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Inhibition of Hepatitis C Virus in Chimeric Mice by Short Synthetic Hairpin RNAs: Sequence Analysis of Surviving Virus Shows Added Selective Pressure of Combination Therapy

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ABSTRACT

We have recently shown that a cocktail of two short synthetic hairpin RNAs (sshRNAs), targeting the internal ribosome entry site of hepatitis C virus (HCV) formulated with lipid nanoparticles, was able to suppress viral replication in chimeric mice infected with HCV GT1a by up to 2.5 $\log_{10}$ (H. Ma et al., Gastroenterology 146:63–66.e5, http://dx.doi.org/10.1053/j.gastro.2013.09.049) Viral load remained about 1 $\log_{10}$ below pretreatment levels 21 days after the end of dosing. We have now sequenced the HCV viral RNA amplified from serum of treated mice after the 21-day follow-up period. Viral RNA from the HCV sshRNA-treated groups was altered in sequences complementary to the sshRNAs and nowhere else in the 500-nucleotide sequenced region, while the viruses from the control group that received an irrelevant sshRNA had no mutations in that region. The ability of the most commonly selected mutations to confer resistance to the sshRNAs was confirmed *in vitro* by introducing those mutations into HCV-luciferase reporters. The mutations most frequently selected by sshRNA treatment within the sshRNA target sequence occurred at the most polymorphic residues, as identified from an analysis of available clinical isolates. These results demonstrate a direct antiviral activity with effective HCV suppression, demonstrate the added selective pressure of combination therapy, and confirm an RNA interference (RNAi) mechanism of action.

IMPORTANCE

This study presents a detailed analysis of the impact of treating a hepatitis C virus (HCV)-infected animal with synthetic hairpin-shaped RNAs that can degrade the virus's RNA genome. These RNAs can reduce the viral load in these animals by over 99% after 1 to 2 injections. The study results confirm that the viral rebound that often occurred a few weeks after treatment is due to emergence of a virus whose genome is mutated in the sequences targeted by the RNAs. The use of two RNA inhibitors, which is more effective than use of either one by itself, requires that any resistant virus have mutations in the targets sites of both agents, a higher hurdle, if the virus is to retain the ability to replicate efficiently. These results demonstrate a direct antiviral activity with effective HCV suppression, demonstrate the added selective pressure of combination therapy, and confirm an RNAi mechanism of action.

Hepatitis C virus (HCV) infection is the most common cause of chronic liver disease and liver transplantation in the United States and a leading cause of liver cirrhosis and hepatocellular carcinoma worldwide (1, 2). HCV has high genetic heterogeneity due to its intrinsically error-prone polymerase, high replication rate, and large number of infected cells in a typical infection. This results in a virus population with a huge number of sequence variations in each infected individual and a high potential for developing resistance to direct-acting antiviral agents (DAA) (3–6). It has been demonstrated that a combination of molecules with nonoverlapping resistance profiles can be successful in treating HCV infection (7–10). A highly potent combination of antiviral agents will be especially important for subpopulations of difficult-to-treat patients, such as interferon nonresponders and patients with high viral load and high quasispecies diversity. RNA interference (RNAi) has the potential to provide multiple agents with nonoverlapping resistance profiles, in that multiple interfering RNAs targeting different parts of the viral RNA can be administered together. This is in contrast to current DAA combination therapy, in which multiple small-molecule drugs, each targeting a different viral protein, are combined.

HCV has a single positive-strand RNA genome that functions as both a template for replication and message for polyprotein synthesis. A single RNAi-induced cleavage of the genomic RNA can abolish both replication and translation of the virus. The 5′ end of the genome contains an internal ribosome entry site (IRES), which is the site of translation initiation and interacts directly with the 40S ribosomal subunit (11). Because the IRES is the most conserved region of the virus (12, 13), it is an attractive target for RNAi-based therapies that are inherently very sequence specific. RNAi is particularly well suited for hepatitis therapy, because the liver is both the site of HCV replication and the tissue most easily targeted by systemically administered oligonucleotides (reviewed in reference 14), and a number of clinical trials of

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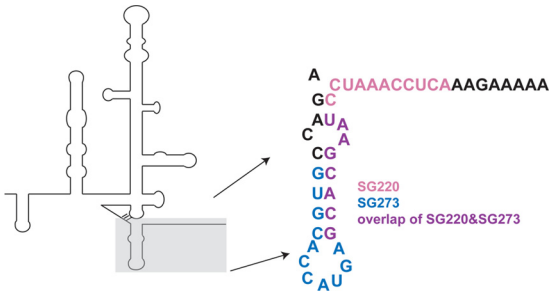

FIG 1 sshRNA target sites in the HCV IRES. Schematic diagram of the HCV IRES secondary structure with the region targeted by sshRNAs in domain IV shown in detail. Residues targeted only by SG273 are shown in blue, residues targeted only by SG220 in pink, and residues targeted by both SG220 and SG273 in purple.

RNAi agents against liver-specific targets are in progress (15). While several other groups have demonstrated RNAi-mediated inhibition of HCV in cell-based reporter gene systems, HCV subgenomic replicon systems, and cell culture-based infectious HCV systems (16–21), RNAi-mediated inhibition of HCV replication in an *in vivo* model system permissive for HCV replication has only recently been described (22).

We have previously characterized a novel class of RNAi agents called short synthetic hairpin RNAs (sshRNAs) that target domain IV of the HCV IRES and are highly potent inhibitors of HCV IRES-dependent luciferase gene expression in cell-based assays (23–27). sshRNAs that are chemically modified with 2′-*O*-methyl (2′-OMe) residues to abrogate immune-stimulatory effects and enhance stability in serum (24) show potential as therapeutic agents. We have shown that sshRNAs induce cleavage of target RNAs by a Dicer-independent RNAi mechanism *in vitro* (25, 28). sshRNAs are predominantly loaded as intact molecules into Argonaute (Ago)-containing complexes without prior processing by Dicer and are activated by Ago2-mediated cleavage of the passenger arm of the hairpin (28). When formulated with lipid nanoparticles (LNP), HCV-directed sshRNAs can be efficiently delivered to the liver, and they show potent and prolonged *in vivo* activity in HCV-luciferase reporter mice with no immune stimulation (29, 30).

