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The siRNA sequence and guide strand overhangs are determinants of *in vivo* duration of silencing

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ABSTRACT

The use of short interfering RNAs (siRNA) in animals for target validation or as potential therapeutics is hindered by the short physical half-life when delivered as unencapsulated material and in turn the short active half-life of siRNAs *in vivo*. Here we demonstrate that the character of the two 3′-overhang nucleotides of the guide strand of siRNAs is a determinant of the duration of silencing by siRNAs both *in vivo* and in tissue culture cells. We demonstrate that deoxyribonucleotides in the guide strand overhang of siRNAs have a negative impact on maintenance of both the *in vitro* and *in vivo* activity of siRNAs over time. Overhangs that contain ribonucleotides or 2′-*O*-methyl modified nucleotides do not demonstrate this same impairment. We also demonstrate that the sequence of an siRNA is a determinant of the duration of silencing of siRNAs directed against the same target even when those siRNAs have equivalent activities *in vitro*. Our experiments have determined that a measurable duration parameter exists, distinct from both maximum silencing ability and the potency of siRNAs. Our findings provide information on incorporating chemically modified nucleotides into siRNAs for potent, durable therapeutics and also inform on methods used to select siRNAs for therapeutic and research purposes.

INTRODUCTION

Over the past quarter century, several generations of potential therapeutic nucleic acid technologies have been brought forward for clinical development. These include antisense oligonucleotides, ribozymes, aptamers and now siRNAs. The ubiquitous presence of nucleases in biological settings and consequent physical instability of RNA and DNA outside cells has led most nucleic acid drug developers to pursue chemical modification of the active nucleic acid entity. These modifications—either to the phosphodiester backbone, the base, or the sugar—can result in dramatic improvement in physical stability in animal or human serum (1,2). However, naked siRNAs, even when extensively chemically modified and highly active *in vitro*, have been shown to have unacceptable pharmacokinetic properties *in vivo*, being rapidly cleared through the kidney in rodents (3). These properties severely limit the use of siRNA for systemic applications without a more sophisticated mode of delivery. A variety of delivery technologies that minimize renal clearance have been explored, including conjugation of ligands to the siRNA to facilitate cellular uptake (4), polymeric cages to encapsulate siRNA and lipid-based nanoparticles, which are designed to be taken up into endosomes and subsequently escape, releasing their cargo into the cytoplasm where the siRNA responsive machinery is located.

Recent approaches with siRNA given systemically have utilized sugar-modified siRNAs encapsulated in lipid-based nanoparticles to efficiently deliver siRNA to therapeutic targets of interest in the liver (5,6). An important question raised by these studies was the interplay between chemical modification of the siRNA and the type of delivery vehicle in determining efficiency of target knockdown *in vivo*. For example, if a siRNA is efficiently encapsulated and protected from serum nucleases, does chemical modification have an impact on duration of silencing *in vivo*? Also, siRNAs have been shown to trigger unwanted inflammatory responses *in vitro* and *in vivo* via interaction with toll-like receptors (TLRs) or other pattern recognition receptors (7). These receptors are located on plasma or endosomal membranes as well as in the cytoplasm where they monitor the presence of pathogen-derived macromolecules. Do chemical modifications that abrogate activation of these receptors (5,8) also have an impact on duration of silencing *in vivo*?

Previous reports have indicated both positive and negative effects of patterns of chemical modification on specific properties of siRNAs such as resistance to serum nucleases, reduction of target mRNA target levels and

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induction of cytokines (2–4,7). The experiments reported below focused specifically on the two 3′-overhang nucleotides of the guide strand of the siRNA to examine the effect of chemically modified nucleotides on the silencing and duration of silencing both *in vitro* and *in vivo*. Within the field, the original motivations for using deoxythymidine nucleotides as the overhang nucleotides in siRNAs were related to cost, availability and ease of synthesis of the siRNAs when utilizing these nucleotides, as well as increased stability of the oligos to serum nucleases (9). In addition, the authors reported no detectable loss of activity of the siRNAs that was dependent upon the character of the nucleotides as long as the length of the overhang was two nucleotides. However, other reports have demonstrated that the character of the 3′-overhang of the guide strand is a determinant of strand selection (10), and that the 3′-overhang on the guide strand interacts with the PAZ domain of RISC in the RNA-binding pocket (11), suggesting that the overhang does in fact have a possible contribution to siRNA activity. In contradiction to the original observation that deoxythymidine nucleotides in the guide strand overhangs have little negative consequence on silencing, other reports have made the observation that these overhangs can reduce maximal silencing activity of siRNAs (12,13).

We demonstrate that the nucleotide content of the guide strand overhang appears to have little or no effect on maximum silencing *in vitro*. However, we also show that minor differences in the character of the overhang nucleotides of a siRNA guide strand have a profound effect on the duration of silencing. Finally, we demonstrate that siRNAs that have very similar capacities for reduction of mRNA levels in tissue culture cells and *in vivo* have distinct durations of silencing that are appear dependent upon the composition of the siRNA.

MATERIALS AND METHODS

Design of siRNAs

The siRNA sequences used in this article were designed using a previously described algorithm (14) developed to predict silencing efficacy in unmodified form with two deoxythymidine nucleotides as the overhang.

