

# Activity of Stabilized Short Interfering RNA in a Mouse Model of Hepatitis B Virus Replication

David V. Morrissey, Karin Blanchard, Lucinda Shaw, Kristi Jensen, Jennifer A. Lockridge, Brent Dickinson, James A. McSwiggen, Chandra Vargeese, Keith Bowman, Chris S. Shaffer, Barry A. Polisky, and Shawn Zinnen

To develop synthetic short interfering RNA (siRNA) molecules as therapeutic agents for systemic administration *in vivo*, chemical modifications were introduced into siRNAs targeted to conserved sites in hepatitis B virus (HBV) RNA. These modifications conferred significantly prolonged stability in human serum compared with unmodified siRNAs. Cell culture studies revealed a high degree of gene silencing after treatment with the chemically modified siRNAs. To assess activity of the stabilized siRNAs *in vivo* initially, an HBV vector-based model was used in which the siRNA and the HBV vector were codelivered via high-volume tail vein injection. More than a 3 log<sub>10</sub> decrease in levels of serum HBV DNA and hepatitis B surface antigen, as well as liver HBV RNA, were observed in the siRNA-treated groups compared with the control siRNA-treated and saline groups. Furthermore, the observed decrease in serum HBV DNA was 1.5 log<sub>10</sub> more with stabilized siRNA compared with unmodified siRNA, indicating the value of chemical modification in therapeutic applications of siRNA. In subsequent experiments, standard systemic intravenous dosing of stabilized siRNA 72 hours after injection of the HBV vector resulted a 0.9 log<sub>10</sub> reduction of serum HBV DNA levels after 2 days of dosing. **In conclusion**, these experiments establish the strong impact that siRNAs can have on the extent of HBV infection and underscore the importance of stabilization of siRNA against nuclease degradation. (HEPATOLOGY 2005;41:1349-1356.)

See Editorial on Page 1220

**R**NA interference (RNAi) is a recently discovered cellular mechanism that detects and destroys double-stranded RNA<sup>1</sup> and seems to play a role in the cell's antiviral defense system.<sup>2</sup> Short interfering RNA (siRNA) molecules are approximately 21-nucleotide, double-stranded RNA intermediates of the RNAi mechanism that guide a unique RNAi protein complex termed RNA-induced silencing complex to target RNA, leading to its subsequent degradation. Although in the natural RNAi pathway, siRNAs are derived from long double strand RNA that is processed by the nuclease Dicer into discrete 21-mers,<sup>3,4</sup> introduction of synthetic siRNAs

into the cell also leads to RNAi-mediated silencing of target gene expression.<sup>5</sup> The use of synthetic siRNAs that use the endogenous cellular mechanism to downregulate the expression of disease-related or viral genes may lead to the development of a new therapeutic approach. In this report, we present evidence that synthetic siRNAs inhibit the replication of the hepatitis B virus (HBV) in cell culture and in a mouse model of HBV replication.

Although siRNA has become an effective research tool to downregulate gene expression in cell culture, the inherent instability of RNA limits its potential use for *in vivo* gene silencing. The development of siRNAs as therapeutic agents will likely require improvements in the stability of siRNAs and the efficiency and specificity of tissue-targeted delivery *in vivo*. Chemical modifications made to synthetic siRNAs for the purpose of stabilization not only must provide resistance to nuclease degradation, but also must be compatible with proper recognition and function of the siRNA by cellular RNAi protein machinery.

A potential clinical use of siRNAs is to combat chronic infection by HBV. Chronic HBV afflicts approximately 350 million people worldwide and accounts for 1.2 million deaths annually. In the United States alone, approximately 1.25 million individuals (0.05%) are chronic carriers of HBV.<sup>6</sup> The natural progression of chronic HBV infection over 10 to 20 years leads to liver cirrhosis

Abbreviations: RNAi, RNA interference; siRNA, short interfering RNA; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HDI, hydrodynamic tail vein injection; siNA, short interfering nucleic acid.

From Sirna Therapeutics, Inc., Boulder, CO.

Received December 7, 2004; accepted February 26, 2005.

Address reprint requests to: David V. Morrissey, Ph.D., Sirna Therapeutics, Inc., 2950 Wilderness Place, Boulder, CO 80301. E-mail: morrisseyd@sirna.com; fax: 303-449-8829.

Copyright © 2005 by the American Association for the Study of Liver Diseases.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.20702

Potential conflict of interest: Nothing to report.

in 20% to 50% of patients, and progression of HBV infection to hepatocellular carcinoma has been well documented.<sup>7</sup> Because of the overlapping nature of the HBV pregenomic RNA and the four major transcripts, an siRNA targeted to a single site is capable of cleaving more than one HBV RNA species, and thus has the potential to interfere with numerous processes in the viral life cycle.

