Targeting a host-cell entry factor barricades antiviral resistant HCV variants from on-therapy breakthrough in human-liver mice.

Journal:	<i>Gut</i>
Manuscript ID:	gutjnl-2014-309045.R1
Article Type:	Original Article
Date Submitted by the Author:	22-Jun-2015
Complete List of Authors:	<p>Vercauteren, Koen; Ghent University and Hospital, Center for Vaccinology Brown, Richard; Institute of Experimental Virology, TWINCORE, Centre for Experimental and Clinical Infection Research; a joint venture between the Medical School Hannover (MHH) and the Helmholtz Centre for Infection Research (HZI), Mesalam, Ahmend; Ghent University and Hospital, Center for Vaccinology Doerrbecker, Juliane; Institute of Experimental Virology, TWINCORE, Centre for Experimental and Clinical Infection Research; a joint venture between the Medical School Hannover (MHH) and the Helmholtz Centre for Infection Research (HZI), Bhujju, Sabin; Genome Analytics, Helmholtz Centre for Infection Research, Geffers, Robert; Genome Analytics, Helmholtz Centre for Infection Research, Van den Eede, Naomi; Ghent University and Hospital, Center for Vaccinology McClure, Patrick; School of Life Sciences and the NIHR Nottingham Digestive Diseases Biomedical Research Unit, University of Nottingham, Queen's Medical Centre, Nottingham NG7 2UH, UK, Troise, Fulvia; Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II, Verhoye, Lieven; Ghent University and Hospital, Center for Vaccinology Baumert, Thomas; Inserm U1110, Farhoudi, Ali; Ghent University and Hospital, Center for Vaccinology Cortese, Riccardo; Keires AG, Ball, Jonathan; The University of Nottingham, Queen's Medical Centre Nottingham Leroux-Roels, Geert; Ghent University and Hospital, Center for Vaccinology Pietschmann, Thomas; TWINCORE, Nicosia, Alfredo; Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II, Meuleman, Philip; Ghent University and Hospital, Center for Vaccinology; Ghent University & Hospital,</p>
Keywords:	ANTIVIRAL THERAPY, HCV, CHRONIC VIRAL HEPATITIS, HEPATITIS C, LIVER

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

SCHOLARONE™
Manuscripts

Confidential: For Review Only

Targeting a host-cell entry factor barricades antiviral resistant HCV variants from on-therapy breakthrough in human-liver mice.

Authors: Koen Vercauteren¹, Richard J.P. Brown², Ahmed Atef Mesalam¹, Juliane Doerrbecker², Sabin Bhuj⁴, Robert Geffers⁴, Naomi Van Den Eede¹, C. Patrick McClure⁵, Fulvia Troise⁶, Lieven Verhoye¹, Thomas Baumert^{7,8}, Ali Farhoudi¹, Riccardo Cortese¹⁰, Jonathan K. Ball⁵, Geert Leroux-Roels¹, Thomas Pietschmann^{2,3}, Alfredo Nicosia^{6,9,10}, Philip Meuleman^{1*}

Affiliations:

¹Center for Vaccinology, Dept. Clinical Chemistry, Microbiology and Immunology, Ghent University, Ghent, Belgium.

²Institute of Experimental Virology, TWINCORE, Centre for Experimental and Clinical Infection Research; a joint venture between the Medical School Hannover (MHH) and the Helmholtz Centre for Infection Research (HZI), Hannover, Germany

³German Centre for Infection Research (DZIF), partner site Hannover-Braunschweig

⁴Genome Analytics, Helmholtz Centre for Infection Research, Braunschweig, Germany

⁵School of Life Sciences and the NIHR Nottingham Digestive Diseases Biomedical Research Unit, University of Nottingham, Queen's Medical Centre, Nottingham NG7 2UH, UK

⁶Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II, Naples, Italy

⁷Institut National de la Santé et de la Recherche Médicale, U1110, Strasbourg, France

⁸Université de Strasbourg, Strasbourg et Pole Hépatite-digestif, Hopitaux Universitaires de Strasbourg, Strasbourg, France

⁹CEINGE, Naples, Italy

¹⁰Keires AG, Basel, Switzerland

* corresponding author

Contact information:

Prof. Dr. Philip Meuleman
Center for Vaccinology – Ghent University
UZ Gent, Building A, 1st floor
De Pintelaan 185
B-9000 Gent, Belgium.
Phone: +32 9 332 02 05
Fax: +32 9 332 63 11
E-mail: Philip.meuleman@ugent.be

One Sentence Summary: HCV entry inhibition improves end-of-treatment antiviral response in human-liver chimeric mice by restricting on-therapy breakthrough of HCV mutants that are resistant to direct-acting antivirals.

Keywords: HCV, entry inhibition, resistance, mouse model, therapy

Word count: 3332

Abbreviations: HCV, Hepatitis C virus; DAA, Direct-Acting Antiviral; RAV, resistance-associated variants; PEG-IFN, pegylated interferon; RBV, ribavirin; PI, protease inhibitor; SR-BI, Scavenger Receptor class B type I; mAb, monoclonal antibody; SVR, sustained virologic response; LOQ, limit of quantification; EOT, end-of-treatment; uPA, urokinase-type plasminogen activator; SCID, severe combined immune deficient; gt, genotype

Abstract:

Objective: Direct-Acting Antivirals (DAAs) inhibit HCV infection by targeting viral proteins that play essential roles in the replication process. However, selection of resistance-associated variants (RAVs) during DAA therapy has been a major cause of therapeutic failure. In this study we wished to address whether such RAVs could be controlled by the co-administration of host-targeting entry inhibitors that prevent intrahepatic viral spread.