The four siRNA sequences used for the *in vivo* studies have the following sequences (all in the 5′–3′ direction)—where XX indicates the various guide strand overhangs described in this work; Seq1 Passenger CUCUCACAUA CAAUUGAAATT, Seq1 Guide UUUCAAUUGUAUG UGAGAGUUXX; Seq25 Passenger CUCCUAUAAUG AAGCAAAATT, Seq25 UUUUGCUUCAUUAUAGG AGUUXX; Seq37 Passenger CUUUAACAAUUCCUG AAAUTT, Seq37 Guide AUUUCAGGAAUUGUUAA AGUUXX; Seq40 Passenger UCAUCACACUGAAUA CCAATT; Seq40 Guide UUGGUAUUCAGUGUGAU GAUUXX. All other siRNA sequences are in Supplementary Table S1.

Cells and reagents

The mouse hepatoma Hepa 1-6 cell line was obtained from the American Type Tissue Collection (Cat # CRL-1830). Cells were grown in Dulbecco's modified Eagle's medium (Mediatech, Cat #10-013-CV) with 4 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate and 4.5 g/l glucose and supplemented with 10% of fetal bovine serum, 100 μg/ml of streptomycin and 100 U/ml penicillin. Cells were cultured at 37°C in the presence of 5% CO_2.

Preparation of synthetic siRNAs (*in vitro* experiments)

SiRNAs for the *in vitro* experiments were ordered from Sigma-Aldrich. A total of 40 sequences were ordered, each being synthesized with four different guide strand overhangs; ribonucleotides complementary to positions −1 and −2 in the target mRNA (rN-rN), 2′-*O*-methylated complementary nucleotides (oN-oN), deoxythymidine overhangs (dT-dT) and 2′-*O*-methyl-uridine overhangs (oU-oU).

Preparation of synthetic siRNAs (*in vivo* experiments)

The siRNAs used in the *in vivo* studies were synthesized by methods previously described (15). For each oligonucleotide, the two individual, complementary strands of the siRNA were synthesized separately using solid phase synthesis, then purified separately by ion exchange chromatography. The complementary strands were annealed to form the double strand siRNA (duplex). The duplex was then ultrafiltered and lyophilized to form the solid drug substance. The duplex material was tested for the presence of endotoxin by standard methods.

Preparation of siRNA–lipid nanoparticle complex

Lipid nanoparticles (LNPs) were made using the cationic lipid CLinDMA (2-{4-[(3b)-cholest-5-en-3-yloxy]-butoxy}-*N*,*N*-dimethyl-3-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yloxy]-propan-1-amine), cholesterol, and PEG-DMG (monomethoxy(polyethyleneglycol)-1,2-dimyristoylglycerol) in 50.3 : 44.3 : 5.4 molar ratio. siRNAs were incorporated in the LNPs with high encapsulation efficiency by mixing siRNA in buffer into an ethanolic solution of the lipid mixture, followed by a stepwise diafiltration process. Cholesterol was purchased from Northern Lipids, PEG-DMG was purchased from NOF Corporation and CLinDMA was synthesized by Merck and Co. The encapsulation efficiency was determined using a SYBR Gold fluorescence assay and the particle size measurements were performed using a Wyatt DynaPro plate reader. The siRNA and lipid concentrations in the LNP were quantified by a HPLC method, developed in house, using a PDA detector.

Transfection of siRNAs for screening

Cells were plated in 96-well plates at 5000 cells/well and transfected at a final siRNA concentration of 10 nM using RNAiMax (Invitrogen, Cat #13778150) according to the manufacturers specifications. Cells were lysed 24 h after transfection.

RNA was isolated by preparing lystates from the cells on a Biomek FX liquid handler using Cells-to-Ct Bulk Lysis and Stop Reagents (ABI, Cat #4391851C) according to the manufacturer's instructions.

In vitro duration assay (RNAiMax transfection)

A plate for transfection of the cells was prepared such that each siRNA was represented in six different random locations on the plate. This was done to address concerns about well-to-well variability in transfection and/or growth of cells over the long time course of the experiment.

Cells were plated in 96-well plates at 1200 cells/well and transfected at a final siRNA concentration of 10 nM using RNAiMax (Invitrogen, Cat #13778150) according to the manufacturers specifications. Cells were lysed 24, 48, 72, 96 and 120 h after transfection.

Transfection of LNP formulated siRNAs into tissue culture cells

Transfections of LNPs were performed in six replicates in two replicate experiments (three replicates/experiment). Cells were plated in 96-well plates at 3500 cells/well. The final concentration of siRNA in each well was 120 nM. The time of incubation with the LNP-siRNA complexes was 24 and 120 h. Media change was performed every 48 h from the time cells were plated by replacing 75 µl with fresh complete growth culture media until the 120 h time point.

RNA was isolated by preparing lystates from the cells on a Biomek FX liquid handler using Cells-to-Ct Bulk Lysis and Stop Reagents (ABI, Cat #4391851C) according to the manufacturer's instructions.

RNA isolation from *in vivo* study samples

C57BL/6 mice were dosed with 3 mg/kg siRNA (in LNP) and sacrificed at various time points. Cohorts for controls and each siRNA tested consisted of five animals. Blood and liver samples were collected immediately following euthanasia.

Total RNA was isolated from liver tissue using the RNeasy 96 Tissue Kit for high-throughput 96-well RNA minipreps (Qiagen, Cat #74881) and a QIAvac 96 vacuum manifold according to the manufacturer's instructions. All RNA samples were treated with DNase I (Qiagen, Cat #79254) on column for 15 min at room temperature. Final RNA eluted was quantified and normalized to a concentration of 50 ng/µl.

