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Formulated Minimal-Length Synthetic Small Hairpin RNAs Are Potent Inhibitors of Hepatitis C Virus in Mice With Humanized Livers

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Abstract

Short synthetic small-hairpin RNAS (sshRNAs) (SG220 and SG273) that target the internal ribosome entry site of the hepatitis C virus (HCV) were formulated into lipid nano-particles and administered intravenously to HCV-infected urokinase plasminogen activator–severe combined immunodeficient mice with livers repopulated with human hepatocytes (humanized livers). Weekly administration of 2.5 mg/kg of each sshRNA for 2 weeks resulted in a maximal mean reduction in viral load of 2.5 $\log_{10}$ from baseline. The viral load remained reduced by more than 90% at 14 days after the last dose was given. The sshRNAs were well tolerated and did not significantly increase liver enzyme levels. These findings indicate the in vivo efficacy of a synthetic RNA inhibitor against the HCV genome in reducing HCV infection.

Keywords

Short shRNA; uPA-SCID Chimeric Mice; RNA Interference; siRNA; HCV

Hepatitis C virus (HCV) infection is a leading cause of chronic liver diseases including cirrhosis and hepatocellular carcinoma.[1] The vast number of HCV quasispecies present in any infected individual results in a high potential for developing resistance to direct-acting antiviral agents, which can lead to treatment failure.[2,3] Thus, most investigational new treatments of chronic HCV infection use a combination of molecules with complementary mechanisms and nonoverlapping resistance profiles. RNA interference (RNAi) potentially offers such a therapeutic approach by directly targeting multiple sites on viral RNA. Here,

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Supplementary Material
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Author names in bold designate shared co-first authorship.

Conflicts of interest
The authors disclose the following: Han Ma and Klaus Klumpp are employees of Hoffmann-La Roche, Inc; Brian Johnston, Anne Dallas, Heini Ilves, and Joshua Shorenstein are employees of SomaGenics, Inc; and Ian MacLachlan is an employee of Tekmira Pharmaceuticals.

we report the potent, durable, and specific inhibition of HCV infection in vivo by 2 short synthetic hairpin RNAs (sshRNAs) formulated with lipid nanoparticles (LNPs).[4]

The sshRNAs (SG220 and SG273), whose in vitro activity was characterized previously,[5,6] target a conserved region within the internal ribosome entry site (IRES) of the HCV genome (Supplementary Figure 1*A*) with perfect target complementarity to genotypes 1a, 1b, 4a, and 5a, a single mismatch to genotype 2a,[7] and more than one mismatch to genotypes 3 and 6. sshRNAs induce RNAi by a noncanonical mechanism in which intact hairpins are loaded into RISC and are activated by Ago2-mediated slicer processing of the passenger arm without Dicer cleavage.[8] sshRNAs also differ from siRNAs in consisting of a single molecular entity and having a loop that hinders off-target activity by the passenger strand.[8] Both sshRNAs induced potent knockdown of IRES-directed firefly luciferase reporter gene activity in HEK-293FT cells without significant in vitro or in vivo immune-stimulatory activity,[5,9] and LNP-formulated SG220 was shown to effect prolonged knockdown in an HCV IRES-dependent bioluminescence mouse model.[9,10]