Design: We investigated the effect of adding an entry inhibitor (the anti-SR-BI mAb1671) to a DAA-monotherapy (the protease inhibitor Ciluprevir) in human-liver mice chronically infected with HCV of genotype 1b. Clinically relevant non-laboratory strains were used to achieve viremia consisting of a cloud of related viral variants (quasispecies) and the emergence of RAVs was monitored at high-resolution using next-generation sequencing.

Results: HCV infected human-liver mice receiving DAA-monotherapy rapidly experienced on-therapy viral breakthrough. Deep sequencing of the HCV protease domain confirmed the manifestation of drug-resistant mutants upon viral rebound. In contrast, none of the mice treated with a combination of the DAA and the entry inhibitor experienced on-therapy viral breakthrough, despite detection of RAV emergence in some animals.

Conclusion: This study provides preclinical *in vivo* evidence showing that addition of an entry inhibitor to an anti-HCV DAA regimen restricts the breakthrough of DAA-resistant viruses. Our approach is an excellent strategy to prevent therapeutic failure caused by on-therapy rebound of DAA-RAVs. Inclusion of an entry inhibitor to the newest DAA combination therapies may further increase response rates, especially in difficult-to-treat patient populations.

Summery Box:

What is known about this subject?

- DAA monotherapy selects therapy resistant HCV variants leading to therapy failure.
- RAVs can be detected in patients that fail the newest highly effective DAA combination therapies
- Entry inhibitors have been shown to potently block the spread of HCV in cell culture and in small animal models.

-In cell culture models, entry inhibitors have additive to synergistic activity to DAAs and effectively block the spread of DAA-resistant HCV mutants.

What are the new findings?

-Deep sequencing technology revealed rapid emergence of drug-resistant HCV mutants coinciding with virus rebound during a DAA therapy in the most relevant small animal model for HCV.

-Humanized mice receiving the DAA monotherapy almost always experienced on-therapy viral breakthrough.

-Addition of an entry inhibitor to this DAA therapy prevented on-therapy rebound of DAA-RAVs.

Impact on clinical practice in the foreseeable future:

RAVs can be detected in difficult-to-treat patients that fail the newest highly potent DAA combination therapies. The addition of an entry inhibitor to a DAA cocktail may further increase response rates in such patients. In addition, our strategy could be used in a liver transplant setting in order to prevent HCV recurrence. Finally this approach could also be utilized in the event of global dissemination of therapy-induced DAA resistant viruses.

In a broader context, an analogous strategy could be implemented for the treatment of other viruses that use spread to neighboring host cells as a means to propagate drug resistance.

Introduction

Approximately 3% of the world’s population is chronically infected with the hepatitis C virus (HCV), a condition that can lead to liver fibrosis, cirrhosis, and hepatocellular carcinoma and has become the leading indication for liver transplantation in developed nations[1].

The frequent occurrence of severe side effects as well as treatment failures of pegylated interferon and ribavirin (PEG-IFN/RBV)-based anti-HCV therapies has driven the development of an increasing range of direct-acting antivirals (DAAs). Although the development of DAAs offers the perspective of viral cure in a large majority of patients, one challenge for the success of DAA therapy has been the emergence of drug resistant mutants[2 3]. While the first generation of licensed protease inhibitors (PIs)[4 5] possessed low barriers to resistance in combination with PEG-IFN/RBV, improved sustained virologic response (SVR) rates have subsequently been achieved with second wave PIs. Nevertheless, despite recent therapeutic advances including IFN-free regimens, selection of resistant strains still occurs in difficult-to-cure patient groups[6], which could become increasingly prevalent in real-life clinical practice due to suboptimal therapy adherence.

The Scavenger Receptor class B type I (SR-BI) is a host factor used by HCV during cell entry[7]. SR-BI targeting monoclonal antibodies (mAbs) have been shown to possess cross-genotypic anti-HCV activity in human-liver mice[8 9] and to inhibit cell-to-cell transmission *in vitro* and intrahepatic spread *in vivo*[10]. By potentially inhibiting the spread of resistant variants selected during DAA therapy, anti-SR-BI mAbs may be able to prevent on-therapy breakthrough of RAVs and improve overall SVR rates in hard-to-treat patient groups. As a proof-of-concept study, we investigated the effect of adding the SR-BI-targeting mAb1671 to PI monotherapy (Ciluprevir) in human-liver mice[11 12] chronically infected with HCV gt1b. Chimeric human-liver mice represent the only small animal model where chronic HCV infection in humans can be mimicked[13 14]. A productive viremia consisting of a cloud of related viral variants (quasispecies) was achieved with clinically relevant non-laboratory strains and the emergence of RAVs monitored at high-resolution using next-generation sequencing.

Results

In vivo virologic response during mono- and combination therapy

During PI monotherapy, five out of seven mice experienced viral breakthrough (defined by a greater than 10-fold increase in HCV RNA over nadir, or when viral RNA becomes quantifiable after having been below the LOQ) (**Fig. 1a-g**). End-of-treatment (EOT) response (HCV RNA below LOQ at day 42) was observed in only two mice (**Fig. 1f and g**). In the combination therapy arm, no viral breakthrough was observed, with EOT responses in four out of five mice (**Fig. 1i-l**).