To test the potential of synthetic siRNA molecules as therapeutic agents for systemic administration *in vivo*, chemical modifications designed to inhibit degradation by nucleases were introduced into chemically synthesized siRNAs targeted to conserved sites in HBV RNA. We have developed a novel combination of siRNA chemical modifications that permit effective gene silencing in the complete absence of 2'-OH residues. The combination of modifications include 2'-fluoro, 2'-O-methyl, and 2'-deoxy sugars, phosphorothioate linkages, and terminus capping chemistries. This combination of modifications conferred significantly prolonged stability to siRNAs in serum, and studies in an HBV cell culture system showed effective gene silencing by the modified siRNAs. To test the *in vivo* potency of the stabilized siRNA, an HBV vector-based mouse model was used with coadministration of the siRNAs with a HBV vector via high-pressure tail vein injection, resulting in a 3 log<sub>10</sub> decrease in serum HBV DNA levels 3 days after a single administration. In subsequent experiments, standard systemic intravenous dosing of stabilized siRNA 72 hours after injection of the HBV vector resulted in an approximately 1 log<sub>10</sub> reduction of serum HBV DNA levels after 2 days of dosing. This first demonstration of *in vivo* activity of a systemically delivered, modified siRNA in an HBV mouse model illustrates the therapeutic potential of siRNAs.

## Materials and Methods

**Oligonucleotide Synthesis and Characterization.** RNA oligonucleotides were synthesized at Sirna Therapeutics, Inc. (Boulder, CO), using ABI 394 synthesizers and standard phosphoramidite chemistry. Modified RNA oligonucleotides were synthesized, deprotected, and purified as previously described.<sup>8</sup> The integrity and purity of the final compounds were confirmed by standard HPLC, capillary electrophoresis, and MALDI-TOF mass spectrometry methodologies. Oligonucleotides used were more than 80% full length. The siRNA sequences for site 263 were: sense strand, 5'-GGACUUCUCUCAAUU-UUCUTT-3'; antisense strand, 5'-AGAAAAUUG-AGAGAAGUCCTT-3'. The negative control sequences are inverted from 5' to 3'.

**siRNA Annealing.** siRNA strands (20  $\mu$ mol/L each strand) were annealed in 100 mmol/L potassium acetate, 30

mmol/L HEPES-KOH at pH 7.4, and 2 mmol/L magnesium acetate. The annealing mixture was first heated to 90°C for 1 minute and then transferred to 37°C for 60 minutes. Annealing was confirmed by nondenaturing PAGE and melting temperature assessment in 150 mmol/L NaCl.

**Serum Stability Assay.** Oligonucleotides were designed such that standard ligation methods would generate full-length sense or antisense strands. Before ligation, standard kinase methods were used with [ $\gamma$ -<sup>32</sup>P]adenosine triphosphate to generate an internal <sup>32</sup>P label. Ligated material was gel purified using denaturing PAGE. Trace internally labeled sense (or antisense) was added to unlabeled material to achieve a final concentration of 20  $\mu$ mol/L. The unlabeled complementary strand was present at 35  $\mu$ mol/L. Annealing was performed as described above. Duplex formation was confirmed by unmodified PAGE and subsequent visualization on a Molecular Dynamics Phosphorimager (Sunnyvale, CA).

Internally labeled, duplexed or single-stranded siRNA was added to human serum to achieve final concentrations of 90% serum (Sigma, St. Louis, MO) and 2  $\mu$ mol/L siRNA duplex with a 1.5  $\mu$ mol/L excess of the unlabeled single-stranded siRNA. Samples were incubated at 37°C. Aliquots were removed at specified time points and quenched using a 5-second Proteinase K (20  $\mu$ g) digestion (Amersham, Piscataway, NJ) in 50 mmol/L Tris-HCl pH 7.8, 2.5 mmol/L ethylenediaminetetraacetic acid, 2.5% SDS, followed by addition of a 6 $\times$  volume of formamide loading buffer (90% formamide, 50 mmol/L ethylenediaminetetraacetic acid, 0.015% xylene cyanol and bromophenol blue, 20  $\mu$ mol/L unlabeled chase oligonucleotide of the same sequence as the radiolabeled strand). Samples were separated by denaturing PAGE and were visualized on a Molecular Dynamics Phosphorimager. ImageQuant (Molecular Dynamics) software was used for quantitation.

**Cell Culture Studies.** The human hepatoblastoma cell lines Hep G2 was grown in minimal essential Eagle media supplemented with 10% fetal calf serum, 2 mmol/L glutamine, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, and 25 mmol/L HEPES. Replication-competent cDNA was generated by excising and relegating the HBV genomic sequences from the psHBV-1 vector. Hep G2 cells were plated (3  $\times$  10<sup>4</sup> cells/well) in 96-well microtiter plates and were incubated overnight. A cationic lipid-DNA-siRNA complex was formed containing (at final concentrations) cationic lipid (11-15  $\mu$ g/mL), religated psHBV-1 (4.5  $\mu$ g/mL), and siRNA (25 nmol/L) in growth media. After a 15-minute incubation at 37°C, 20  $\mu$ L of the complex was added to the plated Hep G2 cells in 80  $\mu$ L of growth media minus antibiotics. The media was removed from the cells 72

hours after transfection for hepatitis B surface antigen (HBsAg) analysis. All transfections were performed in triplicate.