To assess the activity of the HCV sshRNAs in a robust *in vivo* HCV infection model, chimeric human hepatocyte uPA-SCID mice were stably infected with HCV of genotype 1a (GT1a). In this study, the mice received two intravenous injections, given a week apart, of two different LNP-formulated sshRNAs (SG220 and SG273) with overlapping target sequences in the HCV IRES (Fig. 1). Treatment groups included SG220 alone, SG273 alone, a combination of the two, and an irrelevant sshRNA as a control. The results showed substantial and sustained reduction in viral load in the groups treated with the HCV-specific sshRNAs (22). Twenty-one days after the last dose, the viral load of those groups remained about 90% below pretreatment levels. In contrast, there was no reduction of HCV RNA titer in the control sshRNA group during the dosing or follow-up periods. The LNP-formulated sshRNAs were well tolerated, with no treatment-related body weight loss, elevation of liver ALT levels, or reduction in serum human albumin concentration during the dosing and follow-up periods. These results suggested that the reduction of serum HCV RNA concentration by the HCV-targeting sshRNAs was specific and that there was little or no nonspecific loss of human hepatocytes.

To assess to what extent treatment imposed selective pressure on the sequence makeup of the virus and determine whether remaining viruses were resistant to the treatment, we undertook a sequence analysis of HCV that could be recovered from the treated mice, which we present here. We find that almost all of the recovered viral RNA was mutated in the regions targeted by the respective sshRNAs and that these mutations conferred significant resistance to the agents. Use of a combination of SG220 and SG273 selected for mutations that conferred resistance to both sshRNAs, but viral rebound *in vivo* took longer to develop. These findings demonstrate the value of combining sshRNAs targeting different sites on the viral genome in constraining mutational escape, and they provide strong evidence for an RNAi mechanism of action for these agents.

MATERIALS AND METHODS

Reporter plasmids. For *in vitro* cell culture experiments, a dual-luciferase expression plasmid (IRES/fLuc) was used in which the sequence encoding the HCV IRES of genotype 1b is placed between the coding sequences for *Renilla* and *firefly* luciferase (fLuc), such that fLuc expression is dependent on the IRES. We introduced single and double point mutations in the HCV IRES target that result in mismatches to SG220 at position 5, 17, or both 5 and 17, as well as single mutations resulting in mismatches to SG273 at positions 6 and 16 using the QuikChange mutagenesis kit (Stratagene/Agilent, Santa Clara, CA) according to the manufacturer's instructions. The presence of these mutations was confirmed by sequencing (Retrogen, San Diego, CA).

Transfection and *in vitro* assays. The human kidney cell line 293FT (Invitrogen, Carlsbad, CA) was maintained in Dulbecco's modified Eagle medium (DMEM; Cambrex, Walkersville, IN) with 10% fetal bovine serum (HyClone, Logan, UT), supplemented with 2 mM L-glutamine and 1 mM sodium pyruvate. One day prior to transfection, cells were seeded at 23,000 cells/well in a 96-well plate, resulting in ~80% cell confluence at the time of transfection. Transfections were performed using Lipofectamine 2000 (Invitrogen) by following the manufacturer's instructions. Thirteen nanograms of pIRES/fLuc (an IRES-linked firefly luciferase reporter construct), 20 ng of pSEAP2 control plasmid (BD Biosciences Clontech, San Jose, CA) as a transfection control, and the indicated amounts of sshRNAs were cotransfected into the 293FT cells. Forty-eight hours later, the cells were lysed and luciferase activity was measured in a MicroLumat LB 96P luminometer (Berthold Technologies, Bad Wildbad, Germany). All sshRNA samples were tested in triplicate. Percent silencing was calculated relative to transfections with the reporter plasmid in the absence of sshRNA inhibitors.

Formulation of sshRNA into LNP. Monomeric sshRNAs and control short interfering RNAs (siRNAs) were formulated into LNP by the process of stepwise ethanol dilution and spontaneous vesicle formation as previously described (31, 32). LNP were dialyzed against phosphate-buffered saline (PBS) and filter sterilized through a 0.2-μm filter before use. Mean particle sizes were 85 to 90 nm with polydispersity values of <0.1 by dynamic light scattering, and 92 to 98% of the siRNA was encapsulated within the lipid particles.

Treatment of human liver-uPA-SCID chimeric mice and measurements. Male uPA$^{+/+}$/SCID mice with human albumin ($\geq$7 mg/ml) and HCV (genotype 1a) RNA titers between 2.5×10^7 and 5.1×10^8 copies/ml were chosen for the study. All mice received 2 intravenous injections via tail vein of the LNP-formulated HCV sshRNAs or an irrelevant sshRNA at the indicated doses at day 0 and day 7. At the indicated time points before or after the sshRNA dosing, blood samples were collected via the retro-orbital route under isoflurane anesthesia, and serum HCV RNA titer, serum total alanine aminotransferase activity, and human albumin were measured as described previously (33). On day 28, all animals were anesthetized with isoflurane, and a minimum of 300 μl of blood was collected via cardiac

puncture, after which the animals were sacrificed via exsanguination. Animal husbandry and all animal experimental procedures used in this study have been approved by the animal ethics committee of Phoenix-Bio (Hiroshima, Japan, where the injections were performed) in accord with appropriate Japanese regulatory authorities.

Isolation of HCV RNA from serum samples from treated human liver-uPA-SCID chimeric mice. Serum was isolated from blood collected on day 28 of the study (described above) and stored at −80°C until analysis. On the day of analysis, the serum samples were thawed on ice, and 300-μl aliquots were transferred to tubes containing 300 μl of 2× denaturing solution (Ambion). RNA was isolated using the *mir*Vana PARIS kit (Ambion) by following the manufacturer's protocol for liquid samples. RNA was eluted with 100 μl of elution buffer (as supplied by the *mir*Vana kit).