Chimeric urokinase-type plasminogen activator-severe combined immunodeficiency (uPA-SCID) mice with human hepatocyte reconstitution have been used previously to show the antiviral potency of various HCV inhibitors known to be effective in HCV-infected patients.[11–13] To test sshRNAs in this model, we first examined the uptake of the LNP-formulated sshRNA SG220 in chimeric uPA-SCID mice and compared it with that of regular SCID mice. Results obtained from 3 chimeric and 3 regular SCID mice showed similar levels of sshRNA in the livers of the chimeric and regular SCID mice, as determined by a ribonuclease protection assay (RPA) (Figure 1, lanes 4–6 and 8–10). The specificity of the RPA for SG220 was shown by the lack of protection of the probe by total liver RNA from the phosphate-buffered saline (PBS)-treated mice (Figure 1, lane 2). By comparing the band intensities of the protected probe with those of SG220 standards (Figure 1, lanes 11–14), it was determined that 5.9% ± 1.4% (mean ± SD) of the total injected SG220 remained in the chimeric mouse liver 48 hours after treatment, compared with 5.1% ± 0.7% in the SCID mouse liver (Supplementary Table 1). The presence of abundant sshRNA in the liver 48 hours after treatment suggests that the human hepatocytes in the chimeric mouse liver can efficiently take-up LNP-formulated sshRNA and that significant amounts remain intact. This is consistent with previous reports in which LNPs designed for efficient delivery to liver have been shown to accumulate 70%–80% of the total injected dose in the liver of mice within the first 24 hours after intravenous administration[14] and to be readily taken up by hepatocytes by virtue of receptor-mediated endocytosis mediated by the in vivo adoption of endogenous apolipoprotein E, a ligand for the low-density lipoprotein–receptor family of cell surface receptors.[15]

We next assessed the activity of the 2 sshRNAs against HCV replication in chimeric uPA-SCID mice that had high proportions of human hepatocytes (serum human albumin level, ≥8 mg/mL in all mice) and were stably infected with genotype 1a HCV (serum HCV-RNA level, $\geq 4.0 \pm 10^6$ IU/mL in all mice) (Supplementary Table 2). Four mice in each group received 2 intravenous injections, given 1 week apart, of LNP-formulated SG220, SG273, a combination of SG220 and SG273, or an irrelevant (scrambled) sshRNA at 2.5 or 5.0 mg/kg (Figure 2*A* and *B*). Serum HCV viral titer was monitored twice every week. All mice receiving HCV-specific sshRNAs had more than a 90% (1 $\log_{10}$) reduction in serum HCV-RNA concentration after a single dose. Treatment with 2.5 mg/kg SG220 or SG273 led to a 1.8 $\log_{10}$ or 1.2 $\log_{10}$ mean reduction in HCV-RNA concentration, respectively, at 72 hours after the first injection ($P < .01$; Figure 2*A* and *B*). A higher dose of SG220 (5 mg/kg) did not lead to a further reduction in viral load (Figure 2*A*). 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 concentration observed 72 hours after the first dose and an

additional 0.5 $\log_{10}$ reduction 7 days after the second dose ($P < .01$; Figure 3*B*). The inhibition of HCV replication by HCV sshRNA was specific because the serum HCV viral load in mice treated with the LNP-formulated irrelevant control sshRNA at 5 mg/kg did not change significantly at any time point during the study.

The mean viral load reduction in all HCV sshRNA-treated groups was durable. In mice treated with either 2.5 mg/kg SG220 or the combination of SG220 and SG273, the HCV serum viral load remained significantly lower ($P < .01$) than the pretreatment level, up to the last study time point 3 weeks after the second and last doses (Supplementary Table 3). In the case of 2 mice whose pretreatment HCV serum RNA levels were less than 10^7, viral load remained 1.5 and 2.6 $\log_{10}$ below pretreatment levels at 3 weeks after treatment (Supplementary Figure 2). In the mice treated with SG273 alone, the HCV serum viral load remained significantly lower relative to the pretreatment level up to 2 weeks after the second dose ($P < .05$) (Figure 2*B*, Supplementary Table 3).

LNP-formulated sshRNAs were well tolerated. There was no treatment-related increase of liver alanine aminotransferase or aspartate aminotransferase levels in any of the treatment groups or a reduction in serum human albumin concentration (Figure 2*C*), indicating the absence of significant hepatocyte toxicity during the dosing and follow-up periods. There was also no treatment-associated body weight loss and no observations of morbidity or mortality in the study animals. These results, together with the lack of antiviral effect of the irrelevant sshRNAs, suggest that the reduction of serum HCV-RNA concentration by the HCV-targeting sshRNAs was specific.

