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

Koen Vercauteren,1 Richard J P Brown,2 Ahmed Atef Mesalam,1 Juliane Doerrbecker,2 Sabin Bhuju,3 Robert Geffers,3 Naomi Van Den Eede,1 C Patrick McClure,4 Fulvia Troise,5 Lieven Verhoye,1 Thomas Baumert,6,7 Ali Farhoudi,1 Riccardo Cortese,5 Jonathan K Ball,4 Geert Leroux-Roels,1 Thomas Pietschmann,2,8 Alfredo Nicosia,5,9 Philip Meuleman1

ABSTRACT

Objective Direct-acting antivirals (DAAs) inhibit hepatitis C virus (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 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-scavenger receptor class B type I 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 viraemia 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.

Conclusions This study provides preclinical in vivo evidence 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.

INTRODUCTION

Approximately 3% of the world’s population is chronically infected with 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

What is already known on this subject?

▸ Direct-acting antiviral (DAA) monotherapy selects therapy-resistant HCV variants leading to therapy failure.

▸ Resistance-associated variants (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 variants.

What are the new findings?

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

▸ Humanised 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.

How might it 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 used 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 neighbouring host cells as a means to propagate drug resistance.

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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.\textsuperscript{2, 3} While the first generation of licensed protease inhibitors (PIs)\textsuperscript{8} possessed low barriers to resistance in combination with PEG-IFN/RBV, improved sustained virological 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,\textsuperscript{4} 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.\textsuperscript{7} SR-BI targeting monoclonal antibodies (mAbs), and specifically ‘mAb1671’, have been shown to possess cross-genotypic anti-HCV activity in human-liver mice\textsuperscript{8, 9} and to inhibit cell-to-cell transmission in vitro and intrahepatic spread in vivo.\textsuperscript{10} By potently inhibiting the spread of resistant variants selected during DAA therapy, anti-SR-BI mAbs may be able to prevent on-therapy breakthrough of resistance-associated variants (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\textsuperscript{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.\textsuperscript{13, 14} A productive viraemia 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 virological response during mono and combination therapy

During PI monotherapy, five out of seven mice experienced viral breakthrough (defined by a $>10$-fold increase in HCV RNA over nadir, or when viral RNA becomes quantifiable after having been below the limit of quantification (LOQ)) (figure 1A–G). End-of-treatment (EOT) response (HCV RNA below LOQ at day 42) was observed in only two mice (figure 1F, G). In the combination therapy arm, no viral breakthrough was observed, with EOT responses in four out of five mice (figure 1I–L).

The mono and combination therapy arms had similar median viral loads at baseline ($p > 0.99$) and comparable reductions in median viraemia during the first two weeks of treatment ($-2.17 \times \log_{10}$ and $-1.86 \times \log_{10}$, respectively) (figure 2). However, the median viraemia 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 (figure 2A), the median viraemia at EOT was significantly reduced in the combination therapy group ($p = 0.0079$) (figure 2B).

In order to determine whether mAb1671 exerts an antiviral effect as such during chronic infection, three gt1b infected chimeric mice received mAb1671 monotherapy over a 4-week period (figure 3). Although a subtle downwards trend might be perceivable, the HCV RNA levels in the plasma of these mice did not fluctuate more than what is routinely observed in non-treated infected animals. In addition, a 2-week follow-up did not reveal a general rebound after cessation of therapy, underscoring the absence of a pronounced antiviral effect of mAb1671 during chronic infection.

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 online supplementary figure S1A). Viral genomic input into sequencing amplicons was determined empirically and ranged between $1 \times 10^7$ and $3 \times 10^8$ genomes (see online supplementary figure S1B), representing orders of magnitude greater population sampling than conventional clonal sequencing.

As previously described,\textsuperscript{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 (figure 1I), PI-RAVs were not detectable at baseline in the remaining 11 animals (figure 1). However, in all mice of the monotherapy cohort that experienced a viral breakthrough, PI-RAVs consistently became dominant during viral rebound (figure 1A–E). The most frequently identified mutation was the D168V substitution, a mutation known to confer resistance to ciluprevir and other PIs.\textsuperscript{16, 17} The detection of D168V in monotherapy-treated animals 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 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.\textsuperscript{17} In mice achieving EOT response upon PI monotherapy, exclusively wild-type sequences were detected (figure 1F, 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 (figure 1H, I) and one developed a minor D168G population (3.1%) at EOT (figure 1J). Therefore, the occurrence of RAV D168V did not lead to viral breakthrough in the dual therapy arm, indicating the viraemia remained controlled, independent of PI-RAVs development, as long as the entry inhibitor was co-administered.