The mono- and combination therapy arms had similar median viral loads at baseline ($P > 0.99$) and comparable reductions in median viremia during the first two weeks of treatment ($-2.17 \times \log_{10}$ and $-1.86 \times \log_{10}$, respectively) (**Fig. 2**). However, the median viremia diverged markedly between these treatment groups from day 14 to EOT (day 42) ($+2.12 \times \log_{10}$ and $-0.59 \times \log_{10}$, respectively) and also when considering the complete treatment period ($-0.05 \times \log_{10}$ and $-2.45 \times \log_{10}$, respectively). In contrast to the monotherapy group (**Fig. 2a**), the median viremia at EOT was significantly reduced in the combination therapy group ($P = 0.0079$) (**Fig. 2b**).

In order to determine if mAb1671 exerts an antiviral effect in chronic infection, three gt1b infected chimeric mice received mAb1671 monotherapy over a four-week period (**Fig. 3**). HCV RNA in the plasma of these mice did not fluctuate more than what is routinely observed in non-treated infected animals.

Quasispecies analysis during therapy

The observed differences in EOT outcome were primarily due to the occurrence of on-therapy viral rebound in the monotherapy animals. To investigate whether these breakthrough viruses represented drug-resistant variants, we tracked the evolution of the protease region of the viral genome via Illumina deep sequencing technology. Nucleotide coverage across the protease domain was consistent between samples and independent of study phase or treatment regimen (see supplemental Fig. 1a). Viral genomic input into sequencing amplicons was determined empirically and ranged between 1×10^3 and 3×10^6 genomes (see supplemental Fig. 1b), representing orders of magnitude greater population sampling than conventional clonal sequencing.

As previously described [15], the vast majority of variation in the NS3 protease region was detected at silent sites. Besides a minor fraction ($< 2.2\%$) of D168A mutants in the viral population of one mouse (**Fig. 1i**), PI-RAVs were not detectable at baseline in the remaining 11 animals (**Fig. 1**). However, in all mice of the monotherapy cohort that experienced a viral breakthrough, PI-RAVs consistently became dominant during viral rebound (**Fig. 1a-e**). The most frequently identified mutation was the D168V substitution, a mutation known to confer resistance to Ciluprevir and other PIs [16 17]. The detection of D168V in monotherapy treated animals was correlated with viral breakthrough in all cases. In some animals, transient low-level R155Q and A156V/T RAVs were detectable at nadir, but these became undetectable following viral rebound (data not shown). This observation is not surprising since R155Q and A156V/T

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

mutations reduce the replicative capacity of the mutated virus to 10 and 18/30% of that of the wild type, while the fitness of the D168V mutant remains nearly unaffected[17]. In mice achieving EOT response upon PI-monotherapy, exclusively wild type sequences were detected (Fig. 1f and g). In the combination therapy cohort, however, treatment response did not correlate with the absence of detectable PI-RAVs. Indeed, although no on-therapy viral rebounds were observed, two mice presented with RAVs during therapy (Fig. 1h and i) and one developed a minor D168G population (3.1%) at EOT (Fig. 1j). Therefore, the occurrence of RAV D168V was not correlated with viral breakthrough in the dual therapy arm, indicating the viremia remained controlled, independent of PI-RAVs development, as long as the entry inhibitor was co-administered.

Discussion

Our results demonstrate that during PI treatment, plasma HCV RNA levels declined in two phases: an initial rapid decline followed by a more gradual one. During the first two weeks of treatment the mono- and combination therapy arms had a similar reduction in median viremia, whereas during a second phase the median viremia diverged markedly between these two treatment groups. This suggests that addition of an entry inhibitor is probably not increasing the antiviral effect of the DAA during the early phase of therapy. Accordingly, our SR-BI antibody did not significantly influence the viremia in chronically infected control animals (**Fig. 3**). In two recent publications it was shown that monotherapy with other entry inhibitors, an antibody against claudin-1 and an anti-envelope antibody, had antiviral activity in a setting of chronic HCV [18 19]. The cause of this potential discrepancy is currently under investigation but may be related to distinct characteristics of the viral strains used or to differences in the mode-of-action between these entry inhibitors.

Nevertheless, mAb1671 appeared to strongly affect the second phase of viral decline, which was greater and more sustained in mice that received Ciluprevir in combination with mAb1671 than in mice that received Ciluprevir alone. Moreover, during the second phase, the animals from the single therapy group experienced a rebound in median viral titer rather than a continued viral decline (**Fig. 2**). Indeed, the median change in plasma RNA levels from day 14 to day 42 was -0.59-log_{10} and $+2.12\text{-log}_{10}$ for the combination and single therapy group respectively. This difference can be attributed to viral breakthroughs that were solely observed in the PI monotherapy group.

Comparison of the two groups over the complete treatment period clearly indicates the superiority of the combination over the monotherapy regimen. Since our entry inhibitor alone has no effect on the viral load in chronically infected animals, its observed additional antiviral effect must depend on another function. We have previously shown that mAbs against SR-BI can efficiently inhibit intrahepatic viral spread [8 9]. Therefore, we hypothesize that this entry inhibitor is able to protect cured cells from re-infection and prevent the spread of therapy resistant variants, thereby precluding their on-therapy breakthrough. Indeed, during PI monotherapy, rapid emergence of PI-resistant variants occurred that resulted in their immediate breakthrough (**Fig. 1a-e**). In clear contrast, viral breakthrough was not observed in any of the animals receiving combination therapy (**Fig. 1h-l**), not even in those in which the D168V mutation was detected during therapy (**Fig. 1h and i**).