Reverse transcription and PCR

cDNA was generated from lysates and RNA in a 20 µl reaction using reverse transcription reagents from the Ambion Cells-to-Ct Kit (Applied Biosystems, Cat #4368813) according to the manufacturer's instructions. On an ABI 7900 HT real-time PCR System, quantitative real-time PCR was carried out in a 384-well plate. Reactions were set up in duplicate and one well was probed with the Apob Taqman reagents, the other with the GAPDH Taqman reagent in a final volume of 10 µl using TaqMan Gene Expression Master Mix (Applied Biosystems, Cat #4370074). All Taqman probes and primers were supplied as prevalidated sets by Applied Biosystems: mouse GAPDH, Cat #4352339E; mouse Apob, Assay ID Mm01545154_g1.

Taqman data analysis

The Taqman data were analyzed by standard methods on an ABI 7900 instrument. Within each experiment, the baseline was set in the exponential phase of the amplification curve, and based on the intersection point of the baselines with the amplification curve; a Ct value is assigned by the instrument. The expression level of the gene of interest and percentage knockdown was calculated using comparative Ct method:

$$\Delta Ct = Ct_{Target} - Ct_{GAPDH}$$

$$\Delta\Delta Ct = \Delta Ct_{(Target\ siRNA)} - \Delta Ct_{(NTC)}$$

$$\text{Relative expression level} = 2^{-\Delta\Delta Ct}$$

$$\%\ KD = 100 \times (1 - 2^{-\Delta\Delta Ct})$$

The mRNA knockdown was calculated relative to a non-targeting control siRNA in each experiment.

Cytokine quantitation

Cytokine levels were measured in mouse serum using the SearchLight Mouse IR Cytokine Array, 12-plex assay (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Data were acquired with the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) and analyzed with SearchLight Array Analyst Software.

Statistical analysis

Unless otherwise noted, datasets were compared using a two-tailed Student's t-test to generate *P*-values. Also, unless otherwise noted, statistical analyses were performed using the data expressed as log2-fold change rather than in percent expression or percent knockdown of the mRNA transcript, as the logarithm of Taqman error is uniformly distributed, and the logarithm of target-normalized siRNA silencing is normally distributed.

Calculation of retention rate of silencing

To calculate the retention rate of silencing both *in vitro* and *in vivo*, we converted the percent knock-down values at the relevant timepoints to $-\log_2$-fold change (ddCt values from Taqman), subtracted the early timepoint value from the later timepoint value and divided by the number of days between the two timepoints. This gave us a value for the fraction of silencing seen at the first timepoint that was lost per day up to the second timepoint.

RESULTS

In vitro efficacy of chemically modified siRNAs

In this study, we examined the effects of varying the overhanging nucleotides of the guide strand of a standard 21 nucleotide duplex siRNA in several assays. We chose to examine four different guide strand overhangs. We selected deoxythymidime overhangs because they are the de facto industry standard (9), ribonucleotides which are complementary to positions −1 and −2 relative to

the target site in the mRNA as those would form the overhang if the siRNA being examined were to be produced naturally within a cell, and 2′-*O*-methyl modified nucleotides, either uridines to correspond to the thymidines, or complementary nucleotides.

First, we examined the effect of different guide strand overhang modifications on the extent of knockdown of the target mRNA in tissue culture cells 24 h post-transfection. We compared overhangs of ribonucleotides complementary to positions −1 and −2 in the target mRNA (rN-rN), 2′-*O*-methyl modified versions of those complementary nucleotides (oN-oN), dexoxythymidine overhangs (dT-dT) and to 2′-*O*-methyl-uridine (oU-oU) overhangs. In all cases, regardless of the guide strand overhang, the passenger strand overhang was maintained as dT-dT to experimentally isolate the effect of the guide strand overhang. Forty siRNA sequences directed against the murine Apob gene were synthesized with these four guide strand overhangs and tested in Hepa1-6 cells, a mouse hepatoma-derived cell line, for their ability to reduce the level of Apob mRNA.

The average degree of knockdown seen with the dT-dT overhangs was 97.7 ± 1.8%; with oU-oU, 97.0 ± 4.4%; with oN-oN, 98.3 ± .4; and with rN-rN, 97.2 ± 2.3%. These data can be found in Supplementary Table S1. This similarity in knockdown between dT-dT overhangs and rN-rN overhangs is consistent with previously published reports on the activity of siRNA duplexes with deoxyribonucleotides in guide strand overhangs (9) though in disagreement with other published articles which showed a detrimental effect of dT-dT overhangs compared specifically to rN-rN guide strand overhangs (12) or to rU-rU overhangs (13). To compare the data statistically, we converted the knockdown results to $\log_2$-fold change to obtain a normal distribution of variance across silencing levels allowing us to perform standard parametric statistics. The data showed no systematic difference in the ability of siRNAs with these four different guide strand overhangs to reduce the target mRNA level in tissue culture cells at 24 h post-transfection (Student's *t*-test: oU-oU versus dT-dT, $P = 0.20$; rN-rN versus dT-dT, $P = 0.18$; oN-oN versus dT-dT, $P = 0.12$).

In vitro duration assays

In order to assess any differential effect the sequences and the guide strand overhangs had on the rate of recovery of the expression of the Apob mRNA levels *in vitro*, we performed assessments of the levels of mRNA reduction after treatment with each of the 40 siRNA sequences in four chemistries, for a total of 160 siRNAs each day from 1 to 5 days post-transfection. These data are summarized in Supplementary Table S1.