**HBsAg Enzyme-Linked Immunosorbent Assay.** Levels of HBsAg were determined using the Genetic Systems/Bio-Rad (Richmond, VA) HBsAg enzyme-linked immunosorbent assay kit, according to the manufacturer's instructions. The absorbance of cells not transfected with the HBV vector was used as background for the assay, and thus subtracted from the experimental sample values.

**HBV Vector-Based Mouse Model.** To assess the activity of chemically stabilized siRNAs against HBV, systemic dosing of the siRNA was carried out after hydrodynamic tail vein injection (HDI) of HBV vector in mouse strain C57BL/J6. The HBV vector used, pWTD, is a head-to-tail dimer of the complete HBV genome.<sup>9</sup> For a 20-g mouse, a total injection of 1.6 mL containing pWTD in saline, was injected into the tail vein within 5 seconds. For a larger mouse, the injection volume was scaled to 140% of the blood volume of the mouse. For studies in which the HBV vector and siRNA were coinjected, 1  $\mu$ g of vector and 0.03 to 1  $\mu$ g of siRNA were used. In experiments in which systemic dosing of the siRNA by conventional intravenous injection followed the HDI of the HBV vector, 0.3  $\mu$ g of vector was used. Systemic dosing of siRNAs was at 0.3 to 30 mg/kg thrice daily. To allow recovery of the liver from the disruption caused by HDI, systemic dosing was started 72 hours after HDI.

**HBV DNA Analysis.** Viral DNA was extracted from 50  $\mu$ L mouse serum using QIAmp 96 DNA Blood kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. HBV DNA levels were analyzed using an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, CA). Quantitative real-time polymerase chain reaction was carried out using the following primer and probe sequences: forward primer, 5'-CCTGTAT-TCCCATCCCATCGT (HBV nucleotides 2006-2026); reverse primer, 5'-TGAGCCAAGAGAAACGGACTG (HBV nucleotide 2063-2083); and probe FAM, 5'-TTCGCA AAATACCTATGGGAGTGGGCC (HBV nucleotide 2035-2062). The psHBV-1 vector containing the full-length HBV genome was used as a standard curve to calculate HBV copies per milliliter of serum.

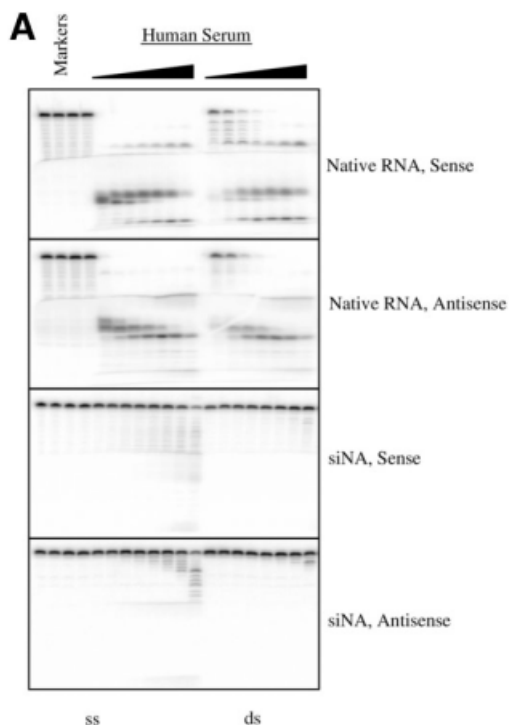
**HBV RNA Analysis.** Total cellular RNA was isolated from approximately 200 mg liver sections using Tri-Reagent (Sigma) according to the manufacturer's instruction. The extracted RNA was digested with DNase I (TURBO DNase, Ambion, Austin, TX) before analysis. Twenty micrograms of total RNA was separated on a 1% agarose-formaldehyde gel and was transferred to a MaxSense hy-

bridization membrane (Enzo Diagnostics, Inc., Farmingdale, NY). A full genome length HBV probe was generated from the psHBV-1 vector. Mouse  $\beta$ -actin probe DNA was obtained from Ambion. DNA probes were labeled with <sup>32</sup>P-dCTP using a random primer mix (DECAPrime II, Ambion). The blot was hybridized overnight at 55°C in PerfectHyb Plus hybridization buffer (Sigma) with a  $1 \times 10^6$  cpm/mL probe. The blots were exposed to phosphor screens and were scanned on Molecular Dynamics Phosphorimager. Band intensity was analyzed using ImageQuant software, and liver HBV RNA levels were expressed as a ratio of HBV to  $\beta$ -actin RNA.