There have been reports of RNAi-mediated inhibition of HCV in HCV subgenomic replicon systems and cell culture–based infectious HCV systems.[16–18] However, it is not known if RNAi can achieve clinically significant levels of antiviral efficacy against chronic HCV infection in vivo. Our results show that the LNP-formulated[4] HCV targeting sshRNAs efficiently inhibit HCV replication in the uPA-SCID chimeric mice, a model that supports high levels of HCV replication. The RNAi effect of LNP-formulated sshRNA treatment was long-lasting, suggesting that such agents could be effective even with infrequent dosing. The fact that SG220 showed higher potency than SG273 both in vitro and in vivo is consistent with a direct-acting antiviral mechanism of action.[5,6,8] This study reports inhibition of HCV virus replication by synthetic RNAs through an RNAi mechanism in a robust HCV infection model, and adds HCV to the small group of viruses shown to be targetable with RNAi.[19]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Brian Johnston, Han Ma, Anne Dallas, Ian MacLachlan, Heini Ilves, and Klaus Klumpp designed the study; Anne Dallas, Heini Ilves, and Joshua Shorenstein performed the research; Ian MacLachlan provided lipid nanoparticle formulation of short synthetic hairpin RNAs; Han Ma, Anne Dallas, Brian Johnston, Klaus Klumpp, and Heini Ilves analyzed and interpreted the data; and Han Ma, Brian Johnston, Anne Dallas, and Klaus Klumpp wrote the manuscript.

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Abbreviations used in this paper

HCV	hepatitis C virus
IRES	internal ribosome entry site
LNP	lipid nanoparticle
PBS	phosphate-buffered saline
RNAi	RNA interference
RPA	ribonuclease protection assay
sshRNA	short synthetic hairpin RNA
uPA-SCID	urokinase-type plasminogen activator-severe combined immunodeficiency

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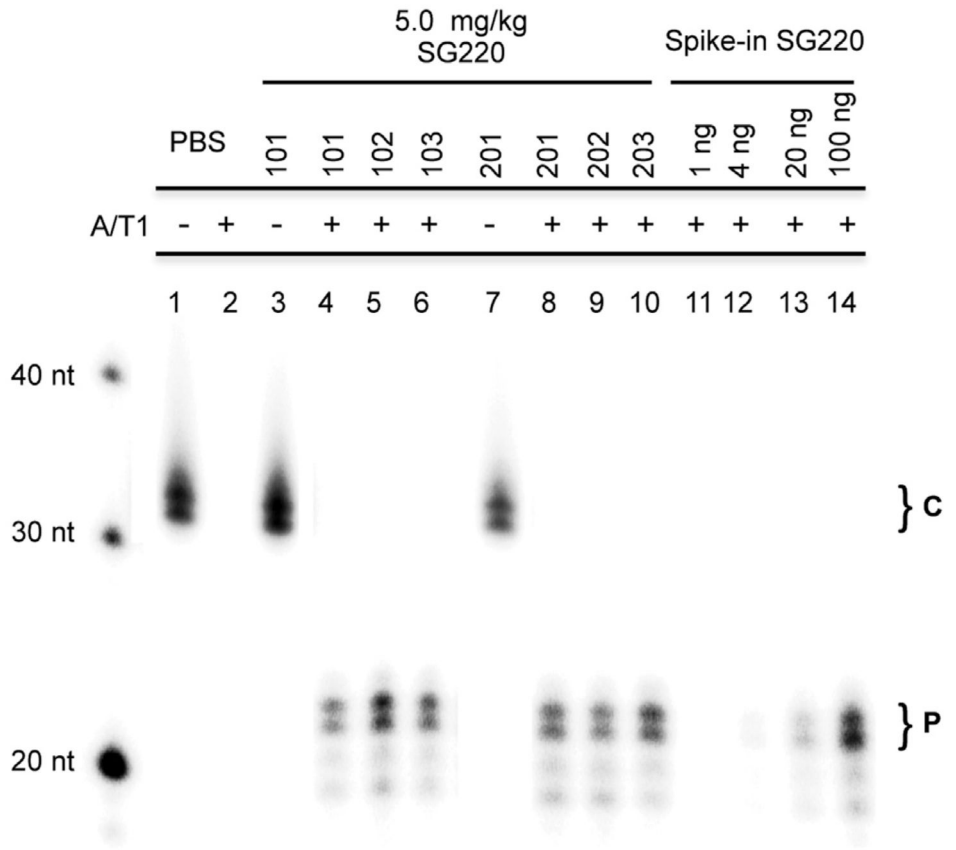

Figure 1.

