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Analysis of hepatitis C virus resistance to Silibinin *in vitro* and *in vivo* points to a novel mechanism involving nonstructural protein 4B

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Abstract

Intravenous silibinin (SIL) is an approved therapeutic that has recently been applied to patients with chronic hepatitis C, successfully clearing hepatitis C virus (HCV) infection in some patients even in monotherapy. Previous studies suggested multiple antiviral mechanisms of SIL, however, the dominant mode of action has not been determined. We first analyzed the impact of SIL on replication of subgenomic replicons from different HCV genotypes *in vitro* and found a strong inhibition of RNA replication for genotype 1a and genotype 1b. In contrast, RNA replication and infection of genotype 2a were minimally affected by SIL. To identify the viral target of SIL we analyzed resistance to SIL *in vitro* and *in vivo*. Selection for drug resistance in cell culture identified a mutation in HCV nonstructural protein (NS) 4B conferring partial resistance to SIL. This was corroborated by sequence analyses of HCV from a liver transplant recipient experiencing viral breakthrough under SIL monotherapy. Again, we identified distinct mutations affecting highly conserved amino acid residues within NS4B, which mediated phenotypic SIL resistance also *in vitro*. Analyses of chimeric viral genomes suggest that SIL might target an interaction between NS4B and NS3/4A. Ultrastructural studies revealed changes in the morphology of viral membrane alterations upon SIL treatment of a susceptible genotype 1b isolate, but not of a resistant NS4B mutant or genotype 2a, indicating that SIL might interfere with the formation of HCV replication sites.

Conclusion—Mutations conferring partial resistance to SIL treatment *in vivo* and in cell culture argue for a mechanism involving NS4B. This novel mode of action renders SIL an attractive

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candidate for combination therapies with other directly acting antiviral drugs, particularly in difficult-to-treat patient cohorts.

Keywords

HCV; NS4B; therapy; antiviral; Legalon-SIL; Silibinin

Worldwide about 170 million people are chronically infected with HCV, a positive-strand RNA virus belonging to the *Flaviviridae* family and leading to severe liver disease in many cases. Only 50% of the patients respond to therapy with pegylated IFNα (pegIFNα) + Ribavirin (RBV) for yet unknown reasons. The newly available direct acting antivirals (DAA) telaprevir and boceprevir enhance sustained viral response rates up to 70–75% during triple therapy (pegIFNα + RBV + DAA) of genotype 1 infected patients, but are accompanied by numerous and potentially severe adverse effects (1). Therefore there is still an urgent need for more potent and better tolerated therapeutic options.

Silymarin is an extract from the milk thistle plant (*Silybum marianum*) and contains a mixture of several compounds with silibinin (SbN) being the major component consisting of the two flavonolignans silybin A and silybin B. In contrast to the oral formulation (2), succinylated silibinin (Legalon-SIL® (SIL)), which is administered intravenously, has antiviral effects in chronic HCV patients. This mixture has been primarily used to prevent re-infection of the graft after liver transplantation (3–5) and for the treatment of IFNα/RBV nonresponders (6–9). Importantly, several individual case reports showed that patients could even be cured from HCV by SIL monotherapy with few adverse side effects, underlining the antiviral potency of this therapeutic (3–5). However, the mode of action (MOA) of SIL is currently under debate. Ahmed-Belkacem *et al.* (10) suggested that SIL targets viral RNA replication by direct inhibition of the HCV RNA dependent RNA polymerase (RdRp). Wagoner *et al.* (11) observed an efficient block of viral RNA replication of HCV genotype 1b (gt1b) replicons by SIL as well, accompanied by additional effects of SIL on viral entry as well as progeny virus particle production at very high SIL concentrations. In this study we aimed to clarify the MOA of SIL by analyzing the effect of SIL on the replication of different viral genotypes as well as emergence of SIL-resistant viruses *in vitro* and *in vivo*.

Experimental Procedures

Reagents

Legalon SIL® (SIL) (lyophilized, Madaus, Cologne) was resuspended to 28.5mg/ml (corresponding to 67.6mM) in sterile water and stored at −70°C. Further dilution was carried out in complete DMEM.

Cell culture and viruses

All amino acid and nucleotide numbers refer to the position of the corresponding amino acid in the complete HCV genomes of JFH1, Con1 and H77 (GenBank accession no. AB047639, AJ238799 and AF011751, respectively). Detailed information can be found in Supplementary Materials and Methods.

SIL-treatment

SIL was diluted in complete DMEM to concentrations ranging from 1μM to 1mM. In assays based on transient transfection of replicon RNA or stable replicon cells SIL treatment occurred 4h after seeding of the cells until cell lysis at 48h. In the infection assay with JcR-2a the drug was added together with the virus. At 6h post infection, medium was replaced with fresh medium containing SIL and incubated for 48h. In transfection assays for

immunofluorescence or electron microscopic analyses SIL was added immediately after transfection until fixation 24h later.

Amplification of HCV sequences from patients

HCV genomes were amplified in two fragments by RT-nested PCR from total RNA purified from the serum of two patients before and after SIL therapy. Sequence data of both patients were deposited in GenBank with following IDs: JQ914271 - patient A before SIL treatment, JQ914272 - patient A after SIL treatment, KC155254 - patient A 21 months after cessation of SIL therapy, JQ914273 - patient B before SIL treatment, JQ914274 - patient B after SIL treatment. Primer sequences and protocols for RNA purification and RT-PCR are given in Supplementary Materials and Methods. Written informed consent was obtained from both patients. No donor organs were obtained from executed prisoners or other institutionalized persons.