## Results

**Modification and Stability of Synthetic siRNAs.** To develop an siRNA molecule with suitable serum stability for therapeutic application, siRNAs were synthesized with chemical modifications. Through a series of sequential rounds of modification and testing for stability and activity, siRNA duplexes were developed in which all 2'-OH groups were substituted. These siRNA duplexes lacking 2'-OH groups are termed a "short interfering nucleic acid" (siNA). Several novel combinations of siNA substitutions were generated in which the sense and antisense strands lack 2'-OH groups and are differentially modified. One of these modified siNA duplexes was used in the experiments in this report. This siNA duplex is composed of a sense strand containing 2'-fluoro substitutions on all pyrimidine positions, deoxyribose in all purine positions, with 5' and 3' inverted abasic end caps. The antisense strand contains 2'-fluoro substitutions in all pyrimidine positions, all purines are 2'-O-methyl substituted, and the 3' terminal linkage is a single phosphorothioate linkage.

The siNA and unmodified siRNA duplexes were assessed for their resistance to degradation in human serum. Throughout the course of the experiments, constant levels of ribonuclease activity were verified. The extent and pattern of unmodified siRNA degradation (3-minute time point) did not change after preincubation of serum at 37°C for up to 24 hours. Figure 1 shows the stability of the unmodified siRNA and the siNA to nucleases as a function of time. The unmodified siRNA single strands or duplexes were extremely unstable in 90% serum, with a half-life of less than 1 minute for the single strands and 3.3 to 5 minutes for the duplexes. In contrast, the chemical modifications of the siNA provided dramatic improvement in stability. As single strands, the siNA sense and antisense strand exhibited half-lives in human serum of 18 and 15.5 hours, respectively. Within the siNA duplex, the sense strand demonstrated a half-life of 2.1 days, whereas the antisense strand had a half-life of 3 days. The



**B**

siRNA	Single strand	Duplex
Native RNA, Sense	<1 min	3.3 min
Native RNA, Antisense	<1 min	5 min
siNA, Sense	18 hrs	2.1 days
siNA, Antisense	15.5 hrs	3 days

Fig. 1. Stability of short interfering RNA (siRNA) and chemically modified sense RNAs in human serum. (A) The top gel panel shows radiolabeled sense strand, either single stranded (ss) or duplexed (ds) to the antisense strand, after incubation in human serum at 37°C. Both single-stranded and duplexed time points are as follows: 1, 5, 15, 30, 60, 120, 300, and 1,310 minutes. Marker lanes from left to right are: single strand incubated in water for 0 or 1,310 minutes followed by duplex at 0 and 1,310 minutes. The subsequent gel panels follow the same loading pattern with the radiolabel present in the RNA antisense, the chemically modified sense strand, and the modified antisense strand, respectively. (B) The fractions of full-length siRNA as a function of time were quantitated and fit to a first-order exponential. The half-lives for single strands and duplexes are shown.

resulting increase in stability of the siNA compared with the unmodified siRNA duplex was approximately 900-fold.

**siRNA Target Site Selection in HBV Cell Culture System.** Potential siRNA sites conserved in more than 95% of available HBV sequences (GenBank) were identified by Blast sequence comparisons. To test siNAs targeted to the HBV RNA for activity in a cell culture model, a replication-competent HBV cDNA, derived from the psHBV-1 vector, was cotransfected along with duplexed siRNA into Hep G2 cells. The initial screening of siNAs to 40 sites in the HBV RNA resulted in the identification of 7 active sites demonstrating more than a 70% decrease in HBsAg levels as compared with an unrelated control

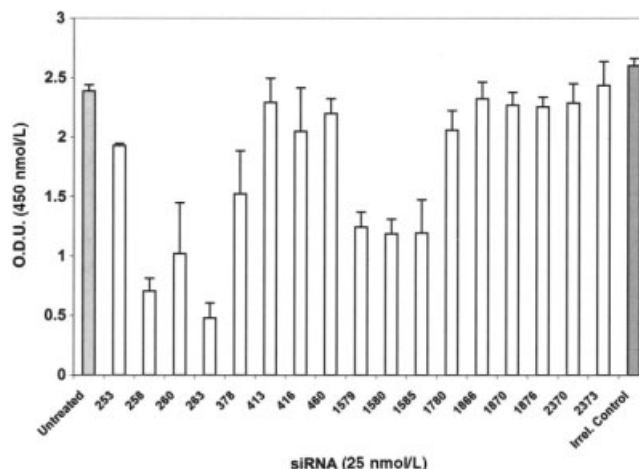


Fig. 2. Screen of conserved short interfering RNA (siRNA) sites in hepatitis B virus (HBV) RNA. Secreted hepatitis B surface antigen (HBsAg) levels were assayed by enzyme-linked immunosorbent assay from Hep G2 cells transfected with HBV expression vector and siRNAs to conserved sites in HBV RNA, or an irrelevant control at final concentrations of 25 nmol/L. HBsAg levels were assayed 3 days after transfection and are expressed as optical density units at 450 nm. Mean levels ( $\pm$ SD) were calculated from three replicate transfections.

siRNA at 25 nmol/L. An example of the siRNA screen is shown in Fig. 2. Subsequent dose response analysis of the active siNAs led to the identification of a highly active site located at starting 5' nucleotide 263 in the HBV genome (data not shown). The 263 site lies within the S-region of the HBV RNA, with the siNA to the site predicted to cleave three of four major transcripts. The sequence and chemical modifications of the HBV site 263 siNA is shown in Fig. 3.

