# Inhibition of Hepatitis C IRES-Mediated Gene Expression by Small Hairpin RNAs in Human Hepatocytes and Mice

HEINI ILVES,[a] ROGER L. KASPAR,[a] QIAN WANG,[b] ATTILA A. SEYHAN,[a] ALEXANDER V. VLASSOV,[a] CHRISTOPHER H. CONTAG,[b,c] DEVIN LEAKE,[d] AND BRIAN H. JOHNSTON[a,c]

[a]*SomaGenics, Inc., Santa Cruz, California 95060, USA*

[b]*Molecular Imaging Program at Stanford (MIPS), and Departments of Radiology, Microbiology & Immunology, Stanford University School of Medicine, Stanford, California, USA*

[c]*Department of Pediatrics, Stanford University School of Medicine, Stanford, California, USA*

[d]*Dharmacon RNA Technologies, LaFayette, Colorado 80026, USA*

**ABSTRACT: The ability of small hairpin RNAs (shRNAs) to inhibit hepatitis C virus internal ribosome entry site (HCV IRES)-dependent gene expression was investigated in cultured cells and a mouse model. The results indicate that shRNAs, delivered as naked RNA or expressed from vectors, may be effective agents for the control of HCV and related viruses.**

**KEYWORDS: hepatitis C virus; shRNA; siRNA; RNA interference**

Viral Hepatitis C is principally a disease of inflammation of the liver, and 70% of patients infected with the hepatitis C virus (HCV) develop chronic liver disease, including cirrhosis and hepatocellular carcinoma. HCV infection afflicts 3.9 million people in the United States (175 million worldwide) and is the primary indication for liver transplants in the United States. RNA interference (RNAi)-mediated gene inhibition has been shown to robustly inhibit gene expression in a number of mammalian systems.[1] Despite its high degree of sequence conservation, the HCV internal ribosome entry site (IRES) would appear *a priori* to be a poor target for RNA interference due to the high proportion of its residues involved in secondary and tertiary folding. Several groups have recently reported, however, some success targeting the HCV IRES in 293FT and Huh7 tissue culture cells (reviewed in Ref. 2).

We have investigated the ability of small hairpin RNAs (shRNAs), delivered directly or expressed from pol III promotors, as well as synthetic siRNAs to

Address for correspondence: Brian H. Johnston, SomaGenics, Inc., 2161 Delaware Ave., Santa Cruz, CA 95060. Voice: 831-426-7700; fax: 831-420-0685.
e-mail: bjohnston@somagenics.com

**Ann. N.Y. Acad. Sci. 1082: 52–55 (2006).** 
**doi: 10.1196/annals.1348.060**

| shRNA abbreviation | Antisense sequence (5'-3') | Target site on HCV IRES | fLuc/SEAP (at 1 nM shRNA) |
|---|---|---|---|
| HCV #1 | UCAUACUAACGCCAUGGCUAGACGC | 75-99 | 62% |
| HCV #2 | CCGGUUCCGCAGACCACUAUGGCUC | 135-159 | 35% |
| HCV #3 | CCUCCCGGGGCACUCGCAAGCACCC | 299-323 | 88% |
| HCV #4 | UGGUGCACGGUCUACGAGACCUCCC | 318-342 | 36% |
| HCV #5 | UGGUGCACGGUCUACGAGACCUC | 320-342 | 40% |
| HCV #6 | CUCAUGGUGCACGGUCUACGAGAC | 323-346 | 42% |
| HCVa-wt (#7) | UCUUUGAGGUUUAGGAUUCGUGCUC | 344-368 | 9% |
| HCV #8 | GGUUUUUCUUUGAGGUUUAGGAUUC | 350-374 | 10% |

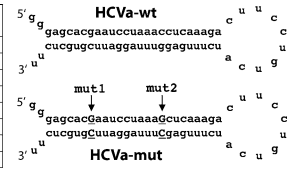


**FIGURE 1.** Left panel: Representative shRNA constructs assayed in the screen for potent inhibitors of HCV IRES-dependent gene expression. Antisense strands are shown with target sites on HCV IRES genotype 1a, along with the inhibitory activity of the constructs at 1 nM (for details see legend to FIG. 2). Right panel: Structure of the most potent shRNA inhibitor found, HCVa-wt. HCVa-mut1 and HCVa-mut2 contains 1 bp change, and HCVmut contains both sets of changes. All shRNAs used in the study contained the same loop sequence as well as 5′-GG and 3′-UU overhangs.