Results

SIL inhibits replication of HCV genotype 1b but not genotype 2a

To elucidate the MOA of SIL, we defined which part of the HCV lifecycle was primarily inhibited by the drug. We first determined the SIL sensitivity of persistent subgenomic reporter replicons of genotype 1b (Luc/neo Con1) and 2a (Luc/neo JFH1) mimicking chronic HCV infection (Fig. 1A, B). Luc/neo Con1 replication was strongly inhibited by SIL, whereas Luc/neo JFH1 replication was not affected (Fig. 1B), in line with recently published data (11) and in absence of cytostatic effects of the drug (Fig. 1D). Similar results were obtained upon transient transfection of luciferase replicons of the same isolates (Fig. 1A, C). In contrast and in agreement with previous reports, Silibinin (SbN) had no specific impact on HCV RNA replication of Con1 or JFH1 ((11); Fig. S1). Since replicons based on isolate JFH1 replicated much more efficiently in cell culture compared to Con1 (12) we tested SIL sensitivity of JFH1 replicons harboring a chimeric 5′UTR or/and X-tail, replicating with comparable efficiency (5′UTR or X-tail Con1) or even less efficiently (5′UTR/X-tail Con1) than Con1 (Fig. 1E, (12). However, none of the replicons bearing the JFH1 nonstructural protein coding sequence were inhibited by SIL treatment (Fig. 1F), suggesting that JFH1 RNA replication is intrinsically unresponsive to SIL treatment. We also found no significant impact of SIL on infection of a chimeric reporter virus (JcR-2a, Fig. 1A, C (13)), arguing against a substantial inhibition of HCV gt2a entry and replication by SIL. The data demonstrate a strong inhibition of persistent and transient HCV genotype 1b RNA replication by SIL and little impact on replication or infection of genotype 2a in cell culture.

Selection for SIL resistance *in vitro*

We next selected gt1b replicon cells for resistance to SIL *in vitro* and obtained a few cell clones with persistent HCV replication in presence of SIL. Sequence analysis of RT-PCR amplicons identified seven mutations (Fig. 2A), which were conserved in at least one of eight SIL resistant replicon cell clone (Table S1). All mutations were introduced individually into a LucCon1 replicon (Fig. 1A) and analyzed for phenotypic effects on SIL sensitivity and replication fitness. Only Q1914R in the C-terminal region of NS4B, present in three SIL resistant replicon clones, significantly reduced HCV sensitivity to SIL (ca. 2fold IC90; Fig. 2B, C; Table 1). Q1914R furthermore impaired replication fitness (Fig. 2D, E), concordant with the high conservation of glutamine at this position across all HCV genotypes, including isolate JFH1 (14).

Collectively, the data indicate that SIL-resistant replicon clones were selected *in vitro* and a mutation located in the C-terminal region of NS4B (Q1914R) conferred HCV gt1b resistance to SIL.

Viral sequence analysis in a SIL-treated patient chronically infected with HCV

Since HCV resistance to SIL could be selected for *in vitro*, we asked whether SIL resistance occurred *in vivo*. We focused on a male patient with chronic HCV genotype 1a infection previously treated unsuccessfully with IFNα monotherapy, IFNα/RBV and pegIFNα/RBV. The patient underwent extended right lobe liver transplantation and during the anhepatic phase SIL therapy was initiated with 20mg/kg body weight/day based on previous reports (4;8;15). After liver transplantation, viral titers dropped rapidly followed by a continuous decrease of viral load until day 13 after onset of treatment (Fig. 3A). From day 18 to 27 viral load increased back to basal level (viral breakthrough) suggesting the emergence of resistance to SIL. To identify mutations potentially conferring SIL resistance, we amplified and directly sequenced the viral genome before and after viral breakthrough in patient A (Fig. 3B). Before SIL treatment we observed several polymorphisms (Fig. 3B, grey lines) and a subpopulation of viral quasispecies carrying a deletion of the coding sequence of E2 and parts of E1 and p7 (nt 1204 to 2639) (Fig. 3C). After treatment the viral sequence lacked polymorphisms and the subgenome was no longer detectable supporting the concept that the viral quasispecies had passed a genetic bottleneck followed by selection of SIL resistant variants. However, seven novel and conserved mutations exclusively within the nonstructural proteins emerged: G963S (NS2), P/T1112S (NS3), F1809L, D1939N, T1946A (NS4B), E2265D (NS5A) and V2431I (NS5B) (Fig. 3B, black lines, Table S2).

In summary, we analyzed a patient with viral breakthrough under SIL treatment, revealing seven conserved mutations potentially contributing to SIL resistance.

Two mutations in NS4B identified *in vivo* contribute to SIL resistance

To characterize phenotypic effects of mutations identified in patient A, we used a highly cell culture adapted variant of the HCV genotype 1a isolate H77, termed H77S (16) (Fig. 4A). Replication fitness and SIL sensitivity of the H77S reporter replicon was similar to LucCon1 (Fig. 4D, 4B, Table 2). We chose F1809L, D1939N and E2265D for further phenotypic analysis due to their high degree of conservation or because they matched to the SIL resistance profile of HCV isolates in cell culture (Table S2). G963S was excluded since NS2 is not required for RNA replication (17). T1946A was already present in H77S, whereas V2431I was found in the SIL sensitive isolate Con1 (Table S2). Mutations chosen for phenotypic analysis of SIL resistance were introduced individually and in combinations into the H77S reporter replicon (Fig. 4A) and analyzed for SIL sensitivity (Fig. 4B, C; Table 2) and replication fitness (Fig. 4D, E). Mutation D1939N located in the C-terminal region of NS4B significantly reduced SIL sensitivity of H77S (ca. 1.7fold IC90, Fig. 4C; Table 2), accompanied by a significant impairment of replication fitness (Fig. 4E). F1809L slightly reduced SIL sensitivity but significantly increased replication fitness (Fig. 4E). Interestingly, double mutant F1809L+D1939N was less sensitive to SIL than the single mutants (ca. 2.5fold IC90; Fig. 4C, Table 2), arguing for a contribution of both NS4B mutations to SIL resistance in the examined patient. Replication fitness was furthermore restored in the double mutant F1809L+D1939N compared to the D1939N single mutant (Fig. 4E) suggesting that the F1809L mutation compensated for the fitness cost associated with D1939N. NS5A mutation E2265D had no impact on the resistance to SIL, neither individually nor in combination (Fig. 4C, Table 2). The involvement of F1809L+D1939N in SIL-resistance was supported by sequence analysis of viral genomes 21 months after cessation of SIL therapy, since both positions were found reverted, in contrast to T1946A, E2265D and V2431I (Fig. 3D). Additional reversion were found at positions 1112 in NS3

and 963 in NS2. However, mutation P1112S did not contribute to SIL resistance *in vitro*, neither as a single mutation introduced in the H77S replicon, nor in combination with F1809L+D1939N (data not shown). Cumulatively, phenotypic analysis of mutations identified *in vivo* supported a mechanism of action of SIL involving NS4B.