**Potency of Stabilized siRNAs in HBV Vector-Based Mouse Model.** Because the siNA to HBV site 263 demonstrated a high level of stability in serum and exhibited effective gene silencing activity in cell culture, the question of how the differences in stability between the unmodified siRNA and siNA duplexes would impact

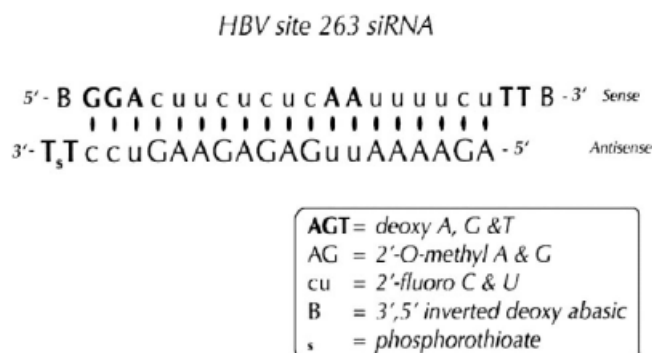


Fig. 3. Sequence and chemical modifications of the hepatitis B virus site 263 short interfering nucleic acid.



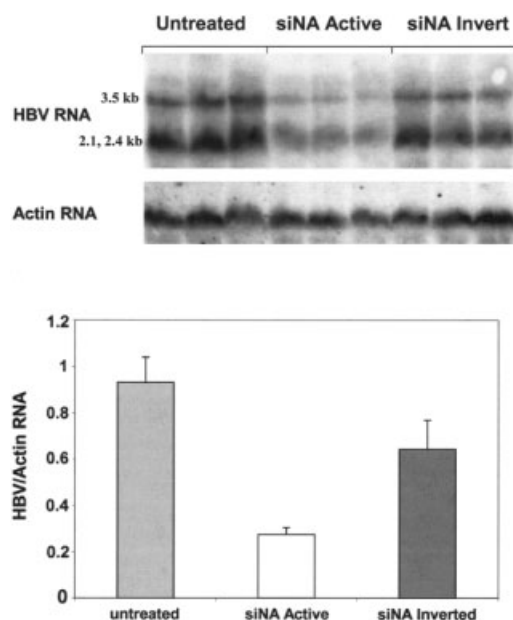


Fig. 4. Reduction of liver hepatitis B virus (HBV) RNA levels after co-hydrodynamic tail vein injection (HDI) administration of stabilized short interfering nucleic acid (siNA). An HDI containing 1  $\mu$ g of the pWTD HBV vector and 1.0  $\mu$ g of active or inverted control siNA was performed on C57BL/6 mice. The levels of liver HBV RNA were determined by Northern blot analysis 72 hours after injection. The HBV RNA levels were normalized to actin messenger RNA (mRNA) and are reported as a ratio of HBV mRNA/actin mRNA ( $\pm$ SD).  $n = 3$  per treatment group.

activity in an *in vivo* model of HBV replication was addressed.

To examine the *in vivo* potencies of the siRNAs, a mouse model was used that uses HDI of a replication-competent HBV vector. To reduce the complicating factors of systemic administration and to focus on only inherent *in vivo* potency of the siRNAs, initial experiments were performed in which the siRNA duplexes were codelivered with the HBV vector via hydrodynamic injection.<sup>10,11</sup> To investigate *in vivo* activity of the stabilized siNAs, 1  $\mu$ g of the pWTD vector and 1  $\mu$ g of active or inverted control siNAs were codelivered to mice by HDI. Three days after HDI, levels of liver HBV RNA were determined by Northern blot analysis. As shown in Fig. 4, a significant reduction in HBV RNA was observed in the active siNA group compared with either the saline-treated control group (71%;  $P < .00126$ ) or the inverted control group (40%;  $P < .0086$ ).