Synthesis, amplification, and cloning of HCV cDNA. The following primers were used to synthesize the HCV cDNA: HCVrev2 (5′-GGATA GGATCCCGTCTACCTCGAGGTTGC-3′), HCVrev3 (5′-AAAAAAGG ATCCCCCAAATTGCGTGACCTGC-3′), and HCVrev4 (5′-AAAAAAG GATCCCGACGAGCGGAATGTACC-3′). Underlined sequences are complementary to HCV-gt1a RNA residues 509 to 526, 682 to 700, and 745 to 762, respectively (relative to reference genome AF009606) (34). Two to five μl of serum RNA was used for cDNA synthesis with Superscript II reverse transcriptase by following the manufacturer's protocol (Invitrogen). Two to 10 μl of HCV cDNA was subjected to PCR amplification with primers HCVfw (5′CCTGATAAGCTTGACACTCCACCAT GAATCA-3′; underlined nucleotides [nt] correspond to nt 21 to 39) and HCVrev2, HCVrev3, or HCVrev4 by using the PfuTurbo Hotstart DNA polymerase by following the manufacturer's protocol (Stratagene). Amplified products were digested with HindIII and BamHI and ligated to plasmid pBluescript SK+ previously digested with HindIII and BamHI. The resulting recombinant plasmids (9 to 13/per mouse for a total of 20 mice) were sequenced using a T7 sequencing primer by Retrogen (San Diego, CA). Approximately 10 clones from each mouse serum sample were sequenced to detect variants. All cloned HCV sequences were aligned against the sequence of the HCV genotype 1a inoculum by using the ClustalW2 multiple-sequence alignment algorithm (www.ebi.ac.uk/Tools/msa/clustalw2/).

HCV 5′-untranslated region polymorphism analysis. A total of 609 confirmed and nonredundant genotype 1 (GT1) HCV sequences were obtained from the EU HCV database (http://euhcvdb.ibcp.fr/euHCVdb/). The 609 GT1 sequences consist of 245 GT1a and 364 GT1b sequences. The confirmed HCV sequences are generally either full-length sequences or near-full-length sequences. The mean length of GT1a sequences is 9,272 nt (standard deviation, 149), and the mean length of GT1b sequences is 9,358 nt (standard deviation, 128). All GT1 sequences contain the complete 30-nt sshRNA target region. The GT1a and GT1b sequences were compared to the 30-nt sequence by blastn (http://www.ncbi.nlm.nih.gov/blast) with a minimal window size of 8. The blastn results were analyzed by a custom script; the nucleotide base variations at each position of the 30-nt reference were counted for GT1a and GT1b sequences separately or in aggregate.

RESULTS

Characterization of drug-resistant HCV variants selected during treatment. HCV has a high mutation rate due to its intrinsically error-prone RNA polymerase and high replication rate (35). The emergence of drug-resistant variants is one of the major causes of the failure of DAA-based treatment regimens to achieve a sustained antiviral response (6, 9, 36). In our efficacy study of two LNP-formulated sshRNAs targeting overlapping sequences in the HCV IRES (SG220 and SG273) (Fig. 1) in chimeric human hepatocyte uPA-SCID mice infected with HCV genotype 1a, the mice were given two intravenous doses a week apart (details of this study are provided in reference 22). We found that after the first injection, all mice receiving HCV-specific sshRNAs showed substantial reduction in serum HCV RNA concentration. Treatment with 2.5 mg/kg of body weight SG220 or SG273 led to a 1.8 $\log_{10}$ or 1.2 $\log_{10}$ reduction in HCV RNA, respectively, at 72 h after the first injection ($P < 0.01$). A higher dose of SG220, 5 mg/kg, did not lead to further reduction in viral load. However, the combination of 2.5 mg/kg SG220 and 2.5 mg/kg SG273 provided the strongest inhibition of HCV replication, with a 2 $\log_{10}$ reduction in HCV RNA observed 72 h after the first treatment and an additional 0.5 $\log_{10}$ reduction 7 days after the second injection ($P < 0.01$). The LNP-formulated irrelevant control sshRNA administered at 5 mg/kg showed no effect. The mean viral load reduction from baseline in all HCV sshRNA-treated groups remained detectable for an extended period of time. In particular, in the mice treated with either SG220 or with the combination of SG220 and SG273, the HCV serum viral load remained >10-fold lower than the pretreatment level ($P < 0.01$) up to the last study time point 3 weeks after the second and last injections. Interestingly, in this study we observed a further reduction in HCV RNA titer after the second dose when a combination of SG220 and SG273 was given, but this was not observed in the case of the individual HCV sshRNAs (22). To determine if this was indicative of the emergence of resistant variants in the case of the individual sshRNAs, we characterized cDNA clones of HCV RNA amplified from the terminal serum (recovered on day 28, 3 weeks after the second dose) of all mice in each of the treatment groups (SG220, 2.5 mg/kg; SG220, 5.0 mg/kg; SG273, 2.5 mg/kg; SG220 plus SG273, 2.5 mg/kg each; SG221 [scrambled control], 5.0 mg/kg). We obtained cDNA clones for all mice with the exception of one mouse in the SG220 2.5-mg/kg dosing group that had terminal serum HCV RNA levels 2.6 $\log_{10}$ below pretreatment levels (22). A total of 169 clones were obtained for the five dose groups, corresponding to 9 to 13 sequences per mouse.