SIL does not affect NS4B self-interactions but probably interferes with NS4B-NS3/4A interactions

NS4B is primarily involved in inducing membrane alterations known as the membranous web which harbors the viral replication complex (18). Previous work suggests NS4B having a complex membrane topology (Fig. 5A, reviewed in (14)). While position 1809 is located in a proposed short ER-luminal loop between predicted transmembrane segments 1 and 2 (TM1 and 2), both major mutations conferring SIL resistance mapped to the C-terminal region of NS4B. Interestingly, substitutions Q1914R and D1939N are located in a region of amphipathic α-helices which are proposed to be embedded in the membrane interface and to point to the cytosol, thereby providing a potential platform for protein-protein interactions (Fig. 5A,B, (19)). Indeed, recent studies have delineated the functional importance of the NS4B C-terminal mediated by homo- and heterotypic interactions between N- and C-termini of NS4B (19–21). We employed a fluorescence resonance energy transfer (FRET)-based assay described earlier (21) to address whether SIL affected self-interaction of NS4B (Fig. 5C). However, SIL had no effect on FRET efficiency, suggesting that SIL did not interfere with NS4B oligomerization. Interactions of NS4B with other HCV nonstructural proteins have been reported as well (reviewed in (14)). We therefore generated chimeric replicons based on the SIL resistant JFH1 isolate, replacing parts of the nonstructural protein coding region by homologous sequences of the SIL sensitive isolate Con1 (Fig. 5D), to analyze which parts of the coding region transferred SIL sensitivity. Exclusive exchange of the NS4B sequence of JFH1 significantly increased SIL sensitivity of the resulting chimera compared to JFH1, but sensitivity did not reach the level of Con1 (NS4B Con1, Fig. 5E,F). We then added the NS3/4A and/or the NS5A coding sequences of Con1 (Fig. 5D). The construct harboring the NS4B and NS5A sequence of Con1 was not replication competent, as well as a replicon containing only NS3/4A of Con1 (NS4B-5A Con1, NS3/4A Con1, respectively; Fig. S3). However, SIL sensitivity of the chimeric replicons NS3-4B Con1 and NS3-5A Con1 was identical to Con1 (Fig. 5D–F), suggesting that NS3/4A is an additional important determinant of SIL response and arguing against a major contribution of NS5A.

SIL alters membranous web morphology

NS4B as well as NS3 have been described to induce membrane alterations (22) and the interaction between both proteins might critically contribute to the formation of functional HCV replication sites. These correspond to accumulations of vesicular structures (18), mainly composed of double membrane vesicles (DMVs) (13). To address a potential interference of SIL on the biogenesis of virus induced membrane alterations we transiently expressed NS3-5B from Con1 or JFH1 in Huh7-T7 cells and analyzed the impact of SIL treatment on the membranous web. SIL treatment had no impact on the distribution of NS4B, NS3 and NS5A and did not change the degree of colocalization of these proteins (Fig. S4 and S5). We also found no alterations in intracellular PI4P levels, suggesting that SIL does not affect the activation of PI4KIIIα, an essential host factor of HCV replication ((13), Fig. S6). However, ultrastructural examination revealed that in cells expressing NS3-5B of the Con1 isolate and treated with SIL, most of the vesicles observed were multi-membrane vesicles (MMVs) and no longer DMVs (Fig. 6A,B), suggesting that SIL indeed modulated the morphology of viral replication sites. In contrast, SbN had no impact on the proportion of MMVs, confirming the specificity of the changes caused by SIL (Fig. S8). Importantly, the morphology of membrane alterations induced by the SIL resistant genotype

1b mutant Q1914R and by the resistant JFH1 isolate was not affected by SIL, supporting the assumption that SIL indeed acts by disturbing viral replication sites (Fig. 6A,C and Fig. S7).

Collectively these data suggest that SIL treatment affects the morphogenesis of viral replication sites by targeting NS4B, probably by modulating a critical interaction with NS3/4A.

Discussion

The mechanisms by which SIL inhibits HCV infection *in vivo* are currently under debate. Previous studies observed a direct inhibition of the viral polymerase *in vitro* by SIL and SbN (10;11;23), suggestive for the mechanism in targeting RNA replication. However, our data confirm that inhibition of viral RNA replication indeed is the primary MOA of SIL, but implicate that NS4B is a candidate target: First we identified *in vitro* and *in vivo* mutations in NS4B conferring partial SIL resistance. Second, SIL modulated the ultrastructure of genotype 1b membrane alterations induced by the viral nonstructural proteins, which are mainly mediated by NS4B (22), but not those generated by gt2a or by a SIL resistant gt1b variant. Third, the NS4B and NS3/4A coding sequence of SIL sensitive isolate Con1 rendered resistant JFH1 fully sensitive to SIL. Taken together, these results suggest that the antiviral activity of SIL is at least in part mediated by NS4B, probably by targeting an interaction with NS3/4A. A critical interaction between NS3 and NS4B has already been suggested by a previous study, based on genetic evidence (24). The fact that we identified mutations conferring SIL resistance only in NS4B might simply reflect restrictions of the isolates included in this study and clearly does not exclude potential alternative sites in NS3 conferring resistance to SIL in other HCV isolates.