inhibit HCV IRES-dependent gene expression in cultured cells and a mouse model. We used a reporter gene plasmid in which firefly luciferase (fLuc) expression is dependent on the HCV IRES.[3] A number of shRNAs directed at various regions of the IRES were generated by *in vitro* transcription using T7 RNA polymerase (FIG. 1, left). All shRNAs had a loop derived from microRNA-23, a duplex stem of 23–25 base pairs (bp), plus 5′-GG and 3′-UU overhangs that may interact to form GU (wobble) bp (FIG. 1, right). Transfection experiments revealed the most effective shRNA, designated HCVa, which has a 25-bp stem and targets the 3′ end of the HCV IRES, near the AUG translation start site. At a concentration of 1 nM, this molecule inhibits HCV IRES-dependent luciferase expression from a cotransfected vector in 293FT cells (∼90% knockdown).[3] As shown in FIGURE 2, HCVa was also highly effective in human Huh7 hepatocytes, with an $IC_{50}$ of approximately 25 pM (FIG. 2, left). Control shRNAs containing a double mutation had little or no effect on fLuc expression, and shRNAs containing single mutations showed partial inhibition (FIG. 2, right).

shRNAs must be processed by Dicer before they can productively enter the RNA-induced silencing complex (RISC) and lead to target inhibition.[1] However, siRNAs of minimal length (∼19 bp), which are most commonly used for routine gene silencing, require no such processing. To determine how shRNAs compare to minimal length siRNAs, we decided to test all possible 19-bp sequences whose target site lies within the 25-nt target site of HCVa (FIG. 3), in both siRNA and shRNA formats. Each of these RNAs showed activity at 1 nM concentration in 293FT cells, with some variability. However, the shRNAs were generally less effective than the siRNAs in this comparison, in contrast to the mouse model where shRNAs show superior efficacy, as discussed below. The poorer performance of the 19-bp shRNAs might be at least partially due to the fact that they still presumably require processing to eliminate the loop, yet may be too short to bind well to Dicer.

The inhibitors found to be active in cell culture were also evaluated in a mouse model by using *in vivo* bioluminescent imaging.[4] The fLuc expression

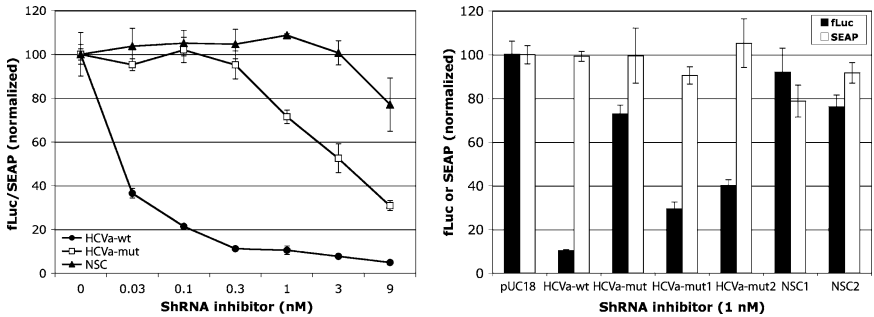


**FIGURE 2.** Inhibition of HCV IRES-dependent gene expression in human Huh7 hepatocytes. Huh7 cells (1.7 × 10(5) cells/well) were cotransfected (LipofectAmine 2000; Invitrogen, Carlsbad, CA) with 40 ng pHCV Dual Luc reporter construct, 50 ng pSEAP2 (as a transfection and specificity control), and the indicated amounts of shRNA inhibitors. pUC18 plasmid was added to the transfection mixture to give a total nucleic acid concentration of 800 ng per well (24-well tissue culture plates). Forty-eight hours later, supernatant was removed for SEAP analysis, and the cells were lysed and fLuc activity was measured. The data are presented as luciferase divided by SEAP activity, normalized to the pUC18 control (100%). All data were generated from individual, independent experiments performed in triplicate. NSC, nonspecific control.

plasmid, the shRNAs (or plasmid vectors expressing them), and a plasmid expressing secreted alkaline phosphatase (SEAP) were delivered to cells in the liver by hydrodynamic injection via the tail vein,[3] which leads to expression primarily in the liver. The animals were imaged following injection of luciferin at time points of 6–120 h post-injection. The results showed that the shRNA HCVa, either introduced directly or expressed by pol III from a plasmid vector, inhibited HCV IRES-dependent fLuc expression at all time points, whereas