Dose-dependent silencing of HBV expression in the mouse model was examined by injection of 1  $\mu$ g of the pWTD HBV vector along with 1, 0.3, 0.1, or 0.03  $\mu$ g of either the unmodified siRNA or siNA duplex. Both active siRNAs and inverted control duplexes were examined in each siRNA chemistry. The animals were killed 72 hours after the injection, and levels of HBV DNA and HBsAg in the serum were assayed. Figure 5A shows the  $\log_{10}$  differ-

ence in serum HBV DNA between the active or inverted siRNA and the saline-treated groups. Both the unmodified siRNA and siNAs displayed a dose-dependent reduction of serum HBV DNA in the mouse model. A 2.2  $\log_{10}$  reduction of serum HBV DNA was observed with the unmodified siRNA duplex after a 1- $\mu$ g dose, whereas a 3.7  $\log_{10}$  reduction was observed with the siNA at the same dose. No difference was observed between the RNA or siNA inverted control groups and the saline group at any siRNA dose level. The analysis of the serum HBsAg levels correlated with the HBV DNA levels in each group (Fig. 5B). There was little or no difference in the activities of the unmodified siRNA and the siNA at the lower dose levels, as seen with either the HBV DNA or HBsAg end points. The pattern of the dose responses and magnitude of HBV reductions for the unmodified siRNA and siNA were highly reproducible, with an average difference of 0.2  $\log_{10}$  between corresponding data points in two independent studies.

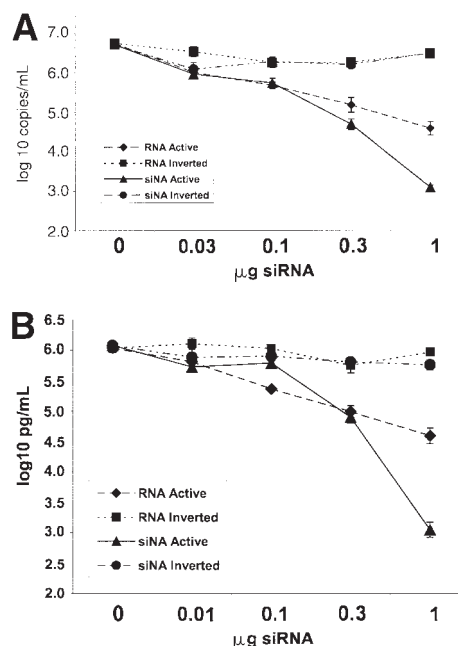


Fig. 5. Activity of stabilized short interfering nucleic acid (siNA) versus unmodified RNA short interfering RNA (siRNA) in a hepatitis B virus (HBV) co-hydrodynamic tail vein injection model. A hydrodynamic tail vein injection (HDI) containing 1  $\mu$ g of the pWTD HBV vector and 0, 0.03, 0.1, 0.3, or 1.0  $\mu$ g siRNA was performed on C57BL/6 mice. Active siRNA duplexes and inverted sequence controls in both unmodified RNA and stabilized chemistry were tested. The levels of serum (A) HBV DNA and (B) hepatitis B surface antigen (HBsAg) were measured 72 hours after injection and are expressed as mean  $\log_{10}$  copies/mL ( $\pm$ SEM) and mean  $\log_{10}$  pg/mL ( $\pm$ SEM), respectively, with  $n = 6$  per treatment group. A dose-dependent reduction in both HBV DNA and HBsAg levels was observed with both the unmodified RNA and stabilized siRNAs. However, the magnitude of the reduction observed in the stabilized siRNAs-treated groups was 1.5  $\log_{10}$  ( $P < .0001$ ) greater for both endpoints at the high-dose level.

The more limited knockdown of HBV RNA levels in the liver as compared with the reductions in serum titers is likely the result of a population of the pregenomic RNA within immature capsids that is sequestered and protected from siRNA-mediated degradation. Because the siRNAs are able to target the 2.1- and 2.4-kb transcripts as well the full-length transcript, it is likely that reductions in HBV protein production would have a negative impact on capsid maturation and release, thus accounting for the more dramatic decreases in serum titers.

This result shows that the improved stability of the modified siRNA results in a more effective level of silencing *in vivo*. Most notably, this is the first demonstration of *in vivo* activity of a modified siRNA completely lacking 2'-OH residues.

**In Vivo Activity of Systemically Dosed Modified siRNA.** As soon as the *in vivo* activity of the siRNAs against HBV was demonstrated by coadministration by HDI, the level of anti-HBV activity of unmodified siRNAs and siNAs was examined by dosing via conventional intravenous injection. Because there have been reports of initial liver damage after hydrodynamic injection,<sup>10</sup> dosing of siNAs was begun 72 hours after HDI. Our own examination of liver alanine aminotransferase and aspartate aminotransferase levels and histopathological analysis after HDI confirmed reports in the literature that the liver returns to near normal status by 72 hours after the initial HDI-induced injury (data not shown). To assess the *in vivo* activity of systemically dosed unmodified siRNAs and siNAs, the pWTD HBV vector was administered by HDI. Seventy-two hours later, the active siRNAs or inverted controls were dosed via standard intravenous injection thrice daily for 2 days. The animals were killed 18 hours after the last dose, and levels of serum HBV DNA were examined. Although no statistically significant activity was observed with the unmodified siRNAs at any dose (data not shown), a dose-dependent reduction in serum HBV DNA levels was observed in the siNA-treated groups in comparison with the inverted control or saline groups (Fig. 6). A statistically significant ( $P < .0006$ ) reduction of 0.91  $\log_{10}$  was observed in the 30-mg/kg group compared with the saline group, whereas a 0.83  $\log_{10}$  reduction ( $P < .0015$ ) was seen in the 10-mg/kg group. These results demonstrate *in vivo* activity of a standard intravenously administered modified siNA and highlight the need for chemical stabilization of siRNAs for *in vivo* applications.