NS4B is the key factor inducing membrane alterations harboring the HCV replication sites, which mainly consist of DMVs and to a lesser extend MMVs (13;20). The biogenesis of these vesicular structures is poorly defined, but they are believed to be generated by a concerted action of the nonstructural proteins, with NS4B as the main actor (22). The fact that SIL strongly reduced the number of DMVs and triggered the formation of MMVs therefore argues for an interference with the morphogenesis of viral replication sites. Mutations conferring resistance were mainly located at the C-terminal amphipathic α-helices (Fig. 5A), and affected conserved residues facing the cytosol (Fig. 5B). Those sites could provide a platform for the interaction with other NS-proteins, like NS3/4A or with host factors. Although our studies suggest that SIL might affect a critical NS4B interaction with NS3/4A, we cannot exclude that SIL in addition impacts on interactions of other NS proteins or host factors. The membrane activity reported for Silibinin (25) might also contribute to the antiviral action of SIL, due to the tight membrane association of all viral proteins. The precise mechanism of action of SIL remains to be determined. However, SIL is not the first drug reported to target NS4B (reviewed in (26)), but resistance profiles and suggested MOAs of other classes of NS4B inhibitors suggest that SIL represents a novel type with a unique mode of action.

In contrast to other studies (10;11), we found no evidence for a substantial inhibition of genotype 2a HCVcc infection by SIL. Still, we might have missed effects on entry due to differences in the experimental design. The fact that mutation G963S at a highly conserved position in NS2 was found after therapy but reverted back later suggests that NS2 might be a target of SIL too. Such additional MOAs complementing inhibition of RNA replication might also explain cure or at least absence of viral breakthrough in SIL monotherapy (3;4;27;28) due to a higher barrier to resistance.

The limited data on SIL therapy outcomes include sustained virological response (3–7), initial suppression of viral replication followed by rebound (relapse or breakthrough, patient A, (5;6)) and non-response (patient B, Fig. S2 (6;15)). Rutter *et al.* suggested low viral load as the most valuable predictor of treatment response in their study (6). This might account for successful therapies post LTx (3;4), since a small pool of virus variants at the onset of therapy limits the chance to select for resistance. The main determinant for successful SIL monotherapy after LTx might therefore be the barrier to resistance of the HCV quasispecies in a patient. In the case of patient A, two mutations in highly conserved residues in NS4B seemed to be sufficient for viral breakthrough; in case of other isolates, more mutations might be required or the fitness costs associated with these mutations might be higher. Our data furthermore indicate that SIL might not be effective in genotype 2 since we found no inhibition of RNA replication of genotype 2a isolate JFH1. Interestingly, this is corroborated by two clinical reports about nonresponse of genotype 2 patients to SIL ((29) and S. Beinhardt, personal communication). However, larger patient cohorts will be required to clarify determinants of treatment failure, particularly since a second gt1a patient treated with SIL (patient B) did not respond to SIL therapy, without having obvious alterations in the NS4B sequence (Fig. S2)

In conclusion, our data indicate that SIL is an efficient inhibitor of HCV RNA replication at least in part by targeting NS4B. The emergence of resistance *in vivo* and *in vitro* suggests that SIL should not be used in monotherapy. Although it seems unlikely that SIL will become a major drug in HCV therapy, it represents a promising component of future combination therapies particularly in difficult-to-treat patient cohorts, due to its novel mode of action and unique resistance profile.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

r.l.u	Relative light units
SIL	Legalon-SIL
SbN	Silibinin
HCV	Hepatitis C virus
IFNα	Interferon alpha
IRES	Internal ribosome entry site
i.v	Intravenously
pegIFNα	pegylated IFNα

RBV	Ribavirin
LTx	Liver transplantation
DAA	directly acting antiviral
EM	electron microscopy
DMV	double membrane vesicle
MMV	multi membrane vesicle
MOA	mode of action
PI4P	Phosphatidylinositol 4-phosphate
PI4KIIIα	Phosphatidylinositol 4-phosphate kinase type III α

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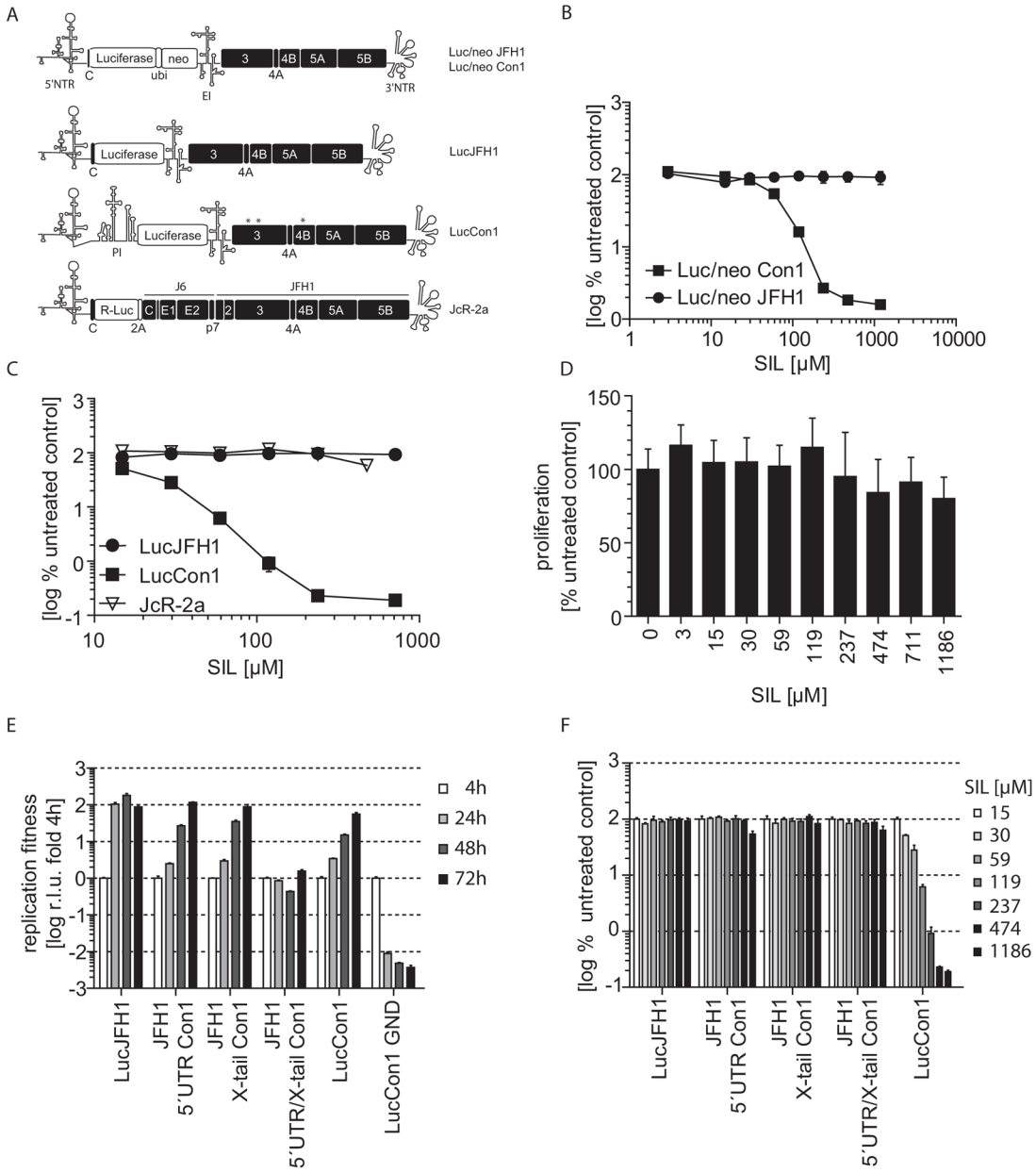