Of note, despite the absence of PI-resistant variants at baseline in all but one mouse, we cannot exclude that these were not present at frequencies below the limit of detection of our deep sequencing detection method [20]. Besides the selection of pre-existing resistant viruses, resistance mutations can rapidly be acquired by wild type virus during therapy[21].

Until now, most companies have been developing DAAs that specifically target the non-structural proteins of HCV. The rationale for this is that such an approach is more targeted causing fewer side effects than when host cell proteins are targeted, where the natural function could become impaired. Nevertheless, a monoclonal antibody very similar to mAb1671 targeting the host cell receptor SR-BI, was shown to be safe in macaques[22]. In addition, evidence for clinical safety of SR-BI inhibition is provided by studies involving the SR-BI-specific small molecule inhibitor ITX-5061[23 24]. The more conserved nature of host factors compared to the viral proteins makes the former interesting potential therapeutic targets. Although also different

HCV variants have been described that are less dependent on SR-BI for cell entry *in vitro*, we recently demonstrated that the anti-SR-BI mAb1671 remains fully effective against such viruses *in vivo*, which could be explained by the increased efficacy of the antibody therapy in the presence of human lipoproteins[10].

It is assumed that entry inhibitors for HCV could be used as a constituent of a drug cocktail together with specifically targeted DAAs, comparable to the use of Maraviroc and Enfuvirtide for the treatment of HIV-1 infections[25]. Therefore it would be interesting to determine how these different drug classes interact or cooperate in the context of HCV infection. HCV entry inhibitors have additive to synergistic antiviral activity to DAAs *in vitro*[26-28] and do not develop cross-resistance with DAAs[29]. One small pilot study suggests synergistic activity of an SR-BI mAb in combination with a DAA in human-liver mice[26].

Although mAb1671 is clearly able to prevent on-therapy viral rebound, SVR was not seen in either treatment group indicating that a longer treatment regimen is required to achieve viral clearance, especially in immune deficient hosts like the SCID mice used here. Considering the recent availability of highly potent anti-HCV DAA combinations, it is foreseeable that the clinical applicability of entry inhibition in the context of HCV infection may initially be limited to specific, difficult-to-treat patient populations. Recent reports highlight that RAVs may still have an impact on the outcome of the newest interferon-free DAA combination therapies. Forns et al. reported that patients with a history of relapse after PEG-IFN/RBV/PI therapy and that harbored NS3 and/or NS5A RAVs at baseline responded less well to Grazoprevir-Elbasvir combination therapy compared to patients without these RAVs (66,7-91,2% versus 100%)[30]. In addition, new RAVs emerged at the NS3 or NS5A loci in the three patients that experienced virologic failure. Lawitz and colleagues reported that the success of a prolonged retreatment of patients, who previously failed Ledipasvir/Sofosbuvir-based regimens, with Ledipasvir/Sofosbuvir was heavily impacted by the baseline presence of NS5A RAVs (50-69% SVR12 in patients with one or more RAVs vs. 100% SVR12 in patients without RAVs)[31]. Interestingly, although none of the patients had baseline NS5B RAVs, 33% of patients that experienced virologic failure had developed one or two NS5B RAVs (S282T and/or L159F). Similarly Poordad et al., showed that about 30% of patients that failed a short-term Grazoprevir/Elbasvir/Sofosbuvir treatment had emerging NS5A RAVs at virologic failure[32]. NS5A RAVs also emerged in the majority of both advanced cirrhotic and post-transplant patients that relapsed after Sofosbuvir/Daclatasvir/RBV therapy[33]. Importantly, in contrast to NS3 RAVs, NS5A RAVs tend to persist at high frequency and for a long time (>96 weeks) after cessation of therapy, hampering subsequent treatment with NS5A-specific inhibitors[34].

In summary, our study shows that SR-BI blockade via mAb1671 administration limits breakthrough of PI-RAVs during PI therapy. These data thus demonstrate the effectiveness of an entry inhibitor targeting a host cell receptor, administered in combination with DAAs, at controlling HCV viremia in a pre-clinical small animal model. Clinical safety and efficacy studies are needed to evaluate whether addition of an entry inhibitor to a cocktail comprised of solely DAAs would suffice to reach SVR, without the need of IFN and RBV.

Our therapeutic approach could also be utilized in the event of global dissemination of therapy-induced DAA resistant viruses. In a broader context and similar to HIV-1, an analogous strategy could be implemented for the treatment of other viruses including hepatitis B virus, measles

virus or human T-lymphotropic virus type 1 (HTLV-1), which may also use spread to neighboring host cells as a means to propagate drug resistance[25 35].