TABLE 1 Proportion of HCV sequences with mutations in the sshRNA target site from serum samples collected 21 days after the last injection

sshRNA(s) (dose)	Proportion (%) of HCV sequences by no. of mutations in target site				Total no. of sequences
	0	1	2	3	
SG220 (2.5 mg/kg)	0	36.1	63.9	0	36
SG220 (5.0 mg/kg)	0	8.7	91.3	0	46
SG273 (2.5 mg/kg)	2.3	95.3	2.3	0	43
SG220 plus SG273 (2.5 mg/kg each)	0	0	86.4	13.6	44
SG221c scrambled control (5.0 mg/kg)	100	0	0	0	42

We found that all HCV cDNA clones from mice receiving the HCV-specific SG220 and almost all receiving only SG273 were mutated in the target region of their respective sshRNA and nowhere else within the surrounding 500 nt sequenced (Table 1). In contrast, there were no sequence changes in the target regions of the two HCV sshRNAs in any of the 42 HCV clones we examined from the mice treated with the irrelevant sshRNA SG221 (Table 1). Positions in the target sequence that were mutated in each dosing group are summarized in Fig. 2, and detailed alignments for each mouse are provided in Fig. S1 in the supplemental material. The mutations in HCV clones from SG220-treated mice occurred within the SG220 target region but not in the region unique to SG273 (Fig. 2A and B) and *vice versa* for mutations in HCV clones from the SG273-treated mice (Fig. 2C). Importantly, the mutations obtained from the mice treated with a combination of SG220 and SG273 occurred predominantly in the overlapping re-

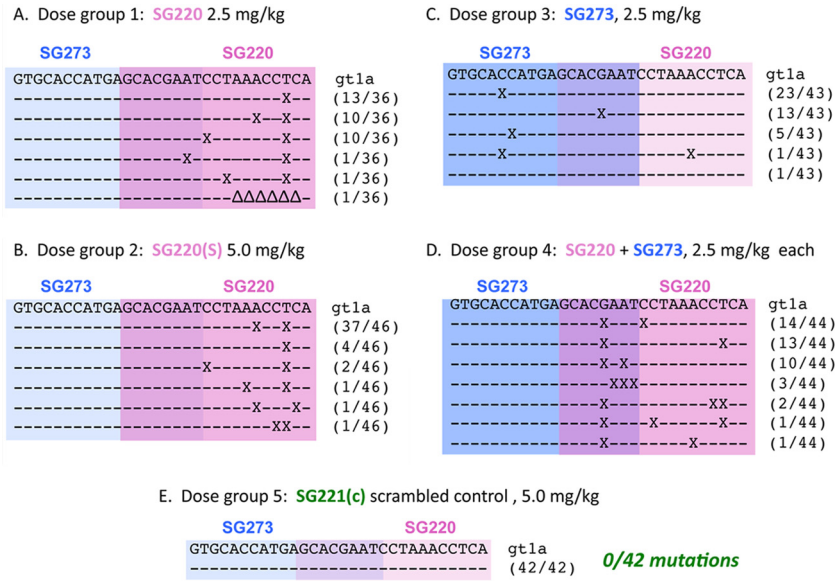

FIG 2 Sequence analysis of cDNA clones of HCV RNA amplified from terminal serum (day 28) of sshRNA-treated mice. The target sites of the 2 HCV sshRNAs with 8-nt overlaps are indicated by colors (blue, SG273; pink, SG220; purple, overlapping sequence). Sequence variants are aligned relative to wild-type genotype 1a virus (gt1a), with each dash representing one wild-type nucleotide of the target region. The positions of mutant variants (X) or deleted nucleotides (Δ) identified for each of the 5 dosing groups are shown aligned with the wild-type sequence (top line). The number of clones obtained out of the total number of cloned sequences is indicated in parentheses beside each sequence variant.

gion of the two sshRNAs and within the target region of SG220 (the more potent of the two sshRNAs characterized *in vitro*) but not in the region only targeted by SG273 (Fig. 2D). The number of mutations in the target site also tended to increase at higher individual doses and in the combination treatment group (Table 1). Mice receiving only SG220 at 2.5 mg/kg had 1 (36%) or 2 (64%) mutations per sequence, whereas those at the 5.0-mg/kg dose mostly had 2 mutations (91%). Most of the mice receiving only SG273, which was less potent than SG220 in *in vitro* screening assays (24, 25) and *in vivo* (22), had only 1 mutation in the target site (95%). In the sequences obtained from the combination group, all mice had 2 to 3 sites mutated in the target region. The overwhelming majority of mutations were point mutations, but 1 clone had a 6-nucleotide deletion instead (Fig. 2A).

The most common mutations in the SG220-treated group were at position 17 of the target site, corresponding to U362 in genotype 1a (SG220/S17; 35 out of 36 clones in the 2.5-mg/kg group and 45 out of 46 clones in the 5.0-mg/kg group), followed by positions 9 and 14. Of the substitutions at U362, 87% were U362C, but mutations to A and G were also observed (see Fig. S1 in the supplemental material). The most common mutations in the SG273-treated mice were at position 6 (C340) (SG273/S6; 24 out of 43), followed by position 16 (G350) (SG273/S16; 13 out of 43). The most frequent mutations in the combination group were at position 5 of the SG220 target (G350) (SG220/S5, which is the same position as SG273/S16) (Fig. 3A), followed by position 17 in the target of SG220. Substitutions at G350 were overwhelmingly G350A (96%) (see Fig. S1), with mutation to U also observed. C340 is in the translation start codon loop of the IRES, whereas G350 and U362 are nucleotides in the IRES just downstream of the start codon and are translated as the N-terminal residues of the core protein. These results indicate that the HCV-targeting sshRNAs were exerting selection pressure on the virus, effectively inhibiting the replication of viruses having the wild-type IRES sequence while allowing replication of HCV variants with certain mutations in the respective target sites. These results are highly suggestive of a specific RNAi mechanism of action, consistent with our previous results obtained in cell culture (25, 28).