Figure 1. SIL sensitivity of different HCV genotypes

(A) Scheme of viral genomes analyzed for SIL sensitivity. Luciferase: firefly luciferase, R-Luc: renilla luciferase, PI: poliovirus IRES, EI: Encephalomyocarditisvirus IRES, NTR: nontranslated region, 2A: 2A peptide-coding region of foot-and-mouth disease virus. (B) Huh7-Lunet cells harboring a persistent Luc/neo replicon of gt1b (Con1) or 2a (JFH1) were treated for 44h with the indicated concentrations of SIL. Mean values and SD from a representative experiment (n=2). (C) Replication or infection efficiency of different HCV genotypes after SIL-treatment. Huh7-Lunet cells were transfected or Huh7.5 cells infected in triplicates with the respective replicons or JcR-2a virus and treated with indicated concentrations of SIL. Mean and SD of one representative experiment (n=2). (D) Impact of SIL treatment on Huh7-Lunet cell proliferation. Data represent mean and SD of triplicate values of two independent experiments. (E, F) Replication fitness (E) and SIL sensitivity (F)

of different HCV subgenomic replicons of gt1b, gt2a and gt2a replicons with chimeric 5′UTR or X-tail derived from gt1b (12). Mean and SD from one representative experiment (n=2).

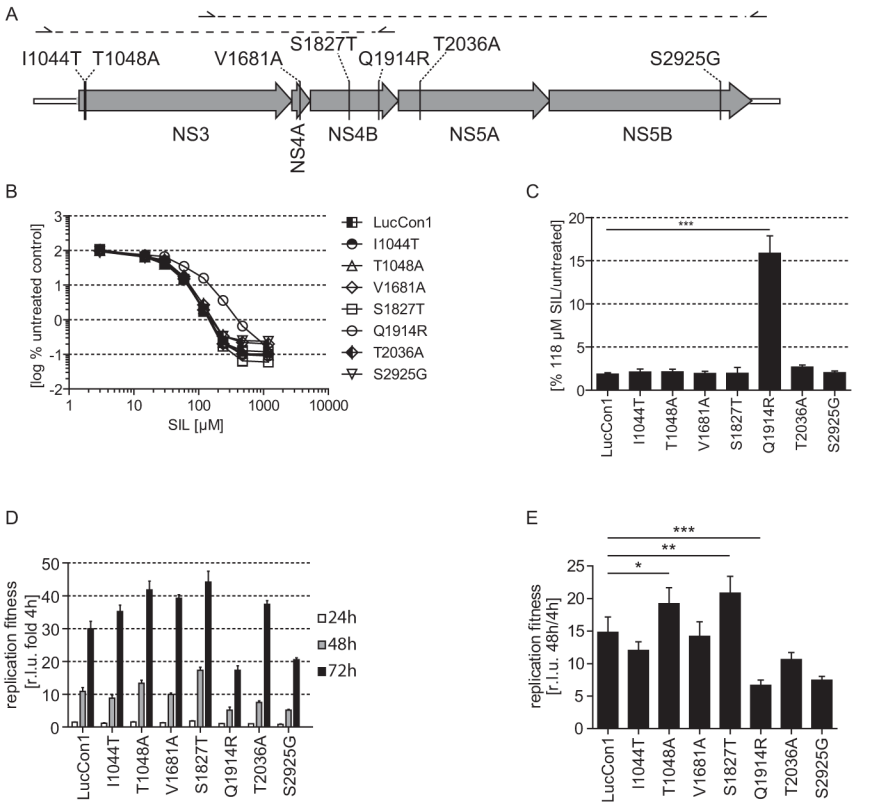

Figure 2. Analysis of SIL resistance *in vitro*

(A) Schematic representation of conserved mutations found in eight SIL resistant Con1 (gt1b) replicon cell clones by direct sequencing of RT-PCR amplicons (dashed lines). (B) Impact of mutations identified in SIL resistant replicon cell clones on SIL sensitivity of the LucCon1 replicon (Fig. 1A). Mean and SD of a representative experiment (n=3). (C) Statistical analysis of SIL sensitivity of mutant Q1914R compared to LucCon1 at 118μM SIL. Mean values and SD from three independent experiments. (D) Replication fitness of LucCon1 replicons containing the indicated mutations identified after SIL selection. Mean and SD of one representative experiment (n=3). (E) Statistical analysis of replication fitness of LucCon1 replicons. Mean and SD of three independent experiments. (*) $p<0.05$ (**) $p<0.01$ (***) $p<0.001$