We observed that even though all 40 sequences showed extremely similar maximum mRNA reductions, the rate at which the silencing activity was lost *in vitro* appeared to have an siRNA-specific component as well as an overhang-specific component. However, we were unable to identify sequence motifs associated with increased or decreased duration of silencing in this data set, perhaps due to the limited number of sequences.

The data from these duration assays are summarized in Figure 1. siRNAs with dT-dT overhangs consistently performed more poorly over time than siRNAs with any of the other tested overhangs. We quantified this by comparing the knockdown results in $\log_2$-fold change space at each of the individual timepoints (Figure 1). Even by 2 days post-transfection the mean silencing of siRNAs with dT-dT overhangs is significantly reduced relative to silencing of the same sequences to the oN-oN overhang ($P = 0.013$). The difference is increased by day 3 and now shows significance to all three overahangs (to rN-rN $P < 0.001$; to oU-oU $P = 0.003$; to oN-oN $P < 0.001$) this significant difference is retained at day 4 (to rN-rN $P < 0.001$; to oU-oU $P < 0.001$; to oN-oN $P < 0.001$) and is slightly reduced by day 5 (to rN-rN $P = 0.001$; to oU-oU $P = 0.017$; to oN-oN $P = 0.01$), probably attributable to an increasing number of sequences having no activity regardless of the guide strand overhang at the latest timepoint.

In vitro dose response curves

To further evaluate the efficacies of siRNAs with different guide strand overhangs, we selected four siRNA sequences (Seq1, Seq25, Seq37 and Seq40) each with dT-dT, rN-rN, oN-oN and oU-oU overhangs. Based on our *in vitro* experiments, we expected rN-rN, oN-oN and oU-oU overhangs to behave equivalently. These four sequences were selected on the basis of having comparable degrees of mRNA reduction in our initial screens regardless of the guide strand overhang tested (see Table 1). We also determined in our subsequent experiments that the sequences demonstrated a range of *in vitro* durations. Seq40 appeared to have the most stable duration in our *in vitro* assays, both Seq25 and Seq37 appeared to be among the least durable siRNAs, and Seq1 fell between those two extremes. We were interested in differences in

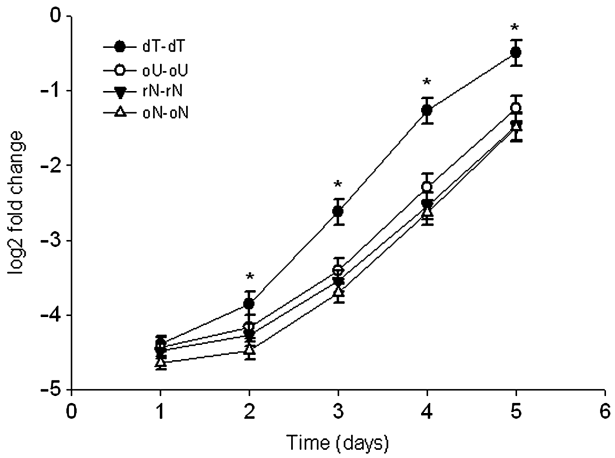

Figure 1. siRNA with different guide strand overhangs were tested over a time course in mouse Hepa1–6 cells. Each data point represents results from: dT-dT $n = 39$, oU-oU $n = 39$, rN-rN $n = 40$ and oN-oN $n = 40$ unique sequences. Asterisk indicates that the dT-dT data point at that particular timepoint has a *P*-value of <0.05 compared with the data points for each of the other three overhangs tested. Data are expressed as mean ± SEM.

Table 1. Comparison of siRNA activities *in vitro* and *in vivo*

Sequence and overhang	Percentage mRNA reduction *in vitro*	Maximum percentage mRNA reduction *in vivo*	Retention rate (*in vivo*) Days 1 to 7 (%)	Retention rate (*in vitro*) Days 1 to 5 (%)	Time to 50% knock-down (days)
Seq1 dT-dT	96.4 ± 2.3	81.4 ± 17.4	85.2	79.3	5
Seq1 oU-oU	97.6 ± 1.8	92.0 ± 7.1	88.8	85.9	10
Seq1 rN-rN	99.1 ± 0.3	89.2 ± 14.4	91.9	88.7	10.5
Seq25 dT-dT	99.4 ± 0.4	78.0 ± 3.8	91.8	78.0	9
Seq25 oU-oU	99.5 ± 0.1	89.3 ± 2.8	91.4	83.8	11.5
Seq25 rN-rN	99.3 ± 0.2	85.7 ± 7.5	93.6	83.9	10
Seq37 dT-dT	99.2 ± 0.1	51.0 ± 8.8	86.3	76.0	<3
Seq37 oU-oU	97.3 ± 1.0	78.5 ± 6.5	91.2	83.1	6.5
Seq37 rN-rN	96.4 ± 0.6	85.9 ± 3.3	95.9	86.7	13
Seq40 dT-dT	97.2 ± 1.0	87.9 ± 18.9	92.3	89.2	11.5
Seq40 oU-oU	96.9 ± 1.3	91.0 ± 8.1	98.9	97.6	>14
Seq40 rN-rN	94.5 ± 1.5	91.9 ± 14.1	94.8	95.3	>14

the concentration required to produce half the maximum degree of reduction of the target and the relationship if any of concentration dependence of silencing to siRNA sequence or guide strand overhang. As shown in Figure 2, siRNAs with the same target sequence but different guide strand overhangs shared similar IC_{50} values. We did observe differences in the IC_{50} values between sequences. Notably Seq25 had an IC_{50} in all chemistries that was ~20-fold higher than that seen with the Seq40, and ~4 times higher than that seen with Seq37 (Figure 2).