Since the region targeted by the two sshRNAs contains nucleotides coding for the core protein N-terminal residues $M_1S_2T_3N_4P_5K_6P_7$, we analyzed the mutated sequences for codon substitutions and resulting amino acid changes (Table 2; also see Table S1 in the supplemental material) and performed protein sequence alignments (see Fig. S2). In the 169 sequences, there were no mutations in the AUG start codon (M_1) or in the serine residue (S_2). For the most frequently mutated codons, T_3 and P_7, virtually all mutations occurred in the third position (at nucleotides G350 and U362), which did not result in an amino acid change. Similarly, 59 out of 60 mutations to the lysine codon also resulted in no change to the amino acid. The one-codon change resulting in an amino acid change was a conservative substitution of a basic residue ($K_6 \rightarrow R$). The N_4 and P_5 codons were less frequently mutated but did result in some codon substitutions. No mutations were observed from nucleotides 345 to 349, which make up codons S_2 and the first 2 nucleotides of T_3 and correspond to a base-paired stem closing the loop with the initiation codon, consistent with its conservation across all genotypes (12, 21). Altogether, 252 codon changes were observed, of which the majority (206 codons) resulted in synonymous substitutions rather than amino acid identity changes (46 codons), suggesting that mutations with minimal impact on viral protein function or ability of the viruses to replicate were preferentially selected.

***In vitro* characterization of sshRNA activity against the most frequently mutated sequences.** To determine whether the identified HCV sequence variants could confer resistance to inhibition

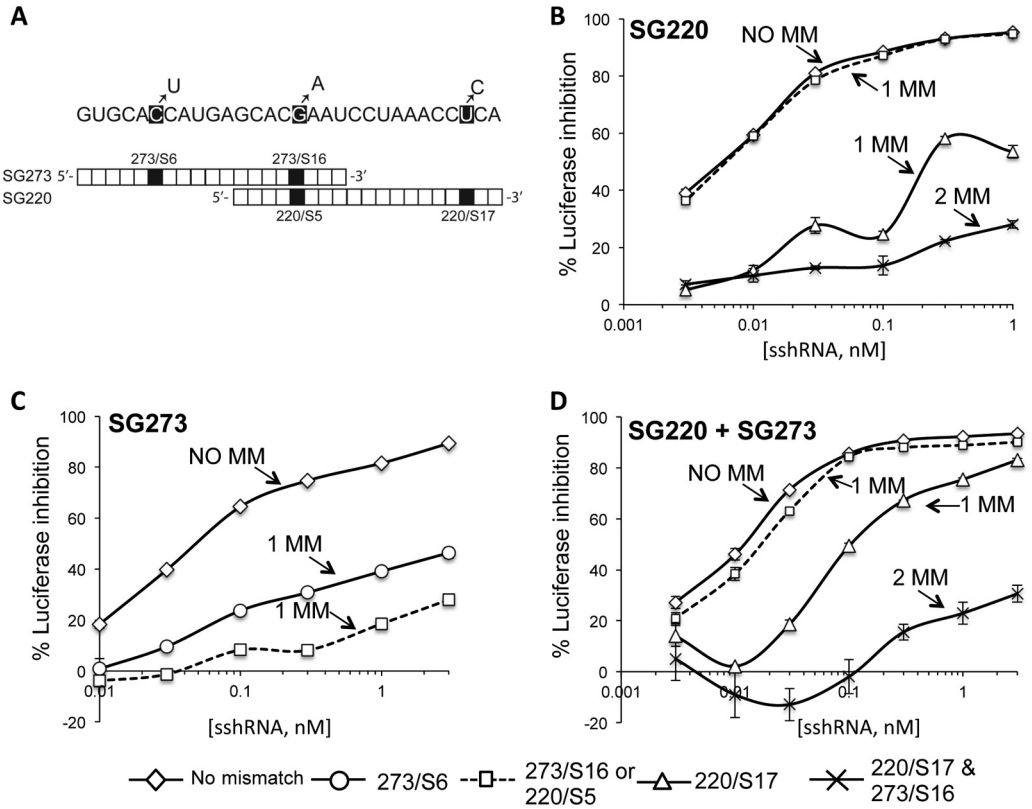

FIG 3 Mutations selected by HCV sshRNA treatments reduced the silencing ability of HCV sshRNA. (A) The three most common mutations among the HCV cDNA clones derived from the terminal serum are shown. Note that SG273/S16 is the same as SG220/S5. (B) Effect of mutations at the indicated sites on the gene-silencing activity of SG220. (C) Effect of mutations at the indicated sites on the gene-silencing activity of SG273. (D) Effect of mutations at the indicated sites on the gene-silencing activity of the SG220 and SG273 combination. The values shown are means and standard deviations from triplicate transfections. MM indicates the number of mutations in the target sequence.

by the HCV sshRNAs, we engineered the mutations most frequently observed in each of the HCV sshRNA treatment groups (C340U, G350A, U362C, and the double mutation G350A-U362C) into the HCV IRES region of an IRES-directed firefly luciferase (fLuc) reporter plasmid (described in Ge et al. [25]) (Fig. 3A). These mutations correspond to target sites SG273/S6, SG273/S16 (identical to SG220/S5), SG220/S17, and double mutation SG273/S16-SG220/S17, respectively (Fig. 3A). HEK-293FT cells were cotransfected with SG220, SG273, or both sshRNAs plus either the wild-type or the mutant plasmid, and luciferase reporter gene activity was measured to determine the activities of the sshRNAs against the mutated IRES.