Liver uptake of sshRNA in chimeric mice and regular SCID mice. RNAse protection assay using total liver RNA from chimeric mice (101–103, *lanes 3–6*) or SCID mice (201–203, *lanes 7–10*). Indicated amounts of SG220 were spiked into 50 μg total RNA from untreated mice (*lanes* 11–14) to generate a calibration curve for quantification of liver uptake of SG220. C, undigested probe; P, RNAse digested probe.

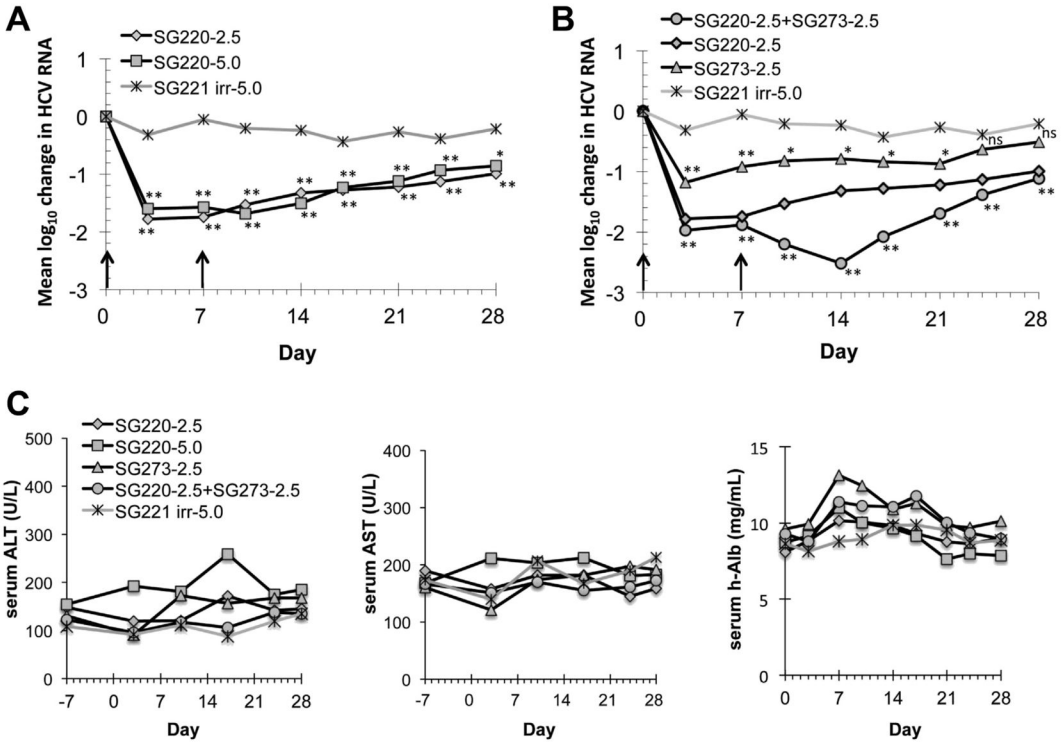

Figure 2.

Specific and durable inhibition of HCV replication in chimeric mice. Chimeric mice (n = 4) infected with genotype 1a HCV were dosed intravenously with each of the indicated sshRNAs on days 0 and 7 (*arrows*). (*A*) Mean reduction in serum HCV-RNA level from 2.5 and 5.0 mg/kg doses of SG220. (*B*) Comparison of SG220, SG273, and a combination of both. (*C*) Lack of effect of sshRNA treatments on liver alanine aminotransaminase (ALT), aspartate aminotransferase (AST), and human albumin (h-Alb) levels. ***P* < .01; **P* = .01–.05.