In vitro duration assay with LNP formulated siRNAs

The four sequences in three chemistries described above were formulated in a LNP-delivery vehicle. The LNP-formulated siRNAs were transfected into cells, and the cells were harvested 24 and 120 h post-transfection (Figure 3). The 12 tested siRNAs showed an average of 96.2 ± 1.3% reduction of the target mRNA at 24 h, but by 120 h a large range in the degree of knockdown was observed.

Comparing the amount of activity lost between the 24-h and 120-h timepoints in siRNAs with dT-dT overhangs, one of the sequences lost 8.9% (from 94.5% mRNA reduction at 24 h to 85.6% mRNA reduction at 120 h) of activity (Seq40), while another (Seq37) showed a loss of 76.2% of activity (from 95.6% mRNA reduction at 24 h to 19.4% mRNA reduction at 120 h) over the same time period. The remaining two sequences (Seq1 and Seq25) showed a loss of activity of 46 and 54%, respectively. Comparing the sequences with oU-oU overhangs, we observed losses of 16 (Seq1), 18.9 (Seq25), 25.1% (Seq37) and no detectable loss in Seq40. When the overhangs were rN-rN, we observed losses of 8 (Seq1), 19.1 (Seq25), 11.2% (Seq37) and again no measurable loss in Seq40. These data indicate that siRNA sequence and overhang chemistry both likely play a role in determining silencing duration.

An overall comparison of the loss of silencing activity dependent on chemistry showed that the oU-oU and rN-rN overhangs showed comparable losses of 14.9 ± 10.9 and 9.9 ± 7.6%, respectively between 24 and 120 h, while the siRNAs containing dT-dT overhangs showed a loss of 46.5 ± 28.0%. The dT-dT loss of activity had a *P*-value of 0.03 compared to oU-oU and 0.05 to rN-rN. The loss of activity with oU-oU overhangs compared to the loss of activity with rN-rN overhangs had a *P*-value of 0.23. In all cases dT-dT overhangs were demonstrably worse in their ability to maintain silencing over the time range tested in this assay.

In vivo efficacy and duration

We had verified *in vitro* that the LNP-encapsulated siRNAs retained the ability to comparably reduce Apob mRNA levels 24 h post-transfection (Figure 3). Furthermore, the IC_{50}s were virtually identical across three guide strand overhangs, although showing some sequence dependence (Figure 2).

To test duration of silencing *in vivo* and investigate the contribution of siRNA sequence and guide strand overhang modification to *in vivo* duration, a single dose of formulated siRNAs were delivered via the tail vein and the degree of knockdown of Apob mRNA in liver was examined at 1, 3, 7 and 14 days (Figure 4). In most cases, siRNAs with all three of the guide strand overhangs showed comparable degrees of knockdown at the shortest timepoint measured (1-day post-treatment). The exception was Seq37, which showed reduced day 1 knockdown *in vivo* with the dT-dT overhang. This sequence had also shown the largest degree of loss *in the vitro* duration assays when the dT-dT overhang (Figure 3) was compared to oU-oU or to rN-rN. As expected, in all cases there was a loss of activity of the siRNAs over time. Differences in the rate of loss of activity were in part due to siRNA sequence. An example of this sequence dependent range is shown in Figure 5, where the dT-dT versions of all four sequences are compared, and the difference between the durations of the sequences is clear. This difference is also seen when comparing the four sequences in the other two tested chemistries, though as we appear to approach the maximum possible duration of action of the siRNAs *in vivo,* the differences become less obvious.

We looked at several parameters of the siRNA activity *in vivo* as a means to discern the differences between the various guide strand overhangs (Table 1). We examined the maximum degree of mRNA reduction and retention rate of silencing activity per day. The values for these parameters appear to be worse for dT-dT overhangs compared to the other two overhangs for each of the