Confidential: For Review Only

Materials and Methods

In vivo HCV treatment

Human liver-uPA-SCID mice (chimeric mice) were produced as previously described[11 12 36 37]. All mice were transplanted with primary human hepatocytes obtained from a single donor (donor HH223; BD Biosciences, Erembodegem, Belgium). The effect of the protease inhibitor, Ciluprevir, was evaluated in 12 human-liver mice that were infected with a clinical HCV isolate of gt1b. Virus was inoculated at least four weeks prior to start of therapy. All mice had over 2 mg/ml of human albumin in their plasma at time of infection. Seven of these mice received Ciluprevir monotherapy while five mice were treated with mAb1671 (produced as described[38]) in addition to the protease inhibitor. Ciluprevir was administered by oral gavage twice daily at 10 mg/kg for a maximum period of six weeks; two mice were treated for only 5 weeks (Fig. 1d and 1l). In total 14 intraperitoneal (IP) injections of mAb1671 were given (400 µg per injection) on day 0, 2, 4, 7, 10, 13, 16, 20, 23, 27, 30, 34, 37, 41. One mouse only received the first 12 injections (Fig. 1l). Two mice in the monotherapy cohort did not reach the end-of treatment (EOT) time point; one was found dead (Fig. 1e, latest available time point: day 20) and the other was sacrificed (Fig. 1b, latest available time point: day 15) for liver and blood collection. In addition, the effect of mAb1671 monotherapy was assessed. Three gt1b infected mice received a 4-week mAb1671 therapy consisting of three IP injections per week (400µg per injection at day 0, 2, 4, 7, 9, 11, 14, 16, 18, 21, 23 and 25). For most infections, a serum-derived virus was used that had been isolated from an HCV-infected liver transplant patient (genotype 1b; P05[39]). Two mice (Fig. 1j and one mouse in Fig. 3) were infected with another gt1b patient isolate. HCV RNA in plasma was quantified using the COBAS Ampliprep/COBAS TaqMan HCV test (Roche Diagnostics, Belgium). Due to dilution of the mouse plasma, the limit of quantification (LOQ = 100% LOD) equaled 750 IU/ml. The study protocol was approved by the Animal Ethics Committee of the Faculty of Medicine and Health Sciences of the Ghent University.

Identification of HCV Ciluprevir-resistant mutations: deep sequencing

RNA extraction and cDNA synthesis

Viral RNA was extracted from mouse EDTA-plasma samples using a MiniElute Viral RNA Spin Kit (Qiagen) according to the manufacturers instructions. Viral RNA was eluted in 30µL buffer AVE and stored at -80°C. Viral RNA (8 µL) was reverse transcribed using the SuperScript III First Strand Synthesis System (Invitrogen) according to the manufacturer's instructions for gene-specific primer (GSP) cDNA synthesis utilising a subtype 1b-specific primer at 20 µM (5' GCTTTTGCCGCTGCCRGTTGG -3'). The 20µL total cDNA synthesis reaction was incubated at 50°C for 1 hour followed by 42°C for 1 hour, prior to heat inactivation at 85°C for 5 min. Input viral RNA was then digested away from cDNA/RNA hybrids using RNase H for 20 min at 37°C. Viral cDNA was stored at -20°C prior to amplicon generation.

Amplicon generation

Amplicon generation for Illumina sequencing was achieved via nested PCR and was performed as previously described[15], replacing subtype 1a-specific primers pairs with 1b-specific primers pairs. Briefly, 1st round amplifications were performed in 50 µl volumes containing 0.2 µM of each 1st round primer (sense primer: 5'-GGACATCATYTYGGGYTRC-3'; antisense primer: 5'-GCTTTTGCCGCTGCCRGTTGG -3'), 1.5 U Platinum Taq High Fidelity polymerase

(Invitrogen), 1x High Fidelity polymerase buffer, 2 mM MgSO₄ and 5 µl of viral cDNA. Cycling parameters comprised initial denaturation at 94°C for 2 minutes followed by ×35 cycles of 94°C for 15 seconds, 50°C for 30 seconds and 68°C for 2 minutes, with a final extension step at 68°C for 10 minutes. Second round amplifications were performed using identical conditions to the 1st round, except 5 µl of 1st round product was used as template and 2nd round primers replaced 1st round primers (sense primer: 5'- GGGGTGGAAGCTCCTYGCGCC -3'; antisense primer: 5'- ACTTGRAATGTCTGCGGTAC -3'). Cycling parameters for 2nd round amplification were identical to the 1st round with minor modifications: cycle number was increased to ×45, a touchdown methodology was employed where annealing temperature was gradually reduced from 58°C to 48°C over the course of the ×45 cycles and the annealing time was reduced from 2 minutes to 1.5 minutes. Amplicons were cleaned-up using QIAquick PCR purification columns (Qiagen) and sequenced using BigDye™ v3.1 (Applied Biosystems) prior to Illumina sequencing.

5' NCR Quantitative PCR

To determine viral cDNA input into Illumina sequencing amplicons, quantitative realtime PCR amplification of viral cDNA was performed using a Stratagene MX3005p instrument in 20 µl volumes containing 0.2 µM forward (5' - GCGCAACCGGTGAGTACA -3') and reverse (5' - ACTCGCAAGCACCTATCAG -3') primers, 1.0 µl viral cDNA and 10 µl of 2X SYBR Green JumpStart Taq ReadyMix (Sigma) reagent with additional ROX reference dye as per the manufacturer's instructions. Ten-fold serial dilutions of the HCV molecular clone JFH-1 from 10⁶ to 10¹ copies were co-amplified to generate a standard curve for absolute quantification. All samples, standards and negative controls were repeated in triplicate. Cycling conditions comprised initial denaturation at 95°C for 2 minutes, followed by 50 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 30 seconds, with a further denaturation for 1 minute at 95 °C followed by a temperature gradient from 55 °C to 95 °C at 30 seconds / °C to determine amplicon melt peaks. Data was analysed using MxPro software (Stratagene) to determine product quantity and specificity.