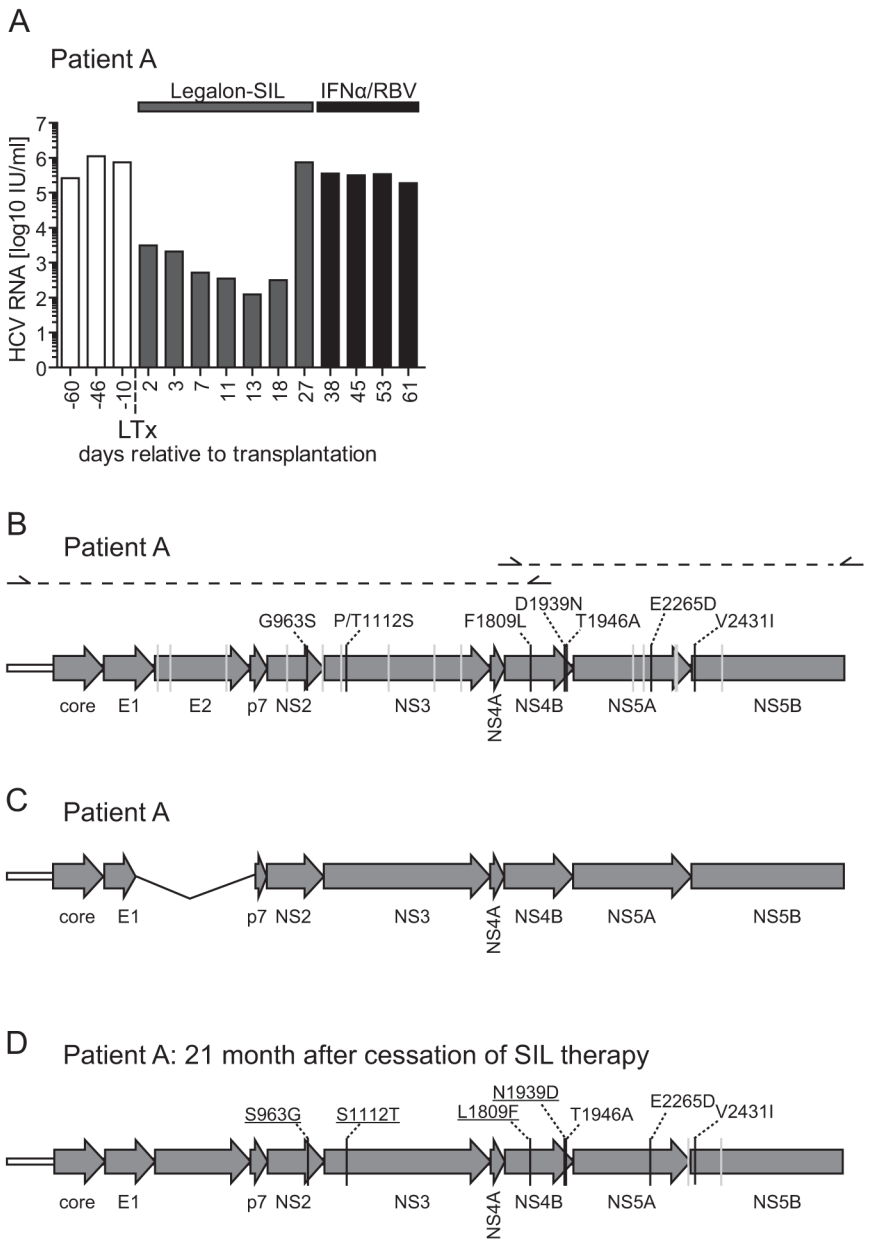

Figure 3. Analysis of viral sequences in a SIL-treated patient

(A) Course of HCV RNA levels [IU/ml serum] in patient A chronically infected with HCV. LTx indicates the time of liver transplantation (day0). (B) Schematic representation of mutations identified in patient A following viral breakthrough during SIL treatment by direct sequencing at day −113 (4.3×10^5 IU/ml) and 45 (3.2×10^5 IU/ml). Black lines indicate new and conserved mutations found after SIL therapy. Grey lines represent polymorphisms found before LTx disappearing after viral breakthrough. (C) Scheme of a subgenome found in a subpopulation of viral quasispecies of patient A before SIL treatment. (D) Changes in the HCV sequence of patient A 21 months after cessation of SIL therapy compared to the sequence at day 45. New polymorphisms are indicated by grey lines, reversions to the pre-treatment sequence are underlined.