DISCUSSION

In the absence of on-therapy viral breakthrough (figure 1F–L), plasma HCV RNA levels declined in two phases: an initial rapid decline followed by a more gradual one, as has been observed and modelled before.\textsuperscript{18} During the first two weeks of treatment, the mono and combination therapy arms had a similar reduction in median viraemia, whereas during the second phase the median viraemia diverged markedly between these two treatment groups (figure 2). This suggests that addition of an entry inhibitor does not increase the antiviral effect of the DAA during the early phase of therapy. Accordingly, our SR-BI antibody did not markedly influence the viraemia in chronically infected control animals (figure 3). In two recent publications however 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. The cause of this 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 in general experienced a rebound in median viral titre rather than a continued viral decline (figure 2). Indeed, the median change in 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 colours.

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A 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 meaningful effect on the viral load in chronically infected animals within 4 weeks of therapy, its observed additional antiviral effect must depend on another function. We have previously shown that mAbs against SR-BI, and specifically also mAb1671, can efficiently inhibit intrahepatic viral spread. Therefore, we hypothesise that this entry inhibitor is able to protect cured cells from reinfection 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 (figure 1A–E). In clear contrast, viral breakthrough was not observed in any of the animals receiving combination therapy (figure 1H–L), not even in those in which the D168V mutation was detected during therapy (figure 1H, 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. Besides the selection of pre-existing resistant viruses, resistance mutations can rapidly be acquired by wild-type virus during therapy.

Until now, most companies have been developing DAAs that specifically target the non-structural proteins of HCV. The
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Figure 2 Comparison of median viraemia between day −1, 14 and 42 in ciluprevir monotherapy (A) and ciluprevir/mAb1671 combination (B) therapy groups. Day −1 represents baseline viraemia prior to start of therapy. End-of-therapy (day 42) viraemias are available for five mice in each group. The Whiskers contain all values. p Values compare median viraemias between baseline and end of therapy in one treatment group using the unpaired non-parametric two-tailed Mann–Whitney test.

Figure 3 Evaluation of the antiviral effect of the scavenger receptor class B type I-specific antibody mAb1671 alone in humanised 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.

DAAs, comparable to the use of maraviroc and enfuvirtide for the treatment of HIV-1 infections. 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 and do not develop cross-resistance with DAAs. One small pilot study suggests synergistic activity of an SR-BI mAb in combination with a DAA in human-liver mice.

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 severe combined immune deficient (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 harboured NS3 and/or NS5A RAVs at baseline responded less well to grazoprevir–elbasvir combination therapy compared with patients without these RAVs (66.7–91.2% vs 100%). In addition, new RAVs emerged at the NS3 or NS5A loci in the three patients that experienced virological 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).

Interestingly, although none of the patients had baseline NS5B RAVs, 33% of patients that experienced virological 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 virological failure. NS5A RAVs also emerged in the majority of both advanced cirrhotic and post-transplant patients that relapsed after sofosbuvir/daclatasvir/RBV therapy. 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.

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 viraemia in a preclinical 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 used 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, which may also use spread to neighbouring host cells as a means to propagate drug resistance.

MATERIALS AND METHODS

In vivo HCV treatment

Human liver-uPA-SCID mice (chimeric mice) were produced as previously described. All mice were transplanted with...
primary human hepatocytes obtained from a single donor (donor HH223; BD Biosciences, Erembodegem, Belgium). The effect of the PI, 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 >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 in ref. 38) in addition to the PI. Ciluprevir was administered by oral gavage twice daily at 10 mg/kg for a maximum period of 6 weeks; two mice were treated for only 5 weeks (figure 1D, L). In total, 14 intraperitoneal injections of mAb1671 were given (400 μg per injection) on days 0, 2, 4, 7, 10, 13, 16, 20, 23, 27, 30, 34, 37 and 41. One mouse only received the first 12 injections (figure 1L). Two mice in the monotherapy cohort did not reach the EOT time point; one was found dead (figure 1E, latest available time point: day 20) and the other was sacrificed (figure 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 intraperitoneal injections per week (400 μg per injection at days 0, 2, 4, 7, 9, 11, 14, 16, 18, 21, 23 and 25) (figure 3). For most infections, a serum-derived virus was used that had been isolated from an HCV-infected liver transplant patient (genotype 1b; P0539). Two mice (figure 1J and one mouse in figure 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 LOQ (= 100% limit of quantitation) equalled 750 IU/mL.

