Original article

Potent immune activation in chronic hepatitis C patients upon administration of an oral inducer of endogenous interferons that acts via Toll-like receptor 7

Andre Boonstra*, Bi-Sheng Liu, Zwie MA Groothuismink, Jilling F Bergmann, Joep de Bruijne, Daphne M Hothen, Bettina E Hansen, Andre A van Vliet, Jeroen van de Wetering de Rooij, Simon P Fletcher, Lisa A Bauman, Mohamad Rahimy, James R Appleman, James L Freddo, Hendrik W Reesink, Robert J de Knecht, Harry LA Janssen

1Department of Gastroenterology and Hepatology, Erasmus Medical Center University Hospital, Rotterdam, the Netherlands
2Department of Gastroenterology and Hepatology, Amsterdam Medical Center, Amsterdam, the Netherlands
3PRA International EDS, Zuidlaren, the Netherlands
4Anadys Pharmaceuticals, Inc., San Diego, CA, USA

*Corresponding author e-mail: p.a.boonstra@erasusmc.nl

Background: ANA773, an oral prodrug of a small-molecule Toll-like receptor (TLR)7 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 (IFN)-α and IFN-γ inducible protein (IP)-10 levels were observed only in virological responders (≥1 log10 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 virological responders showed a high frequency of IFN-α-producing plasmacytoid DC upon stimulation in vitro with ANA773, whereas no IFN-α 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 IFNs and IFN-stimulated gene products (IFN-α and IP-10) upon TLR7 ligation.

Introduction

HCV is a major cause of chronic liver disease, affecting >170 million individuals globally. In approximately 80% of individuals 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 (IFN)-α plus ribavirin. This combination therapy has many adverse effects, and a sustained viral response is only observed in approximately 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. Indeed, 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 Toll-like receptor (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 dendritic cells (DC) express TLR7 messenger RNA (mRNA), and respond to specific TLR7 agonists, such as single-stranded RNA and R848, to produce type I IFNs [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 0.14–3.10 log at the highest dosing group receiving 2,000 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 1,600 mg or 2,000 mg ANA773 were evaluated for immune status, as well as a cohort of healthy controls receiving 1,600 mg ANA773. The highest dose cohorts were examined since considerable reductions of serum HCV RNA load were observed in these cohorts. In the 1,600 mg group, six chronic HCV infected patients received oral ANA773 and two received placebo. In the 2,000 mg group, eight patients received ANA773 and two received placebo. Blood samples of the 1,600 mg group were drawn on day 0, 5, 13, 27 and 41 and the blood samples of the 2,000 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 peripheral blood mononuclear cells (PBMC) from five patients in the 1,600 mg group and from seven patients in the 2,000 mg group. The patient details have been described previously [16]. In addition, the IL28B SNP rs12979860 was determined for all patients using competitive allele-specific PCR (KASP; KBioscience, Hoddesdon, UK). In the 1,600 mg group, the patients unresponsive to ANA773 had the TC, TC and CC genotype, while the responsive patients both had the CC genotype. In the 2,000 mg group, both non-responders to ANA773 were TC, while in patients responding to ANA773 two individuals had the CC genotype and three individuals the TC genotype. Patients were dosed with oral ANA773 every other day for either 28 days (1,600 mg group) or 10 days (2,000 mg group). Study medication (100 mg capsules) and placebo capsules were supplied by Anadys Pharmaceuticals, Inc. (San Diego, CA, 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/m2, treatment-naïve or relapse from prior IFN-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 ≥75×103 IU/ml. Key exclusion criteria included decompensated liver disease (consistent with Child Pugh B/C liver cirrhosis), and coinfection 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
Intracellular cytokine staining
PBMC were isolated from peripheral blood of patients prior to treatment with ANA773 (2,000 mg group only). Cells were isolated from peripheral blood by density centrifugation on Ficoll-Hypaque (GE Healthcare, Diegem, Belgium). Fresh PBMC were stimulated on the day of blood collection with medium, ANA773 (300 μM) or R848 (1 μg/ml; Alexis, San Diego, CA, USA) in RPMI-1640 medium (BioWhittaker, Lonza, Verviers, Belgium) supplemented with 10% human serum for 5 h, with brefeldin-A (10 μg/ml; Sigma–Aldrich, Steinheim, Germany) 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, San Jose, CA, USA), BDCA4-APC (AD5-17F6, Miltenyi Biotech), tumour necrosis factor (TNF)-PE-Cy7 (MAb11, eBioscience), and IFN-α-FITC (MMHA-1, PBL InterferonSource, Piscataway, NJ, USA). Cytokine-producing plasmacytoid DC and monocytes were detected by flow cytometry (Canto-II, BD).

Immunoassay for detection of cytokines
The levels in serum of IFN-α and IP-10 during the course of treatment with ANA773 were detected by ELISA by Alta Analytical Laboratory (San Diego, CA, USA). 2,5'-Oligoadenylate synthetase (OAS) was analysed by radio immunoassay at PRA International (Assen, the Netherlands).

Statistics
Values are expressed as mean values, unless indicated otherwise. Data was analysed with Prism 5.0 (Graphpad software, La Jolla, CA, USA) using the Mann–Whitney t-test to compare variables between two independent groups. In all analyses, a two-tailed P-value of <0.05 (95% CI) was considered statistically significant.