SG220/S17, which lies in the seed region (nt 2 to 8 of the guide sequence) of SG220 and is the most frequent mutation to emerge from SG220 treatment, significantly attenuated the gene silencing activity of SG220 (Fig. 3B). SG220/S5, a mutation outside the seed region, did not reduce the silencing activity of SG220, consistent with the fact that this mutation did not emerge in the SG220-only

TABLE 2 Nucleotide and amino acid changes in the core protein residues contained within the sshRNA target sites

	Sequence change for core protein residue[a]:							
Codon type	M_1	S_2	T_3	N_4	P_5	K_6	P_7	Q_8
GT1a	AUG	AGC	ACG	AAU	CCU	AAA	CCU	CAA[c]
Substitution[b]			ACA (T; 52/169)	CUC (L; 3/169)	UCU (S; 26/169)	AAC (K; 58/169)	CCC (P; 81/169)	AAA(K; 1/169)
			ACU (T; 2/169)	ACU (T; 10/169)	CUU (L; 1/169)	AAG (K; 1/169)	CCA (P; 9/169)	
				AUU (I; 1/169)		AGA (R; 1/169)	CCG (P; 3/169)	
							CUC (L; 2/169)	
							CAA (Q; 1/169)	
Total no. of substitutions	0/169	0/169	54/169	14/169	27/169	60/169	96/169	1/169

[a] Sequences were obtained from viral cDNA isolated on day 28 of the study. Codon changes resulting in amino acid substitutions are shaded gray. Changed codons resulting in a synonymous substitution are not shaded. Results are combined from all dose groups.
[b] The amino acid substitution is in parentheses with the frequency listed.
[c] Only the first 2 nucleotides of this codon are within the target sequence.

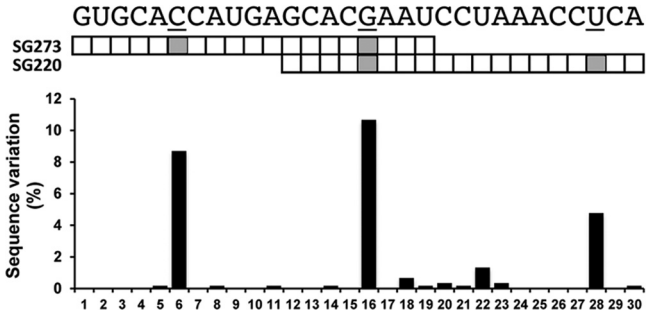

FIG 4 Natural sequence polymorphism of HCV GT1 clinical isolates within the target region of the overlapping HCV sshRNAs SG220 and SG273. The percentage of HCV sequences deviating from the HCV sshRNA reference sequence at any particular nucleotide position is shown. The shaded squares indicate the three most frequently mutated sites found in HCV isolated from mice treated with the active sshRNAs.

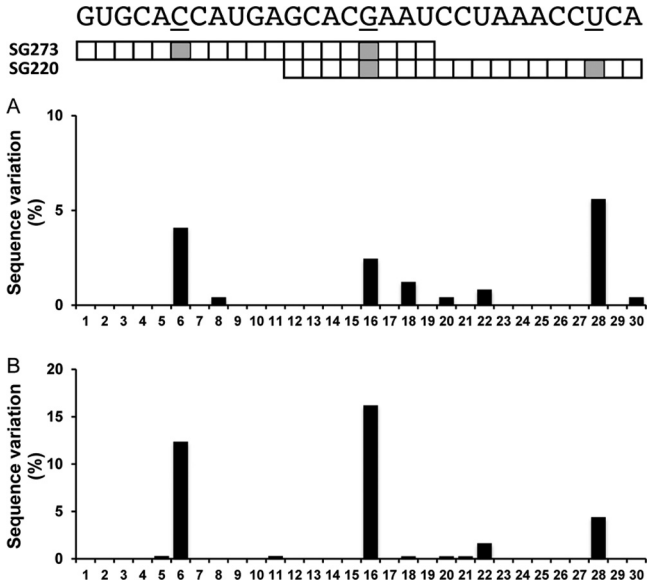

FIG 5 Comparison of natural polymorphism within the sshRNA target region for GT1a (245 confirmed isolates) (A) and GT1b (364 confirmed isolates) (B). Analyses were performed and are displayed as described for Fig. 4.

treatment groups. As expected, the double mutation of SG220/S5 and SG220/S17 did attenuate SG220 silencing.

In contrast to SG220, the less potent SG273 was vulnerable to inactivating mutations outside as well as inside its seed region: both SG273/S16 (within the SG273 seed region) and SG273/S6 (outside the seed region) attenuated SG273 silencing activity, although the seed mutation S16 had a stronger effect (Fig. 3C). This finding is consistent with the chimeric mouse results, where mutations emerged at either position upon SG273 treatment, indicating that each mutation was sufficient to confer resistance to SG273 *in vivo*.

Treatment of HCV-infected mice with the SG220-SG273 combination induced the selection of sequence variants at the SG220 binding site, including the region overlapping the SG273 binding site (where mutations selected by SG220 alone were rare), but not the region unique to SG273 (Fig. 2). Of these variants, the SG220/S17 mutation affected the *in vitro* silencing activity of the sshRNA combination to an extent similar to that of SG220 alone (Fig. 3D). As noted above, the SG220/S5 (equivalent to SG273/S16) mutation alone had no measurable impact on the activity of SG220 (Fig. 3B) and did not significantly reduce the silencing effect of the sshRNA combination (Fig. 3D), whereas it did confer resistance to SG273 (Fig. 3C). The SG220/S5/S17 double mutant reduced the silencing activity of the sshRNA combination to an extent similar to that of SG220 alone (compare Fig. 3B to D). Taken together with the clustering of mutations in the overlap region with the combination treatment, these results suggest that the gene-silencing activity of the sshRNA combination was due predominantly to SG220, the more potent of the two sshRNAs, but SG273 helped narrow the possibilities for mutational escape.