Illumina MiSeq Sequencing and Data Analysis

Sequencing library preparation was then performed using TruSeq DNA PCR-Free Sample Preparation Kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. Prior to Illumina MiSeq sequencing, quality control of libraries was performed with an Agilent Bioanalyzer HS Chip (Agilent Technologies) and quantification performed using qPCR (KAPA Library Quantification Kit). Bidirectional sequencing of fragmented amplicon libraries was then performed (300 cycles) using a MiSeq Reagent Kit v3 (Illumina Inc.) according to the manufacturer's protocol. Generated sequence data was analyzed as previously described[15], with minor modifications: read quality checking, contig assembly against a reference sequence, coverage plotting and population variant calling were performed using CLC Genomics Workbench Version 8 (Qiagen).

Graphs and statistics

All graphs were prepared with GraphPad Prism v5 (GraphPad Software Inc., La Jolla, CA, USA). To analyze whether differences in median viremia were statistically significant, the unpaired nonparametric two-tailed Mann-Whitney test was performed using GraphPad InStat v3.06 (GraphPad Software Inc.).

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Funding.

This project was funded by the Ghent University (Concerted Action Grant 01G01712), The Research Foundation – Flanders (FWO-Vlaanderen; project 1.5.009.10N), the Belgian state (IUAP P7/47-HEPRO-2), and the European Union (FP7, HepaMab). T.P. was supported by grants from the DFG (PI 734/2-1 and CRC 900 project A6), the Helmholtz Association SO-024 and the ERC (ERC-2011-StG_281473-VIRAFRONT). AAM is a recipient of a PhD Fellowship provided by the Egyptian Government.

Competing interests.

Dr. Cortese and Dr. Nicosia are listed as inventors on the patent of the anti-SR-BI antibody used in this work and are shareholders of JVBio srl, a company that has commercial rights to these patents.

Author contributions.

P.M., G.L-R. and T.P. initiated and supervised the study. K.V. and P.M. designed *in vivo* experiments and analyzed data. P.M., K.V., T.P. and R.J.P.B. designed deep sequencing analyses. R.J.P.B., J.D., S.B., R.G.,C.P.M and J.K.B performed deep sequencing experiments and analyzed data. K.V., A.A.M. and L.V. performed *in vivo* experiments. A.F. and N.V.D.E. performed *ex vivo* experiments and analyses. A.N., R.C., F.T. and T.B. provided key reagents. K.V., R.J.P.B and P.M. wrote the manuscript.

Figure legends:

Fig. 1. HCV viremia and protease population variant composition at residue 168 prior to, during and following Ciluprevir therapy with and without the addition of an entry inhibitor (the SR-BI-specific antibody mAb1671) in human-liver mice. Twelve gt1b-infected mice were divided over two study groups, each panel represents a single animal. Seven received Ciluprevir monotherapy (**a-g**, shown in red), and five received the combination of Ciluprevir with mAb1671 (**h-l**, shown in green). The shaded area indicates the duration of therapy. Each data point represents the plasma HCV RNA level (IU/ml) of an individual subject at a given time. The limit of quantification (LOQ) equals 750 IU/ml. Illumina deep-sequencing analysis of the HCV protease domain in NS3 was performed at indicated time points (black and red arrows indicate successful and unsuccessful generation of sequencing amplicons respectively). The amino acid population frequency at position 168 is presented as horizontal bars accompanying the graphs. The wild type amino acid, Aspartic acid (D), is represented in blue, whereas mutants have been assigned other colors.

Fig. 2. Comparison of median viremia between day -1, 14 and 42 in Ciluprevir monotherapy (**a**) and Ciluprevir/mAb1671 combination (**b**) therapy groups. Day -1 represents baseline viremia prior to start of therapy. End-of-therapy (day 42) viremias are available for five mice in each group. The Whiskers contain all values. P values compare median viremias between baseline and end-of-therapy in one treatment group using the unpaired nonparametric two-tailed Mann-Whitney test.

Fig. 3. Evaluation of the antiviral effect of the SR-BI-specific antibody mAb1671 alone in humanized mice chronically infected with gt1b. Three gt1b infected mice received mAb1671 three times weekly during four weeks. Each data point represents the plasma HCV RNA level (IU/ml) of an individual chimeric mouse at a given time point. The limit of quantification (LOQ) equals 750 IU/ml.

Reference list:

1. Hajarizadeh B, Grebely J, Dore GJ. Epidemiology and natural history of HCV infection. *Nat Rev Gastroenterol Hepatol* 2013;**10**(9):553-62 doi: 10.1038/nrgastro.2013.107[published Online First: Epub Date].

2. Pawlotsky JM. Treatment failure and resistance with direct-acting antiviral drugs against hepatitis C virus. *Hepatology* 2011;**53**(5):1742-51 doi: 10.1002/hep.24262[published Online First: Epub Date].

3. Chung RT, Baumert TF. Curing chronic hepatitis C--the arc of a medical triumph. *N Engl J Med* 2014;**370**(17):1576-8 doi: 10.1056/NEJMp1400986[published Online First: Epub Date].

4. Kieffer TL, Sarrazin C, Miller JS, et al. Telaprevir and pegylated interferon-alpha-2a inhibit wild-type and resistant genotype 1 hepatitis C virus replication in patients. *Hepatology* 2007;**46**(3):631-9 doi: 10.1002/hep.21781[published Online First: Epub Date].