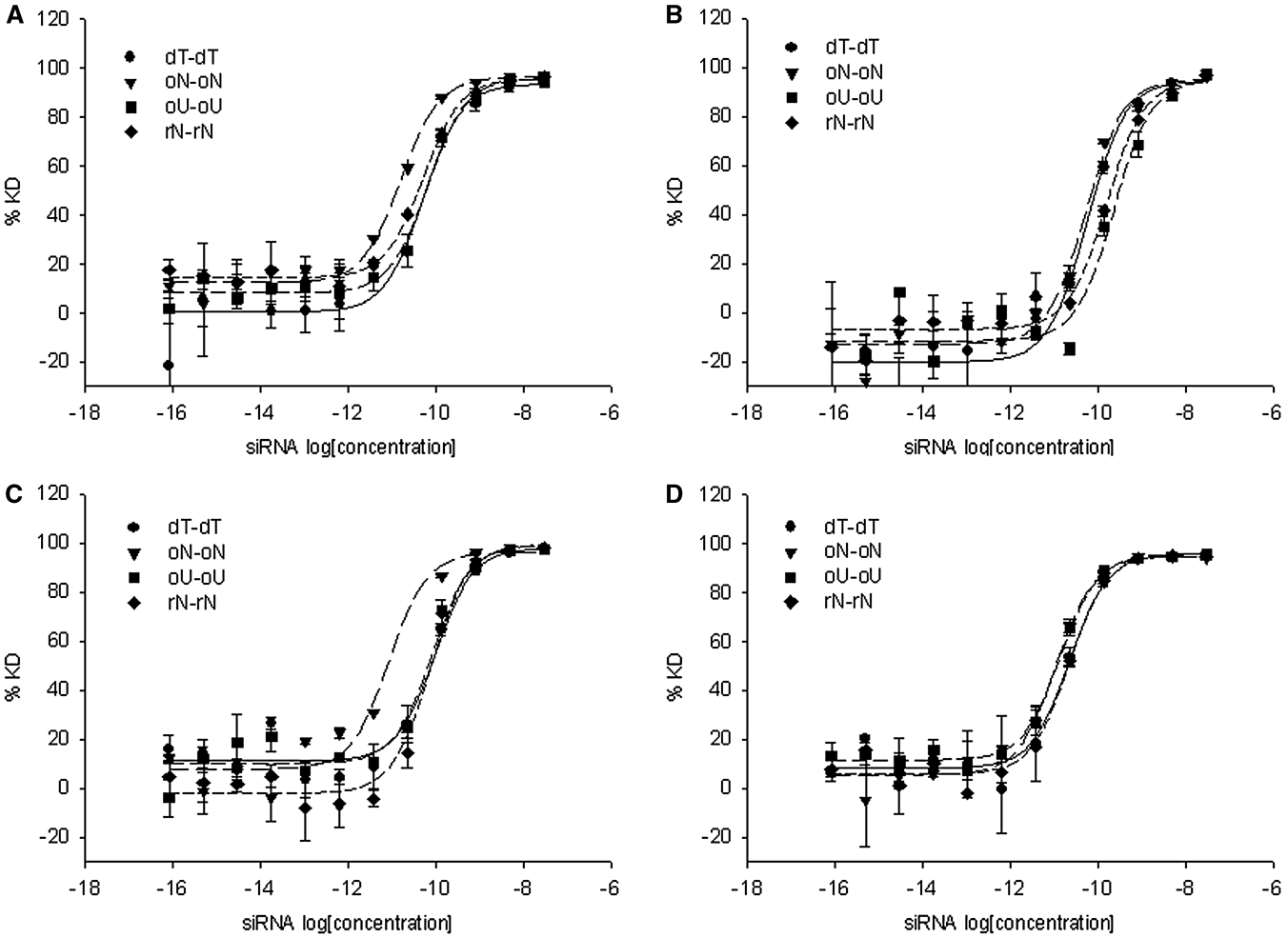

Figure 2. Testing of siRNAs for maximum reduction of mRNA levels and the potency. siRNAs were tested in a range of concentrations on mouse Hepa1–6 cells to compare both the maximum reduction of mRNA levels with each of the siRNAs and the potency. In each panel, the filled circles are for siRNAs with dT-dT overhangs, filled squares are siRNAs with oU-oU overhangs, filled diamonds are siRNAs with rN-rN overhangs and filled triangles are siRNAs with oN-oN overhangs. Panel (**A**) is Seq1, (**B**) Seq25, (**C**) Seq37 and (**D**) Seq40.

individual sequences. These parameters are expected to be independent, but are both critically important for therapeutic dosing. We attempted to estimate the duration of therapeutic effect by calculating the length of time for which 50% knockdown was achieved, which is a parameter which includes both the maximum knockdown achieved and the amount of silencing lost each day. This last parameter demonstrates a clear difference between the siRNAs with dT-dT overhangs and siRNAs with rN-rN and oU-oU overhangs. For all the sequences rN-rN and oU-oU overhangs yielded similar results, in all cases having a longer duration than the respective siRNAs with dT-dT overhangs. The improvement in duration relative to the same sequence with dT-dT overhangs varied from 11 to >100% in the length of time for which 50% knockdown was achieved.

Comparison of *in vitro* and *in vivo* data

We compared the level of mRNA expression at the four *in vivo* timepoints to the level of mRNA expression at both timepoints tested in tissue culture cells to determine how well the *in vitro* assay was capable of predicting the duration of siRNA activity *in vivo*. Comparing any of the *in vivo* timepoints to the mRNA expression at the 24-h timepoint of the *in vitro* assay produced no obvious correlation between the two datasets (data not shown). A comparison of the *in vitro* 120-h data with each of the available sets from the *in vivo* experiment showed an R^2 value of 0.65 at 1 day, 0.89 at 3 days, 0.84 at 7 days and 0.54 at 14 days. This indicates that the *in vitro* assay as performed here provides a very strong correlation with observed data from the *in vivo* studies. Additionally, the percent of silencing retained per day, calculated over Days 1–7 *in vivo* and from 24 to 120 h *in vitro*, was well correlated ($R^2 = 0.60$; $P < 0.003$).

Effect of guide strand overhangs on cytokine release

An additional advantage of using chemically modified siRNAs is the ability of those siRNAs to prevent or reduce the release of cytokines by the treated cells (8). We wanted to be certain that the differences we observed in the loss of activity that were dependent on chemistry or sequence were not the result of variable cytokine responses.

