werd opzij gezet en we mochten mee met de fase 1 Fyra die onze patiënten vaak naar PRA in Zuidlaren of Groningen bracht. Het was beslist geen sinecure om keer op keer de keiharde deadline te halen en een cohort geschikte patiënten aan te leveren. En dan zat er toch weer eentje tussen die de avond voor start van de behandeling een lijntje coke had gedaan: urine screening onverbiddelijk, weer een kandidaat minder… Maar liefst 4 artikelen hebben we samen geschreven en er liggen nog een aantal op de plank. Joep, ik kijk uit naar jouw 60 minutes of scientific fame!

Ik wil alle leden van de promotiecommissie bedanken voor hun bereidheid om plaats te nemen in deze commissie.
Beste Bart, (dr. Haagmans), ik wil je nog apart noemen gezien je teaching en coaching vanuit virologische hoek. Veel dank voor al het werk dat jullie hebben gedaan voor de SCIN-C studie.

Beste André (dr. Boonstra), veel dank voor jouw basic science ondersteuning. Tjonge, het zal niet makkelijk voor je geweest zijn: de eerste jaren bleven dendritsche cellen voor mij toch mysterieuze wezens van een verre planeet. Dankzij jou is het gelukt om de vertaalslag naar de klinische praktijk te maken en om samen begrijpelijke papers te schrijven. Eén van de lessen die ik van je geleerd heb is dat statistiek toch meer iets voor clinici is (laat Bettina het niet horen)...
Tevens wil ik Hanneke (dr. van Vuuren) en haar team bedanken voor ondersteuning vanuit het laboratorium en voor de verwerking en opslag van duizenden samples in ijskoude vriezers.

Bettina (dr. Hansen), jij bent dus onmisbaar gebleken. De coolste statisticus ever (zeg dat maar tegen de kinderen)! Zeer veel dank voor het mogelijk maken van dit proefschrift. Tak for det.
En ik was dus niet de enige die onderzoek deed… Ik wil alle mede provendi en MDL-assistenten (dakpoli, flexplekkers en basalisten) bedanken voor tips, tricks, morele support en inspiratie: Manon, Leonieke, Frank, Jan-Maarten, Claudia, Pieter, Geert, Bart, Sarwa, Hajo, Martijn, Jolanda, Sanna, Joyce, Marjolein, Mark, Erik, Jurriën, Edith, Lieke, Vincent, Jildou, Daphne, Ad, Paul, Nicoline, Desirée, Judith en anderen. Wetenschap bleek goed te combineren met borrels, ski-trips en congressen (en andersom)!
Beste Robert, wat mooi dat we uiteindelijk op dezelfde dag in het zweetkamertje mogen zitten! Ik wil je hartelijk danken voor het afronden van de SCIN-C studie. Zonder jou had ik het niet gered.

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Mar en Mar (Marion en Margriet): hepatologie secretaresses, organizers en insiders. Jullie staan garant voor eindeloos submitten, ingevulde formulieren, email (vrijblijvend tot dwingend) en allerlei andere belangrijke zaken waarvan ik blij ben dat jullie je daar mee bezig houden. Voor het laatste nieuwtje was er gelukkig altijd tijd! Many thanks.

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mocht ik zelf een exotisch oord uitkiezen om de eerste 4 jaar door te brengen. Gezien mijn voorliefde voor het verre oosten koos ik voor de Hanzestad Deventer…

En daar wordt gelukkig goed voor mij gezorgd! De beginselen van internal medicine zijn mij reeds vakkundig bijgebracht. Beste Martin (dr. Gerding) en vakgroep interne geneeskunde, dank voor enthousiasme en inspiratie tijdens de vooropleiding. Daarnaast was het erg prettig dat ik altijd in de gelegenheid werd gesteld om aan artikelen te werken of om in het kader van mijn onderzoek congressen en meetings te bezoeken. DZ-assistenten: vrijwillig of minder vrijwillig zijn we aan elkaar overgeleverd, maar gelukkig vergeten we niet om op gezette tijden de patiëntenronde in te ruilen voor een rondje op de Brink! Veel dank voor jullie collegialiteit en interesse.

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Vele vrienden, vriendinnen en familieleden hebben mij al die jaren gesteund. Uiteraard kan ik mijn meest dierbaren niet ongenoemd laten zo aan het einde van dit boek.

Lieve Weddingsensations (Linda, Mir, Saar, Jap, Joost, Piet, Mark, PP, Guido), sinds 1997 staan wij on stage: een onwaarschijnlijk avontuur. Als HHB veroverden we de East-Indies en de West-Indies, en onze Afrikaanse ambassade trips langs donkere schurkenstaten en vieze barretjes zal niemand van ons ooit vergeten. De afgelopen jaren hebben we elke zichzelf respecterende huwelijkslocatie in ons eigen kikkerland gezien: the show must go on. Ik dank jullie voor jullie vriendschap en support en voor rock'n roll buiten het ziekenhuis. Paul en Joost, fantastisch dat jullie mij bijstaan als paranimfen op de dag des oordeels!

Lieve Eline, ik ben er trots op dat jij mijn zusje bent. Medicine is art, maar art is ook medicine. Jouw betoverende pianospel brengt mensen in vervoering. Misschien niet gedegen onderzocht, maar ik ben overtuigd van de therapeutische werking! Het leven van een pianiste gaat echter niet altijd over rozen. Speciaal voor jou heb ik nog een aantal vrijgezellen uitgenodigd voor het promotiefeest.

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Curriculum Vitae

De auteur van dit proefschrift werd geboren op 18 oktober 1977 te Groningen. Na het behalen van zijn eindexamen in 9 vakken aan het Willem Lodewijk Gymnasium te Groningen startte hij in 1996 met de studie geneeskunde aan de Erasmus Universiteit te Rotterdam. Tijdens zijn studie was hij in het Erasmus MC werkzaam als student-assistent op de afdelingen Psychiatrie, Plastische Chirurgie en Medische Microbiologie & Infectieziekten.
Daarnaast speelde hij van 1997 tot 2001 als gitarist in de Rotterdamse studentenband Hermes House Band.
De coschappen werden gelopen in Rotterdam en omstreken. Na het behalen van het artsexamen werd in 2005 gestart met zijn promotieonderzoek naar verbetering van de behandeling van chronische hepatitis C op de afdeling Maag-, Darm- en Leverziekten van het Erasmus MC te Rotterdam onder begeleiding van prof.dr. S.W. Schalm (in 2006 opgevolgd door prof.dr. H.L.A. Janssen) en copromotor dr. R.J. de Knegt.
Van februari 2009 tot januari 2011 volgde hij zijn vooropleiding Interne Geneeskunde in het Deventer Ziekenhuis (opleider: dr. M. Gerding).
Vanaf februari 2011 tot heden vervolgt hij zijn opleiding tot Maag-, Darm- en Leverarts in het Deventer Ziekenhuis (opleider: dr. F. ter Borg).
Hij is getrouwd met Francesca Pisetzky en zij wonen in Utrecht met hun twee prachtige kinderen Sara en Maurits.

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