Polymorphism analysis of target site revealed potential mechanism of resistant variant selection. To estimate the relative barriers to resistance for the two sshRNAs tested here, we investigated the conservation of the target sequences of the HCV sshRNAs in clinical isolates. A total of 609 nonredundant and confirmed genotype 1 (GT1a and GT1b combined) HCV sequences were collected from the EU HCV database (http://euhcvdb.ibcp.fr/euHCVdb/). These sequences were analyzed with respect to the 30-nt sequence representing the target sequences of the two sshRNAs used in this study, using a custom script to count the polymorphisms at each position of the 30 nucleotides. The results are shown in Fig. 4. Strikingly, the three nucleotide positions with the highest frequency of sequence polymorphism in clinical isolates in the 30-nt region coincide with the three most frequent mutations induced by sshRNA treatment: 8.7% of the GT1 sequences in the databases contain variants at the SG273/S6 position (the most common mutation in the SG273-treated group), 4.8% of the sequences contain variants at the SG220/S17 position (the most common mutation in the SG220-treated group), and 10.7% of the sequences contain variants at the SG220/S5 (SG273/S16) position (the most common mutation in the combination treatment group). These three nucleotide positions remain the most polymorphic sites when GT1a and GT1b sequences are separately analyzed against the 30-nt sequence, although the three sites are slightly more conserved in GT1a (Fig. 5A) than GT1b (Fig. 5B). The natural polymorphism at these sites suggests that sequence changes at these sites have less impact on replication fitness than changes at the more conserved sites; therefore, they are preferentially selected for. This, together with the reverse genetic characterization results presented in Fig. 3, indicates that the mechanism of selection of HCV sshRNA resistance *in vivo* is similar to that of other direct-acting antivirals: mutation of the DAA binding site leads to reduced drug binding affinity or binding effectiveness and, as a result, drug resistance.

DISCUSSION

In recent studies, we investigated the *in vivo* efficacy of LNP-formulated sshRNAs targeting the HCV IRES (22, 29). Our results showed that, consistent with their potent *in vitro* inhibition of HCV IRES activity, these sshRNAs induced potent and rapid inhibition of HCV IRES-dependent reporter gene expression in the mouse liver (29) and also efficiently inhibited HCV replication in the uPA-SCID human hepatocyte chimeric mouse model (22). Several features of these mice make them a good model for the human disease. First, the uPA-SCID mice used in this study supported high levels of HCV replication. The HCV RNA titer in these mice (serum HCV RNA in the range of 10^7 to 10^8 copies/ml) was comparable to or higher than that typically observed in pa-

tients infected with HCV genotype 1. It has been reported that the human hepatocytes in the uPA-SCID chimeric mouse model have the capacity to produce HCV virions at a rate comparable to that of the hepatocytes in chronically infected patients (33). Second, data generated with direct-acting antivirals in this mouse model have correlated well with the intrinsic clinical antiviral activity of those compounds in HCV-infected patients (33, 37). The *in vivo* efficacy of the HCV sshRNAs reported previously (22, 29), together with the resistance selection profile described here, establishes sshRNAs as a novel class of direct-acting antiviral agents and represents, to the best of our knowledge, the first demonstrations of HCV inhibition by synthetic RNAs acting through an RNAi mechanism in a robust HCV infection model. Importantly, the RNA interference effect of LNP-formulated sshRNA treatment was long lasting, suggesting that such compounds could be effective even with infrequent dosing. In the chimeric mice treated with SG220 (at both 2.5 and 5.0 mg/kg) or with the combination of SG220 and SG273, the serum HCV viral load increased slowly over the 3 weeks of follow-up after the end of dosing. The mean viral load remained about 1 $\log_{10}$ lower than the baseline and the control groups 21 days after the second and last treatments (22).

Treatment of the HCV-infected chimeric mice with HCV-targeted sshRNA effectively selected for drug-resistant HCV variants. The selected sequence changes were restricted to the target regions of the sshRNAs, demonstrating that the effect was sequence specific. Interestingly, treatment with the less potent molecule SG273 selected mainly HCV variants with single point mutations in the sshRNA binding site (Table 1 and Fig. 2C), whereas with the more potent SG220 molecule (or the combination of both sshRNAs) the majority of the clones obtained had acquired 2 or even 3 mutations (Table 1 and Fig. 2A, C, and D). In the combination treatment group, the mutations clustered to the region of overlap of the target regions of SG220 and SG273 as well as the region unique to SG220. These results are consistent with SG220 having exerted a higher selection pressure on HCV than SG273, as expected based on its greater potency both *in vitro* and *in vivo* (22, 24), but with SG273 contributing additional selection pressure in the combination so as to require that escape mutations be directed to the overlap region. Interestingly, mutations in the overlap region that were selected by the combination treatment appeared infrequently (1 out of 82 clones) in mice treated with SG220 alone, although they were more common in the mice receiving the less potent SG273 alone (13 out of 43 clones had the G16 position of SG273 mutated). This correlates with the *in vitro* assay showing that a single mutation of G350A in the overlap region had no observable effect on the silencing activity of SG220 but severely attenuated the silencing activity of SG273 (Fig. 3B and C).

The target region of SG220 and SG273 lies within the sequence common to both the IRES and the 5′ end of the coding region of the core protein. Together with the 5′ nontranslated region (NTR), these sequences are among the most conserved regions of HCV (13, 38). The HCV core protein forms the viral nucleocapsid. Its N-terminal domain is highly basic and has been shown to have RNA binding activity (38, 39). Whereas most IRES require only the 5′-NTR for translation initiation, several studies have shown that efficient initiation of translation with the HCV IRES is dependent upon the sequence downstream of the initiation AUG codon (40–43). The N-terminal sequence of the core protein has a cluster of basic residues that are important for HCV infectivity (44). While extensive mutagenesis of these residues to alanine knocks down core protein expression to undetectable levels, alanine-scanning mutants limited to amino acids in the sequence targeted by SG220/SG273 had only a slight effect on core expression (44). In addition, the nucleotide sequence that forms the N terminus of the core protein contains an adenosine-rich region, which has been shown to interact directly with an IRES *trans*-acting factor called NSAP1. In addition to binding to this A-rich region, NSAP1 also interacts with the 40S subunit and is important for correct positioning of the start codon in the translation initiation complex (45, 46). Interestingly, introduction of silent mutations that convert A residues in this residue to G dramatically reduced binding activity of NSAP1 to HCV RNA (45). Since the region targeted by SG220 and SG273 comprises only a part of the A-rich element, the mutations of A residues we observe may not be sufficient to severely reduce the binding affinity of NSAP1. There were not any observed mutations from nucleotides 345 to 349, which make up codons S_2 and the first 2 nucleotides of T_3. The amino acids coded by these residues not only are conserved (38) but also are part of the base-paired stem that closes the loop containing the initiation codon, suggesting that this conserved stem structure is important for translation initiation. Indeed, Honda et al. found in a mutagenesis study that the internal stability of this stem loop is inversely correlated with efficiency of initiation of translation and may serve a regulatory function (47). Interestingly, we did not find any mutations in the nucleotides that comprise the other strand of the stem (335 to 338) that are targeted by SG273, so no double compensatory mutations were observed in sequence space targeted by 2 different sshRNAs. Nevertheless, most (206/252) of the observed escape mutations occur at the third positions of core codons and do not alter the amino acid sequence. This is also consistent with the previous observation that, across all genotypes, most of the frequently observed changes in the coding region occur at synonymous sites (13).