## Discussion

The potential use of siRNAs as therapeutic agents is likely to require improvements in siRNA stability and efficiency of delivery to specific target tissues. As a first

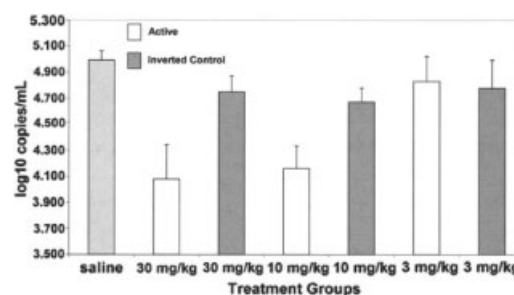


Fig. 6. Activity of systemically administered short interfering RNA (siRNA) in a hepatitis B virus (HBV) mouse model. A hydrodynamic tail vein injection (HDI) was administered that contained 0.3  $\mu$ g of the pWTD HBV vector. Stabilized active short interfering nucleic acid (siNA) and inverted control were administered via standard intravenous injection beginning 72 hours after HDI. The siRNAs were dosed at 30, 10, or 3 mg/kg thrice daily for 2 days. The animals were killed 18 hours after the last dose was administered, and the levels of serum HBV DNA were determined by quantitative real-time polymerase chain reaction. Serum HBV DNA levels are expressed as mean  $\log_{10}$  copies/mL ( $\pm$ SEM), with  $n = 6$  per treatment group. A dose-dependent decrease in serum HBV DNA levels was observed with the stabilized siRNAs in comparison with the saline or inverted control groups. In the high-dose group, a reduction of 0.91  $\log_{10}$  ( $P < .0006$ ) was observed compared with the saline group.

step in this process, we have developed a unique combination of chemical modifications of synthetic siRNAs that provide greatly increased stability in serum as well as maintain effective levels of gene silencing.

Given that RNAi requires recognition of siRNAs by the RNA-induced silencing complex protein complex, it is not surprising that siRNA function is sensitive to chemical modification. The interplay between internucleotide linkage changes, base and sugar modifications, and effects on helical character are likely to be complex and may preclude simple rules about which modifications are permissible. In addition, modifications are likely to have dissimilar impact on different primary sequences. Recently, several studies have explored some of these variables. Global replacement of ribose residues with either 2'-deoxyribose or 2'-O-methyl substitution in either strand has been reported to block or to reduce silencing activity.<sup>12,13</sup> It has been reported that when all uridine positions were present as 2-fluoro modifications, activity is significantly reduced, and when additional 2'-fluoro substitutions are added, further severe reduction in activity is seen.<sup>14</sup> In contrast, we and others<sup>13,15</sup> have found no reduction in activity when 2'-fluoro substitutions were made on all pyrimidine residues on both the sense and antisense strands. Layzer et al.<sup>16</sup> observed no difference in activity between unmodified RNA and 2'-fluoro pyrimidine modified siRNAs in a co-HDI mouse model targeting a luciferase reporter gene. This is in contrast to our results reported here in which there is a marked

improvement in the *in vivo* activity of the fully stabilized siNAs compared with unmodified siRNAs. In addition to 2'-fluoro substitution on pyrimidines, we found that the remaining purine positions were amenable to substitutions of 2'-deoxyribose or 2'-O-methyl with minimal reduction in silencing activity. These differences between our results and the previously reported effects of modifications on silencing activity may be because of our use of unique combinations of modifications and the addition of terminal capping structure.

The hydrodynamic delivery of nucleic acids in the mouse was described by Liu et al.,<sup>10</sup> who showed that the vast majority of the injected nucleic acid is delivered to the liver by this technique. Yang et al.<sup>11</sup> first demonstrated that hydrodynamic injection of a replication-competent HBV vector resulted in high levels of HBV replication in the livers of injected mice. In the vector-based model, HBV replicates in the liver of immunocompetent mice for 7 to 10 days, resulting in detectable levels of HBV RNA and antigens in the liver and of HBV DNA and antigens in the serum. Several reports have documented the use of the HBV vector model to examine the *in vivo* activity of co-HDI administered HBV-targeted unmodified siRNAs<sup>17,18</sup> or vector-expressed short hairpin RNAs<sup>19</sup> in silencing HBV gene expression.