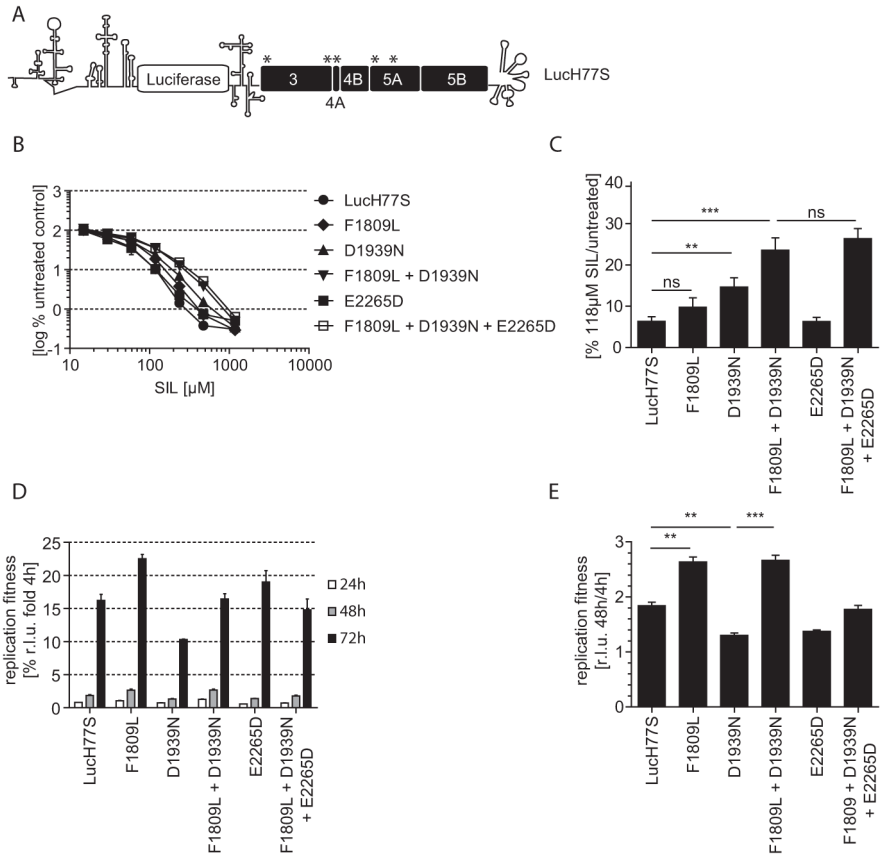

Figure 4. Phenotypic analysis of mutations found after viral breakthrough during SIL treatment

(A) Scheme of the gt1a reporter replicon based on isolate H77 and containing five cell culture adaptive mutations indicated by asterisks (H77S, (16)). (B) Impact of mutations identified *in vivo* on SIL sensitivity of mutant LucH77S replicons as indicated. Mean and SD from a representative experiment (n=3). (C) Statistical analysis of SIL sensitivity of mutant LucH77S replicons compared to the wildtype at 118μM SIL. Mean and SD of three independent experiments. (D) Replication fitness of mutant LucH77S replicons. Mean and SD of one representative experiment (n=3). (E) Statistical analysis of the replication fitness of mutant H77S replicons compared to LucH77S wildtype. Mean and SD of three independent experiments. (ns) not significant (**) $p<0.01$ (***) $p<0.001$

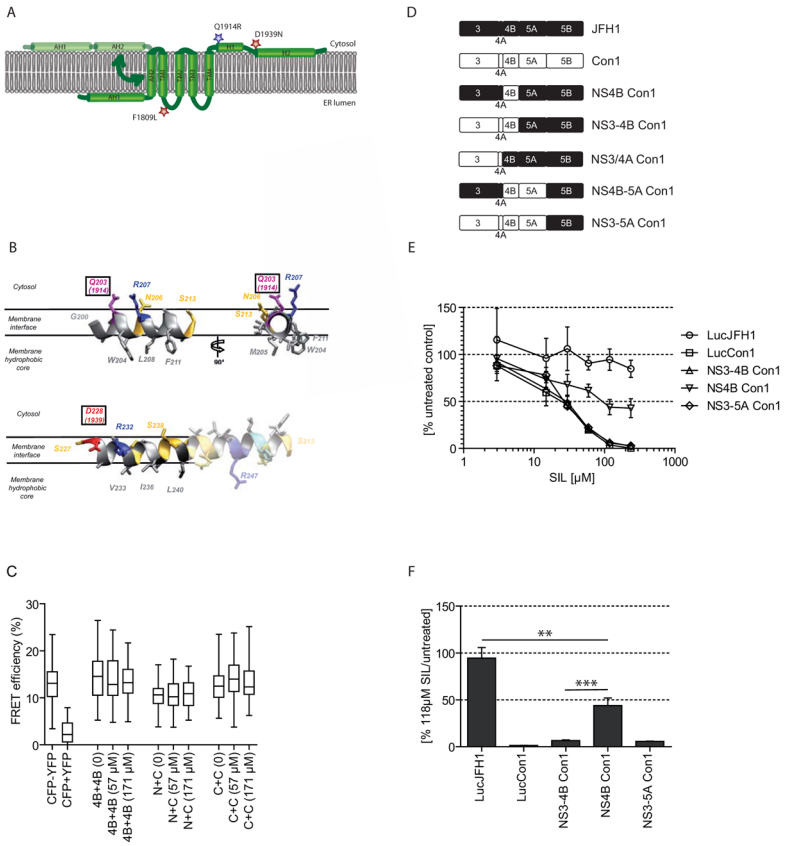

Figure 5. Position of SIL-resistance mutations in NS4B and analyses of NS4B self-interactions and SIL sensitivity of chimeric replicons

(A) Predicted membrane topology of NS4B and localization of mutations contributing to SIL resistance *in vivo* (red stars) or *in vitro* (blue star). AH1, AH2, amphipatic helices; TM1-4 predicted transmembrane regions; H1, H2 C-terminal α-helices (14). (B) Tentative membrane location of the two C-terminal amphipathic α-helices H1 and H2 ((19), PDB entry: 2KDR) and orientation of amino acid side chains at position 1914 (upper panel) and 1939 (lower panel), respectively. Hydrophobic residues are shown in light gray, polar residues in yellow, Gln in magenta, His in cyan and basic and acidic residues are blue and red, respectively. Figures were generated from structure coordinates using VMD (http://www.ks.uiuc.edu/Research/vmd/).

(C) Influence of SIL on homo- and heterotypic NS4B self-interactions. Full-length (4B), C-terminal (C) or N-terminal (N) constructs fused to Cerulean cyan fluorescent protein (CFP) or Venus yellow fluorescent protein (YFP) were cotransfected into US-OS cells and analyzed for fluorescence resonance energy transfer (FRET) in presence and absence of SIL. The results are shown as box plots representing 56 measurements performed under each condition. (D) Schematic representation of the nonstructural protein regions of chimeric replicon constructs. Portions of the SIL-resistant isolate JFH1 and the SIL-sensitive isolate Con1 are given in black and white, respectively. (E) SIL sensitivity of chimeric reporter replicons and parental constructs as indicated in (D). Mean and SD of triplicate values from a representative experiment (n=3). Note that chimeras NS4B-5A Con1 and NS3-4A Con1 were not replication competent (Fig. S3). (F) Statistical analysis of SIL resistance of replicon chimeras compared to LucJFH1 at 118µM SIL. Mean values and SD from two independent experiments.