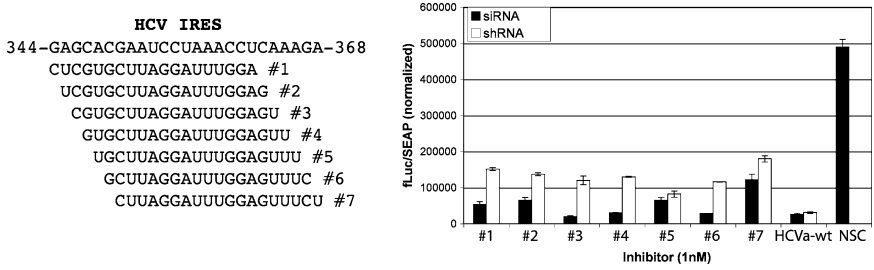


**FIGURE 3.** Inhibitory efficacy of all seven possible 19-bp synthetic siRNAs and *in vitro*-transcribed 19-bp shRNAs contained within the 25 nt target site for HCVa. Left panel: Target sites on the HCV IRES for the seven assayed 19-bp siRNAs and shRNAs. Right panel: 293FT cells were cotransfected with pHCV Dual Luc reporter construct, pSEAP2, and 1 nM of the indicated si/shRNA and assayed for fLuc expression (for details see legend to FIG. 2). NSC shRNA was similar to NSC siRNA.

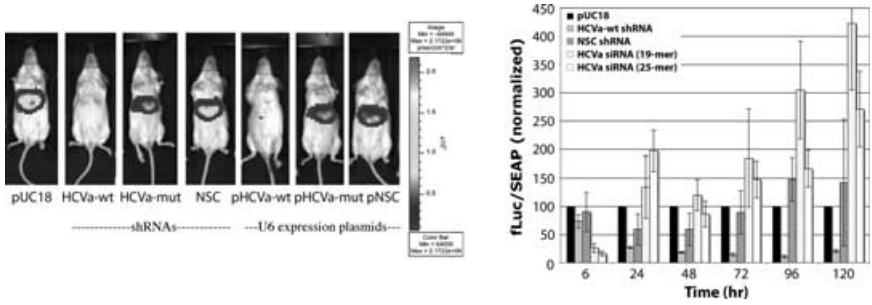


**FIGURE 4.** Inhibition of HCV IRES-mediated reporter gene expression in mice. pHCV Dual Luc reporter (10 μg) and pSEAP2 plasmids were coinjected at constant high pressure into the tail veins of mice with 100 μg of the indicated shRNA inhibitors or their expression plasmids. pSEAP2 provided constitutive expression of SEAP (used as a control for injection and nonspecific effects). NSC is irrelevant shRNA, and pUC18 is used as an additional control. At various time points postinjection, luciferin was administered intraperitoneally and the mice were imaged using the IVIS *in vivo* imaging system (Xenogen, Alameda, CA).[3] Representative mice from the 84 h time point are shown in the left panel; luciferase expression is shown in shaded contours. Images were quantitated using LivingImage software (Xenogen) and the time course is plotted in the right panel (4–5 mice were used per group). A total of 96 h following injection, the mice were bled and the amount of SEAP activity was determined by pNPP assay. The data are presented as luciferase divided by SEAP activity, normalized to pUC18 control mice (100%).

doubly mutated HCVa-mut or irrelevant (NSC) shRNAs had little or no effect (FIG. 4). In contrast, siRNAs showed inhibition at the earliest time point (6 h), but no inhibition was observed at later times. The lack of sustained inhibition by the siRNAs tested might be explained by the limited stability of siRNAs in blood.[1] The presence of the loop structure in the shRNAs may increase stability or may facilitate blood transport and/or cellular uptake. Taken together, these results indicate that shRNAs, delivered as naked RNA or expressed from viral or nonviral vectors, may be effective agents for the control of HCV and related viruses.

## REFERENCES

1. DORSETT, Y. & T. TUSCHL. 2004. siRNAs: applications in functional genomics and potential therapeutics. Nat. Rev. Drug Discov. **3:** 318–329.
2. RADHAKRISHNAN, S.K., T.J. LAYDEN & A.L. GARTEL. 2004. RNA interference as a new strategy against viral hepatitis. Virology **323:** 173–181.
3. WANG, Q. *et al.* 2005. Small hairpin RNA efficiently inhibit hepatitis C IRES-mediated gene expression in human tissue culture cells and a mouse model. Mol. Ther. **12:** 562–568.
4. MCCAFFREY, A.P. *et al.* 2002. RNA interference in adult mice. Nature **418:** 38–39.