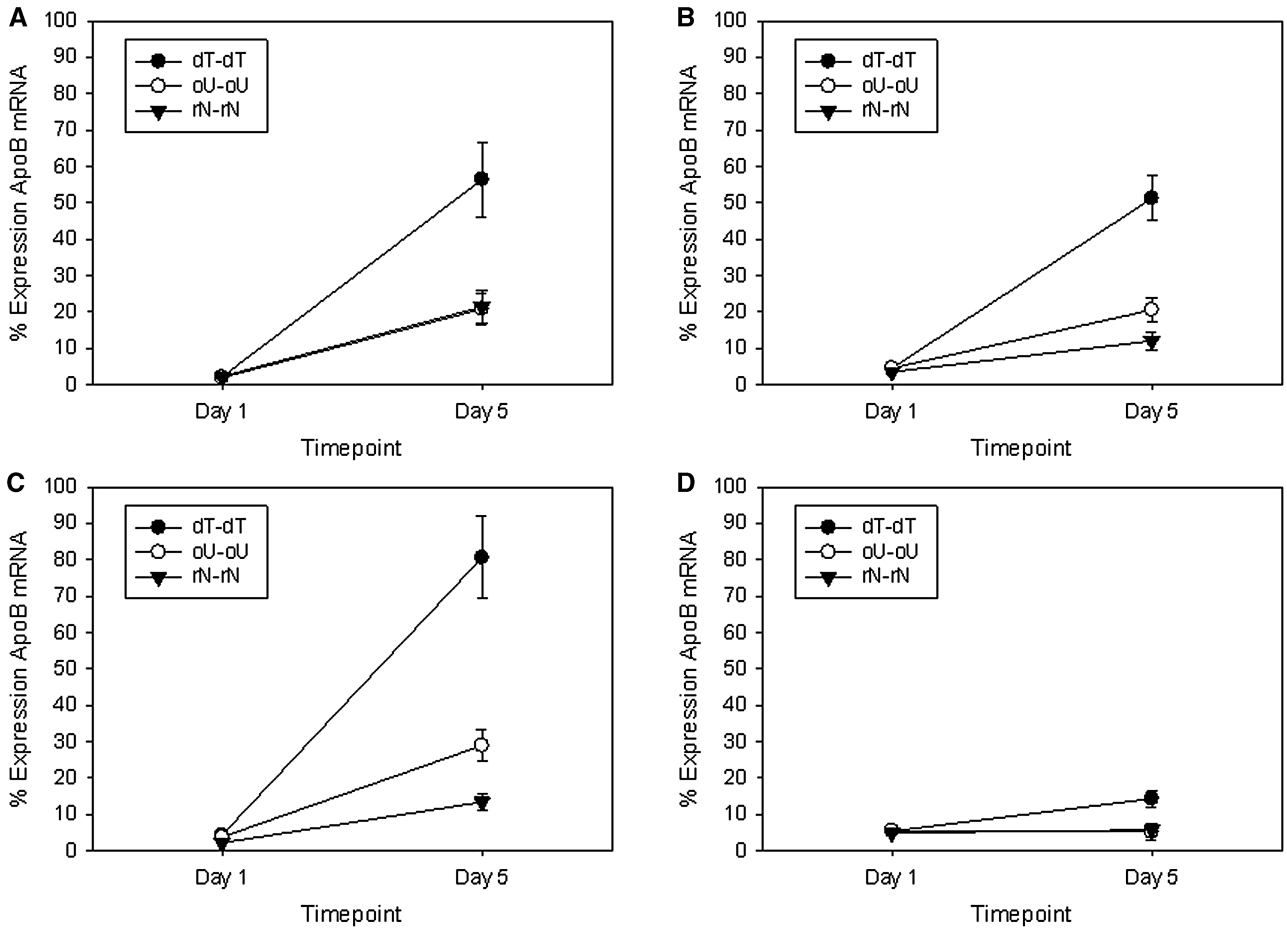

Figure 3. Comparison of activity of siRNAs at 24 and 120 h post-transfection. LNP-encapsulated siRNAs were tested in mouse Hepa1–6 cells to compare the reduction of mRNA levels for each of the siRNAs over time. In all panels the filled circles indicate dT-dT overhangs, open circles oU-oU overhangs and filled triangles indicate rN-rN overhangs. (**A**) Seq1, (**B**) Seq25, (**C**) Seq37 and (**D**) Seq40.

We compared the circulating levels of five different cytokines (IL-1α, KC, IL-6, IFNγ and TNFα) at 4 h post-dosing with the 12 duplexes tested *in vivo* in LNP forumation, to determine if the variation in the guide strand overhangs had any effect on immunostimulation (Supplementary Figure S1). For the siRNAs examined in this study, there was no apparent dependence of the degree of cytokine release on the guide strand overhangs or on the underlying sequence of the siRNAs.

DISCUSSION

A critical aspect of chemical modifications of siRNAs is to improve their drug-like properties. To date, almost all siRNAs used in animals have been chemically modified in some form (3–6), but the exact impact of many of these modifications on performance *in vivo* relative to unmodified siRNAs has not been established. While most studies have focused on maximal silencing achieved by siRNAs *in vitro* (16,17), for therapeutic utility an extended duration of action is in practice a required corollary. Currently available delivery mechanisms necessitate intravenous delivery of the therapeutic material, thereby increasing the need for maximizing the dosing interval.