5. Susser S, Welsch C, Wang Y, et al. Characterization of resistance to the protease inhibitor boceprevir in hepatitis C virus-infected patients. *Hepatology* 2009;**50**(6):1709-18 doi: 10.1002/hep.23192[published Online First: Epub Date].

6. Feld JJ, Kowdley KV, Coakley E, et al. Treatment of HCV with ABT-450/r-ombitasvir and dasabuvir with ribavirin. *N Engl J Med* 2014;**370**(17):1594-603 doi: 10.1056/NEJMoa1315722[published Online First: Epub Date].

7. Scarselli E, Ansuini H, Cerino R, et al. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J* 2002;**21**(19):5017-25

8. Meuleman P, Catanese MT, Verhoye L, et al. A human monoclonal antibody targeting scavenger receptor class B type I precludes hepatitis C virus infection and viral spread in vitro and in vivo. *Hepatology* 2012;**55**(2):364-72 doi: 10.1002/hep.24692[published Online First: Epub Date].

9. Lacek K, Vercauteren K, Grzyb K, et al. Novel human SR-BI antibodies prevent infection and dissemination of HCV in vitro and in humanized mice. *Journal of hepatology* 2012;**57**(1):17-23 doi: 10.1016/j.jhep.2012.02.018[published Online First: Epub Date].

10. Vercauteren K, Van Den Eede N, Mesalam AA, et al. Successful anti-scavenger receptor class B type I (SR-BI) monoclonal antibody therapy in humanized mice after challenge with HCV variants with in vitro resistance to SR-BI-targeting agents. *Hepatology* 2014;**60**(5):1508-18 doi: 10.1002/hep.27196[published Online First: Epub Date].

11. Meuleman P, Libbrecht L, De Vos R, et al. Morphological and biochemical characterization of a human liver in a uPA-SCID mouse chimera. *Hepatology* 2005;**41**(4):847-56 doi: 10.1002/hep.20657[published Online First: Epub Date].

12. Mercer DF, Schiller DE, Elliott JF, et al. Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* 2001;**7**(8):927-33 doi: 10.1038/90968[published Online First: Epub Date].

13. Vercauteren K, de Jong YP, Meuleman P. Animal models for the study of HCV. *Current opinion in virology* 2015;**13**:67-74 doi: 10.1016/j.coviro.2015.04.009[published Online First: Epub Date].

14. Vercauteren K, de Jong YP, Meuleman P. HCV animal models and liver disease. *Journal of hepatology* 2014;**61**(1 Suppl):S26-33 doi: 10.1016/j.jhep.2014.07.013[published Online First: Epub Date].

15. Irving WL, Rupp D, McClure CP, et al. Development of a high-throughput pyrosequencing assay for monitoring temporal evolution and resistance associated variant emergence in the Hepatitis C virus protease coding-region. *Antiviral research* 2014;**110**:52-9 doi: 10.1016/j.antiviral.2014.07.009[published Online First: Epub Date].

16. Verbinen T, Van Marck H, Vandenbroucke I, et al. Tracking the evolution of multiple in vitro hepatitis C virus replicon variants under protease inhibitor selection pressure by 454 deep sequencing. *Journal of virology* 2010;**84**(21):11124-33 doi: 10.1128/JVI.01217-10[published Online First: Epub Date].

17. Lenz O, Verbinen T, Lin TI, et al. In vitro resistance profile of the hepatitis C virus NS3/4A protease inhibitor TMC435. *Antimicrobial agents and chemotherapy* 2010;**54**(5):1878-87 doi: 10.1128/AAC.01452-09[published Online First: Epub Date].

18. Mailly L, Xiao F, Lupberger J, et al. Clearance of persistent hepatitis C virus infection in humanized mice using a claudin-1-targeting monoclonal antibody. *Nature biotechnology* 2015;**33**(5):549-54 doi: 10.1038/nbt.3179[published Online First: Epub Date].

19. de Jong YP, Dorner M, Mommersteeg MC, et al. Broadly neutralizing antibodies abrogate established hepatitis C virus infection. *Science translational medicine* 2014;**6**(254):254ra129 doi: 10.1126/scitranslmed.3009512[published Online First: Epub Date].

20. Rong LB, Dahari H, Ribeiro RM, et al. Rapid Emergence of Protease Inhibitor Resistance in Hepatitis C Virus. *Science translational medicine* 2010;**2**(30):30ra32 doi: ARTN 30ra32