We have optimized this model further by greatly reducing the amount of HBV vector injected per mouse, permitting the examination of dose-dependent siRNA activity at levels that are potentially more clinically relevant. Whereas previous reports used either unmodified RNA siRNAs or vector-expressed shRNAs, we have demonstrated the importance of stabilizing siRNA for *in vivo* activity. With co-HDI administration of the siRNAs, we have demonstrated a 1.5 log<sub>10</sub> greater reduction in HBV replication with the stabilized siNA compared with unmodified siRNA. The reductions we have observed in serum titers with the stabilized siNAs are far more than the 70% to 88% decreases previously reported with unmodified RNA siRNAs or expressed siRNAs.

Most notably, we have demonstrated *in vivo* activity of stabilized siNAs via standard intravenous administration. This is the first demonstration of siRNA *in vivo* activity in a hepatitis animal model with a clinically viable route of administration. Although we have seen a nearly 1 log<sub>10</sub> reduction of serum HBV levels with siNAs in an HBV mouse model, the high dose level and frequency of administration required to produce this antiviral effect clearly indicates that improved efficiency of delivery will be required to develop siNAs

into a clinically viable treatment approach. This is consistent with recently reported work showing reduced expression from an endogenous gene in the livers of mice treated with high doses of an siRNA.<sup>20</sup> Although the results show the reductions in HBV titers to be siRNA sequence specific, the potential contribution of toxicity from the stabilized siRNAs has not been ruled out strictly and will need to be investigated further. With the advent of effective siRNA delivery strategies, characterization of potential toxicity and immunostimulation from siRNAs will be critical for development of the molecules into a clinically viable strategy.

In conclusion, based on our analysis of the pharmacokinetic properties of unformulated siNAs, less than 1% of a 30-mg/kg intravenously administered dose reached the liver (data not shown). This suggests that we are achieving significant reduction in HBV replication with extremely small amounts of the siNAs in hepatocytes. Progress currently is being made in the delivery of siNAs to hepatocytes, which is likely to result in increased antiviral activity with clinically viable dose levels and frequency.

**Acknowledgment:** We thank Roger Aitchison for statistical analysis and Aleem Siddiqui of the University of Colorado Health Sciences Center for the gift of vectors.

## References

1. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 1998;391:806-811.
2. Zamore PD. Ancient pathways programmed by small RNAs. *Science* 2002;296:1265-1269.
3. Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* 2000;404:293-296.
4. Zamore PD, Tuschl T, Sharp PA, Bartel DP. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 2000;101:25-33.
5. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001;411:494-498.
6. Centers for Disease Control (CDC). Viral Hepatitis B Fact Sheet. Bethesda, MD: CDC, 2002.
7. Beasley RP. Hepatitis B virus. The major etiology of hepatocellular carcinoma. *Cancer* 1988;61:1942-1956.
8. Wincott F, DiRenzo A, Shaffer C, Grimm S, Tracz D, Workman C, et al. Synthesis, deprotection, analysis and purification of RNA and ribozymes. *Nucleic Acids Res* 1995;23:2677-2684.
9. Buckwold VE, Xu Z, Chen M, Yen TS, Ou JH. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. *J Virol* 1996;70:5845-5851.
10. Liu F, Song Y, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther* 1999;6:1258-1266.
11. Yang PL, Althage A, Chung J, Chisari FV. Hydrodynamic injection of viral DNA: a mouse model of acute hepatitis B virus infection. *Proc Natl Acad Sci U S A* 2002;99:13825-13830.

12. Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W, Tuschl T. Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *EMBO J* 2001;20:6877-6888.
13. Chiu YL, Rana TM. siRNA function in RNAi: a chemical modification analysis. *RNA* 2003;9:1034-1048.
14. Parrish S, Fleenor J, Xu S, Mello C, Fire A. Functional anatomy of a dsRNA trigger: differential requirement for the two trigger strands in RNA interference. *Mol Cell* 2000;6:1077-1087.
15. Capodici J, Kariko K, Weissman D. Inhibition of HIV-1 Infection by small interfering RNA-mediated RNA interference. *J Immunol* 2002;169:5196-5201.
16. Layzer JM, McCaffrey AP, Tanner AK, Huang Z, Kay MA, Sullenger BA. In vivo activity of nuclease-resistant siRNAs. *RNA* 2004;10:766-771.
17. Giladi H, Ketzinil-Gilad M, Rivkin L, Felig Y, Nussbaum O, Galun E. Small interfering RNA inhibits hepatitis B virus replication in mice. *Mol Ther* 2003;8:769-776.
18. Klein C, Bock CT, Wedemeyer H, Wustefeld T, Locarnini S, Dienes HP, et al. Inhibition of hepatitis B virus replication in vivo by nucleoside analogues and siRNA. *Gastroenterology* 2003;125:9-18.
19. McCaffrey AP, Nakai H, Pandey K, Huang Z, Salazar FH, Xu H, et al. Inhibition of hepatitis B virus in mice by RNA interference. *Nat Biotechnol* 2003;21:639-644.
20. Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, et al. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* 2004;432:173-178.