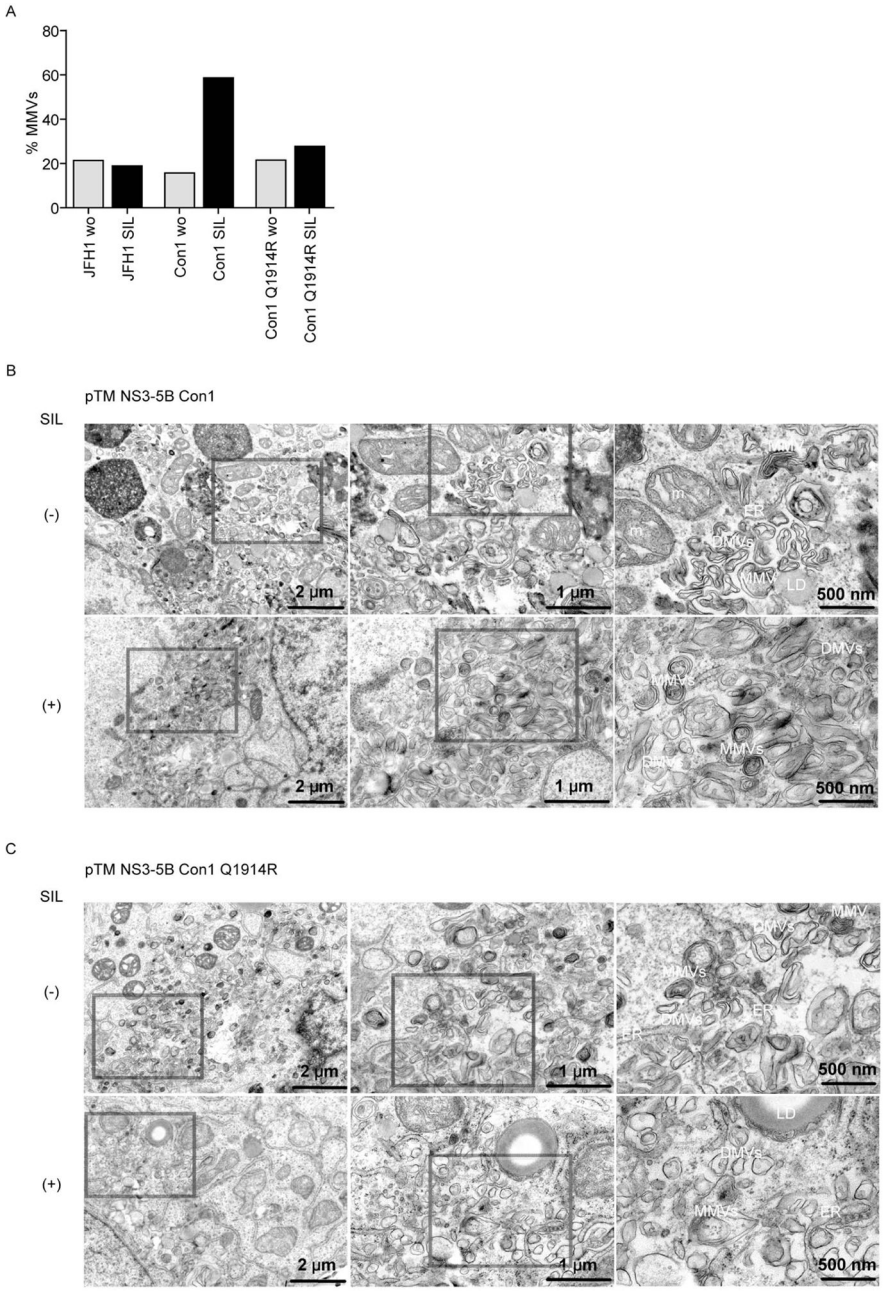

Figure 6. Impact of SIL on NS4B-induced membranous web morphology

(A) Percentage of multi-membrane vesicles (MMV) in Lunet-T7 cells transfected with pTM plasmids encoding NS3-5B of HCV JFH1, Con1 or Con1 Q1914R, respectively, in presence and absence of SIL (n=60). (B,C) Ultrastructural analysis of the morphology of the membranous web (MW) in presence and absence of SIL upon expression of NS3-5B of isolate Con1 (B) or Con1 Q1914R (C).

Table 1

Mutations identified in replicon cell clones after SIL-selection and corresponding IC_{90}

Mutation	Protein (aa)	IC_{90} [μM SIL]	95% confidence interval	Frequency[A]
Con1WT		92	67 to 126	
I1044T	NS3 (18)	94	58 to 153	2/8
T1048A	NS3 (22)	85	67 to 108	1/8
V1681A	NS4A (24)	89	69 to 114	1/8
S1827T	NS4B (116)	94	59 to 150	2/8
Q1914R	NS4B (203)	191	144 to 254	3/8
T2036A	NS5A (64)	102	80 to 130	1/8
S2925G	NS5B (506)	85	68 to 107	1/8

[A]Number of SIL-resistant replicon cell clones containing the mutated sequence (n=8)

Table 2

SIL sensitivity of H77S replicons containing single or multiple mutations identified in patient A after SIL therapy

Mutation	Protein	IC_{90} [μM SIL]	95% confidence interval
H77S		104	81 to 133
F1809L	NS4B	137	102 to 184
D1939N	NS4B	172	129 to 228
F1809L + D1939N	NS4B	252	167 to 381
E2265D	NS5A	102	86 to 120
F1809L + D1939N + E2265D	NS4B+NS5A	263	186 to 370