**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 manufacturer’s instructions. Viral RNA was eluted in 30 μL 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 cDNA synthesis using a subtype 1b-specific primer at 20 μM (5′-GCTTTCGCGGCTGCRGTGGGG-3′). The 20 μL total cDNA synthesis reaction was incubated at 50°C for 1 h followed by 42°C for 1 h, 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,13 replacing subtype 1a-specific primers pairs with 1b-specific primers pairs. Briefly, first-round amplifications were performed in 50 μL volumes containing 0.2 μM of each first-round primer (sense primer: 5′-GGACATCATYTYGGGYYTRC-3′; antisense primer: 5′-GCTTTTGCGGCTGCRGTGGGG-3′), 1.5 U Platinum Taq High Fidelity polymerase (Invitrogen), 1× high-fidelity polymerase buffer, 2 mM MgSO4 and 5 μL of viral cDNA. Cycling parameters comprised initial denaturation at 94°C for 2 min followed by ×35 cycles of 94°C for 15 s, 50°C for 30 s and 68°C for 2 min, with a final extension step at 68°C for 10 min. Second-round amplifications were performed using identical conditions to the first round, except 5 μL of first-round product was used as template and second-round primers replaced first-round primers (sense primer: 5′-GGGGTGGAAGCTCCTYGGGCC-3′; antisense primer: 5′-ACTTGRAATGTCTGGGTATC-3′). Cycling parameters for second-round amplification were identical to the first 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 min to 1.5 min. 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 amplifiers, quantitative real-time PCR amplification of viral cDNA was performed using a Stratagene MX3000P instrument in 20 μL volumes containing 0.2 μM forward (5′-GCGCAACCGGTGAGTTACA-3′) and reverse (5′-ACTTGGCAAGCACTCTATCCAG-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. Tenfold serial dilutions of the HCV molecular clone JFH-1 from 105 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 min, followed by 50 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, with a further denaturation for 1 min at 95°C, followed by a temperature gradient from 55°C to 95°C at 30 s/°C to determine ampli- con melt peaks. Data were analysed using MxPro software (Stratagene) to determine product quantity and specificity.

**Illumina MiSeq sequencing and data analysis**

Sequencing library preparation was then performed using TrueSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, California, 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) according to the manufacturer’s protocol. Generated sequence data were analysed as previously described,13 with minor modifications: read quality checking, contig assembly against a reference sequence, coverage plotting and population variant calling were performed using CLC Genomics Workbench V8 (Qiagen).

**Graphs and statistics**

All graphs were prepared with GraphPad Prism V5 (GraphPad Software, La Jolla, California, USA). To analyse whether differences in median viraemia were statistically significant, the unpaired non-parametric two-tailed Mann–Whitney test was performed using GraphPad InStat V3.06 (GraphPad Software).

**Author affiliations**

1Department Clinical Chemistry, Microbiology and Immunology, Center for Vaccinology, 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 (H2iZ), Hannover, Germany

3Genome Analytics, Helmholtz Centre for Infection Research, Braunschweig, Germany

4School of Life Sciences and the NIHR Nottingham Digestive Diseases Biomedical Research Unit, University of Nottingham, Queen’s Medical Centre, Nottingham, UK
Contributors PM, GL-R and TP initiated and supervised the study. KV and PM designed in vivo experiments and analysed data. PM, KV, TP and RJPB performed deep sequencing analyses. RJPB, JD, SB, RG, CPM and JKB performed deep sequencing experiments and analysed data. KV, AAM and LV performed in vivo experiments. AF and IVDE performed ex vivo experiments and analyses. AN, RC, FT and TB provided key reagents. KV, RJPB and PM wrote the manuscript.

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Competing interests RC and AN 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.

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