Our analysis of over 600 sequences of HCV genotype 1 revealed that the resistant variants selected in the chimeric mice coincide with the most highly polymorphic sites within the target region. Other recent studies using the chimeric mouse HCV infection model also generated drug-resistant variants similar to those observed in treated patients with chronic HCV infection (37, 48, 49). Preferential selection of resistance mutations at polymorphic sites suggests that those mutants are associated with reasonable replication fitness, a feature that could be tested in competitive replication assays (6, 50). The frequency of sequence polymorphism in regions encoding the targets of direct-acting antiviral agents can have a clear impact on the rate of resistance selection and the clinical efficacy of these drugs.

It has been shown that siRNAs and shRNAs can inhibit gene expression nonspecifically by mechanisms such as activation of innate immunity, which can result in cytotoxicity from induction of interferon and inflammatory cytokines (51–54). Abrogation of such unwanted effects is important for the development of RNAi agents for therapeutic use, so care must be taken to avoid the misinterpretation of gene silencing resulting from nonspecific or off-target effects (reviewed in reference 55). The induction of innate immunity is influenced by both RNA structure and sequence (52, 56). Because sshRNAs have a novel structural design, it is critical to verify that these molecules act through an RNAi mechanism of action and carefully examine their potential to induce unwanted immune stimulation if they are to be considered for therapeutic applications. We have previously shown that this is

the case in cultured cells, where chemically modified sshRNAs specifically knock down their intended target genes via an RNAi mechanism without immune stimulation (24). Furthermore, the RNA target was cleaved at the site expected for an RNAi mechanism in cells treated with these sshRNAs, and the sshRNAs could be immunoprecipitated in association with Ago2 (28), a key component of RNA-induced silencing complex (RISC). We next showed that LNP-formulated sshRNAs induce sequence-specific inhibition of HCV with little or no immune stimulatory effect *in vivo* (22, 29). In this study, we provide further, definitive evidence that this *in vivo* anti-HCV activity is a specific, RNAi-mediated effect by showing that mutations of drug-resistant HCV variants selected during *in vivo* treatment are limited to sequences targeted by the sshRNAs, and that these mutations confer resistance to the sshRNAs *in vitro*. Together, these results demonstrate convincingly that the sshRNAs used act specifically on the HCV RNA through an RNAi mechanism *in vivo*.

The ability of viruses with error-prone polymerases to escape inhibition of siRNA and shRNA agents *in vitro* is well known (57–65). Strategies for mitigating resistance selection include targeting highly conserved target sites, increasing the inhibitory quotient, and using combination therapy. The latter approach could combine drugs of different mechanisms, such as small-molecule DAAs and interfering RNA, or employ a mixture of interfering RNAs (66). In the present context, additional sshRNAs could be combined with SG220 and SG273 to target the most common escape mutants revealed in our study or to target independent, conserved sites. Such combinations of siRNAs targeting different conserved regions have been shown to mitigate the risk of resistance *in vitro* (60, 67). In the case of morbilliviruses, a combination of three siRNAs targeting conserved regions was found to prevent viral escape, but two were insufficient (67). In that study, the emergence of escape mutants was delayed by 6 to 13 passages when two siRNAs were simultaneously delivered in the cell culture, and the association of three siRNAs prevented any escape over 20 passages in cell culture. A similar result was obtained by Kusov et al. (68), who efficiently prevented the selection of resistant HIV-1 variants by using siRNAs targeting various sites in the HIV-1 nonstructural genes.

Because even treatment of HCV-infected chimeric mice with a combination of 2 HCV-targeted sshRNAs effectively selected for drug-resistant HCV variants, a successful sshRNA-based therapy against HCV will probably require a cocktail of more than 2 sshRNAs targeting highly conserved sequences. Oligonucleotide delivery agents are continually being improved, with the latest generation of LNP effective at doses well below 1 mg/kg (15, 69). This effectively reduces nonspecific toxicity and allows more agents to be combined while maintaining an effective dose of each component. Whether such cocktails consist only of sshRNAs or sshRNAs combined with small-molecule DAAs, the potent and effective reduction of HCV replication and the favorable tolerability profile we observe with one or two doses of LNP-formulated sshRNAs suggest that sshRNAs have the potential to become effective components of highly active treatment regimens for HCV infection.

In summary, we have investigated the *in vivo* efficacy of LNP-formulated sshRNAs targeting the HCV IRES (22, 29) as a novel RNAi-based therapeutic modality. Our results showed that, consistent with their potent *in vitro* inhibition of HCV IRES activity, these sshRNAs induced potent and rapid inhibition of HCV IRES-dependent reporter gene expression in mouse liver (29) and also efficiently inhibited HCV replication in the uPA-SCID human hepatocyte chimeric mouse model (22). The *in vivo* efficacy of the HCV sshRNAs reported in the studies described above (22, 29) together with the resistance selection profile described here establish sshRNAs as a novel class of DAA and represent, to the best of our knowledge, the first demonstrations of HCV inhibition by synthetic RNAs acting through an RNAi mechanism in a robust HCV infection model.

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