Results
Administration of ANA773 leads to a transient reduction of the number of lymphocytes in blood of healthy and HCV-infected individuals
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 h after the first administration by comparing paired blood samples. As shown in Figure 1, treatment of healthy individuals with a dose of 1,600 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 1,600 mg or 2,000 mg ANA773 every other day, except for the number of monocytes, which declined within 6 h following administration of 2,000 mg TLR7 agonist. The absolute numbers of lymphocytes was significantly reduced 6 h after start of treatment in both healthy individuals (dose 1,600 mg) and chronic HCV patients (dose 1,600 and 2,000 mg).

The reduction in the number of lymphocytes 6 h after administration of ANA773 was transient, since the number of leukocytes, lymphocytes and monocytes was similar to their pretreatment 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 virological 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 1,600 mg ANA773 showed the same trend with respect to the numbers of plasmacytoid 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 1,600 mg group, multiple dosing of 2,000 mg ANA773 showed the same trend with respect to the decline of the numbers of DC, which was not significant.

Administration with 2,000 mg ANA773 resulted in a viral load reduction of >1 log in five out of seven 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...
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 1,600 mg or 2,000 mg ANA773. Blood was collected before and 6 h after administration. The absolute numbers of cells were expressed as 10^9 cells/l and shown for individual patients.
Figure 2. 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 48 h for a period of 28 days (1,600 mg), and blood was collected at the indicated time points. Leukocyte subpopulations were determined in whole blood by automated analyses and flow cytometry as described in Methods. (B) The effect of ANA773 on dendritic cell populations was determined in whole blood of patients treated with 1,600 mg ANA773 (as described above) or 2,000 mg, which was administered every 48 h for 10 days.
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 two non-responder patients as compared to the responder patients, which
was also observed when examining the non-responder patients of the 1,600 mg group (Additional file 1).

Differential effects of TLR7-induced responses in virological 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 IFN-stimulated genes IFN-α, IFN-γ inducible protein (IP)-10 and mRNA levels for 2,5-OAS. As presented in Figure 4A, IFN-α 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 h after start of treatment.

In addition, we examined the activation status of NK cells in treated patients. By performing flow-cytometry, we observed that 6 h 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 patients’ subsequent virological responses to ANA773 treatment. As shown in Figure 5, a high frequency of IFN-α-producing plasmacytoid DC upon stimulation in vitro was detected in PBMC from patients who were subsequently virological responders, whereas no IFN-α 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 have antiviral activity initiated by the induction of endogenous IFN-α as well as by specific activation of various
Figure 4. *Ex vivo* analysis demonstrates stronger activation of immunity in virological responders to ANA773 as compared to non-responders.

(A) The serum levels of interferon (IFN)-α and IFN-γ inducible protein (IP)-10 were determined by ELISA, and the 2,5-oligoadenylate synthetase (OAS) levels in serum by radio immunoassay 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. mRNA, messenger RNA.
Immune effects of TLR7 agonists in HCV-infected patients

Antiviral Therapy 17.4

leukocyte populations by TLR7, such as plasmacytoid DC. Direct stimulation of the immune system may be an important advantage over the use of exogenous IFN-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 proinflammatory cytokines other than IFN-α (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 IFN-α and IFN-α-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 IFN-α 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 IFN-α 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

Figure 5. The frequency of interferon-α-producing plasmacytoid dendritic cells in vitro was higher in PBMC from patients who were subsequently virological responders, whereas interferon-α was not induced in non-responders.
accounted for the different efficacy of ANA773. Interestingly, evaluation of the effect of ANA773 on PBMC from healthy individuals showed induction of IFN-α 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 cannot 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 signalling 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 IFN-α levels that are induced during therapy. TLR ligation as well as exogenous administration of IFN-α in mice also showed a transient lymphopaenia 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 IFN-based therapy in chronic HCV-infected patients [22], and by gender differences, which are known to influence the levels of IFN-α produced upon TLR7 ligation [23]. Furthermore, although the clinical outcome of IFN-based therapy is strongly dependent on specific IL-28B gene polymorphisms, our study cohort was too small to draw firm conclusions on the importance of the IL28B SNP in the response to ANA773.

Among individuals responsive to ANA773, both the CC and TC rs12979860 genotypes were found (see Methods section). 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 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 IFNs and IFN-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 finding 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 antiviral 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 reinfection.

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 Medical Center) and Martine Peters (Academic Medical Center). Furthermore, we would like to thank Boreth Eam, Maria
Sergeeva, and Tim Harding with their help during various stages of this project.

This study was sponsored by Anadys Pharmaceuticals Inc.

Disclosure statement

HLAJ has served as a speaker, a consultant and an advisory board member for Roche, Novartis, Gilead, Schering-Plough and Bristol-Myers Squibb, and has received research funding from Roche, Novartis, Gilead, Schering-Plough and Bristol-Myers Squibb. RJdK has served as a speaker, a consultant and an advisory board member for Schering-Plough, and has received research funding from Schering-Plough, Roche and Medtronic. HWR has served as a speaker, a consultant and an advisory board member for Roche Molecular Diagnostics, Anadys, Merck, Arrows, Gilead, PRA-International, Tibotec, GlaxoSmithKline, Chiron Novartis and Roche Therapeutics, and has received research funding from Schering-Plough, PRA International and Roche. SPF, LAB, MR, JRA and JLF are employees of Anadys Pharmaceuticals. JFB, JdB, DMH, BEH, AB, B-SL, ZMAG, JvdWdR and AAVV declare no competing interests.

Additional file

Additional file 1: A figure illustrating plasmacytoid dendritic cells in non-responders at different time points after start of treatment can be found at http://www.intmedpress.com/uploads/documents/AVT-11-OA-2060_Boonstra_Add_file1.pdf

References


Accepted 28 August 2011; published online 19 December 2011