- DOI 10.1126/scitranslmed.3000544[published Online First: Epub Date]].
21. Sato M, Maekawa S, Komatsu N, et al. Deep sequencing and phylogenetic analysis of variants resistant to interferon-based protease inhibitor therapy in chronic hepatitis induced by genotype 1b hepatitis C virus. *J Virol* 2015;**89**(11):6105-16 doi: 10.1128/JVI.03127-14[published Online First: Epub Date]].
22. Flores MV, Corbau RG, Guionaud S. Pharmacokinetics and safety in cynomolgus monkeys of a monoclonal antibody targeting human scavenger receptor class B type-1 for hepatitis C treatment. *Antiviral therapy* 2013;**18**(6):775-84 doi: 10.3851/IMP2570[published Online First: Epub Date]].
23. Masson D, Koseki M, Ishibashi M, et al. Increased HDL cholesterol and apoA-I in humans and mice treated with a novel SR-BI inhibitor. *Arterioscler Thromb Vasc Biol* 2009;**29**(12):2054-60 doi: 10.1161/ATVBAHA.109.191320[published Online First: Epub Date]].
24. Wong-Staal F, Syder AJ, McKelvy JF. Targeting HCV entry for development of therapeutics. *Viruses* 2010;**2**(8):1718-33 doi: 10.3390/v2081718[published Online First: Epub Date]].
25. Haqqani AA, Tilton JC. Entry inhibitors and their use in the treatment of HIV-1 infection. *Antiviral research* 2013;**98**(2):158-70 doi: 10.1016/j.antiviral.2013.03.017[published Online First: Epub Date]].
26. Xiao F, Fofana I, Thumann C, et al. Synergy of entry inhibitors with direct-acting antivirals uncovers novel combinations for prevention and treatment of hepatitis C. *Gut* 2014 doi: 10.1136/gutjnl-2013-306155[published Online First: Epub Date]].
27. Uprichard SL, Sainz B. Inhibition of hepatitis C entry: too soon to dismiss while many are still being denied treatment. *Gut* 2014 doi: 10.1136/gutjnl-2014-308396[published Online First: Epub Date]].
28. Xiao F, Fofana I, Heydmann L, et al. Hepatitis C virus cell-cell transmission and resistance to direct-acting antiviral agents. *PLoS pathogens* 2014;**10**(5):e1004128 doi: 10.1371/journal.ppat.1004128[published Online First: Epub Date]].
29. Zhu H, Wong-Staal F, Lee H, et al. Evaluation of ITX 5061, a scavenger receptor B1 antagonist: resistance selection and activity in combination with other hepatitis C virus antivirals. *The Journal of infectious diseases* 2012;**205**(4):656-62 doi: 10.1093/infdis/jir802[published Online First: Epub Date]].
30. Forns X, Gordon SC, Zuckerman E, et al. Grazoprevir/Elbasvir plus Ribavirin For Chronic HCV Genotype-1 Infection After Failure of Combination Therapy Containing a Direct-Acting Antiviral Agent. *J Hepatol*. 2015 doi: 10.1016/j.jhep.2015.04.009[published Online First: Epub Date]].
31. Lawitz E, Flamm S, Yang JC, et al. RETREATMENT OF PATIENTS WHO FAILED 8 OR 12 WEEKS OF LEDIPASVIR/SOFOSBUVIR-BASED REGIMENS WITH LEDIPASVIR/SOFOSBUVIR FOR 24 WEEKS. *J Hepatol*. 2015;**vol.62:S192**
- abstract # O005**
32. Poordad F, Lawitz E, Gutierrez JA, et al. C-SWIFT: GRAZOPREVR/ELBASVIR + SOFOSBUVIR IN CIRRHOTIC AND NONCIRRHOTIC, TREATMENT-NAIVE PATIENTS WITH HEPATITIS C VIRUS GENOTYPE 1 INFECTION, FOR DURATIONS OF 4, 6 OR 8 WEEKS AND GENOTYPE 3 INFECTION FOR DURATIONS OF 8 OR 12 WEEKS. *Journal of hepatology* 2015;**2015, vol.62:S192**
- abstract # O006**
33. Poordad F, Schiff ER, Vierling JM, et al. DACLATASVIR, SOFOSBUVIR, AND RIBAVIRIN COMBINATION FOR HCV PATIENTS WITH ADVANCED CIRRHOSIS OR POSTTRANSPLANT RECURRENCE: PHASE 3 ALLY-1 STUDY. 2015;**vol.62:S261**
- abstract # LO8**
34. Dvory-Sobol H, Wyles D, Ouyang W, et al. LONG-TERM PERSISTENCE OF HCV NS5A VARIANTS AFTER TREATMENT WITH NS5A INHIBITOR LEDIPASVIR. *Journal of hepatology* 2015;**vol.62:S221**
- abstract O059**
35. Petersen J, Dandri M, Mier W, et al. Prevention of hepatitis B virus infection in vivo by entry inhibitors derived from the large envelope protein. *Nature biotechnology* 2008;**26**(3):335-41 doi: 10.1038/nbt1389[published Online First: Epub Date]].
36. Meuleman P, Vanlandschoot P, Leroux-Roels G. A simple and rapid method to determine the zygosity of uPA-transgenic SCID mice. *Biochemical and biophysical research communications* 2003;**308**(2):375-8
37. Vanwolleghem T, Libbrecht L, Hansen BE, et al. Factors determining successful engraftment of hepatocytes and susceptibility to hepatitis B and C virus infection in uPA-SCID mice. *J Hepatol* 2010;**53**(3):468-76 doi: 10.1016/j.jhep.2010.03.024[published Online First: Epub Date]].
38. Catanese MT, Graziani R, von Hahn T, et al. High-avidity monoclonal antibodies against the human scavenger class B type I receptor efficiently block hepatitis C virus infection in the presence of high-density

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

lipoprotein. Journal of virology 2007;**81**(15):8063-71 doi: 10.1128/JVI.00193-07[published Online First: Epub Date]].

39. Fafi-Kremer S, Fofana I, Soulier E, et al. Viral entry and escape from antibody-mediated neutralization influence hepatitis C virus reinfection in liver transplantation. The Journal of experimental medicine 2010;**207**(9):2019-31 doi: jem.20090766 [pii] 10.1084/jem.20090766[published Online First: Epub Date]].

Confidential: For Review Only

278x192mm (300 x 300 DPI)

99x105mm (300 x 300 DPI)

173x99mm (300 x 300 DPI)

79x41mm (300 x 300 DPI)