We first investigated a set of 40 sequences directed against the mouse Apob gene in assays in tissue culture cells. From these experiments, we were able to demonstrate that the guide strand overhang has no measurable effect on the maximal capacity of a given siRNA to reduce the expression of the target. However, we also demonstrated that over time, differences in the capablities of the siRNAs to mediate silencing begin to manifest themselves. Specifically, the dT-dT overhangs, which are a common industry standard, have a consistently negative effect on the duration of action of siRNAs in tissue culture cells. Interestingly, there are reports in the literature that see no effect of the guide strand overhang at the tested timepoint (9) and those where a difference is clearly evident (12,13). We suggest that both of these results can be explained as the result of the timepoints under which the experiments were carried out. Essentially, the dependence of silencing on guide strand overhangs is a direct result of the timepoints used in the experiments.

We noted that duration of silencing has some sequence dependence, and sequences with naturally long durations of silencing may not show much overhang dependence of silencing *in vitro*. We observed that an siRNA with naturally long duration of silencing lost virtually no activity

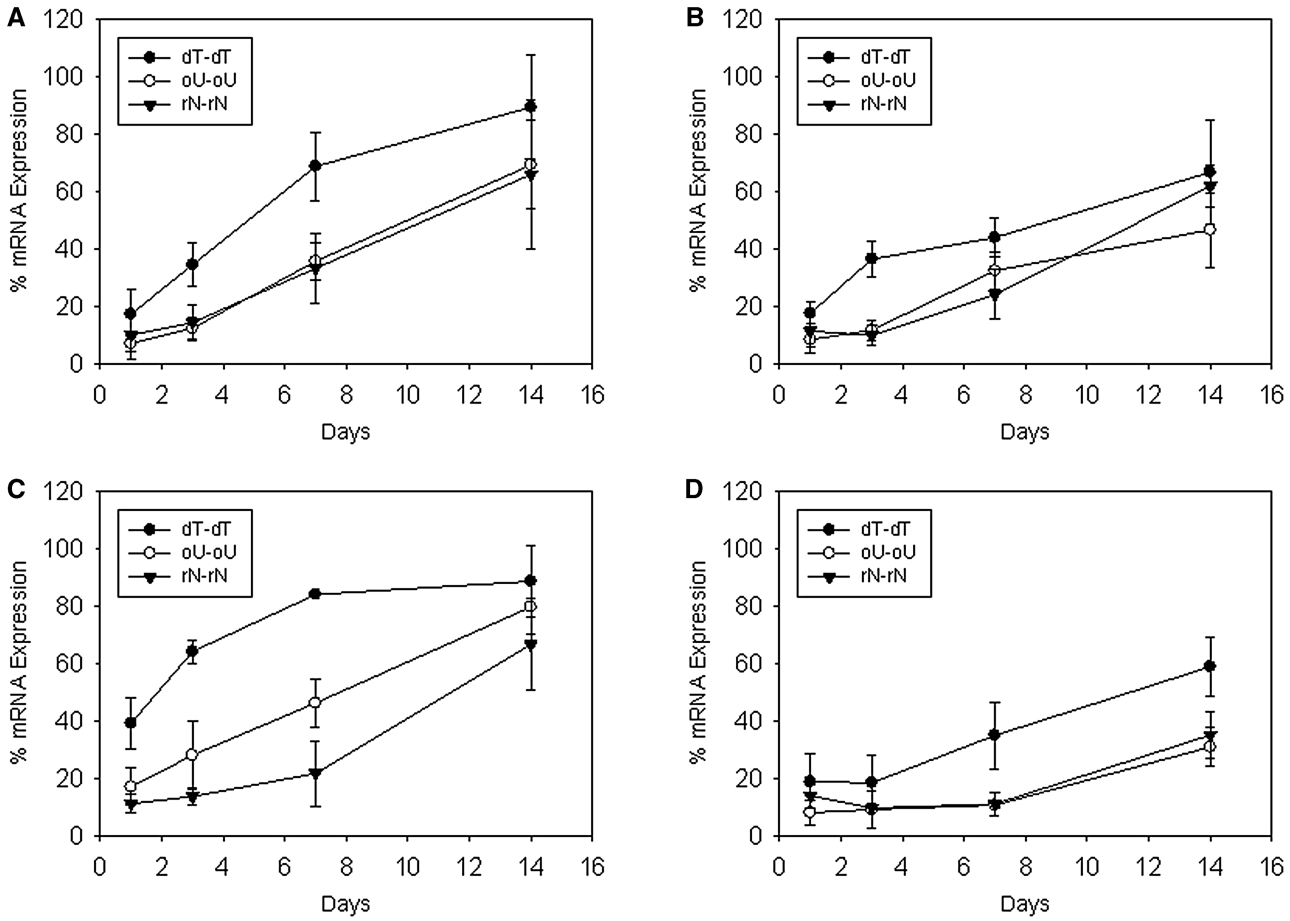

Figure 4. Liver Apob target mRNA reduction by siRNAs with different guide strand overhangs. C57Bl/6 mice were intravenously injected with LNP formulated siRNAs at a dose of 3 mg/kg. Livers were harvested at various timepoints between 1 day and 14 days post-dosing, and the mRNA level of Apob relative to GAPDH was determined. siRNAs targeting the four Apob target sites with three different guide strand overhangs are compared to each other. In each panel the filled circles indicate dT-dT guide strand overhangs; open circles—oU-oU guide strand overhangs; filled triangles—rN-rN guide strand overhangs. (**A**) Seq1, (**B**) Seq25, (**C**) Seq37 and (**D**) Seq40.

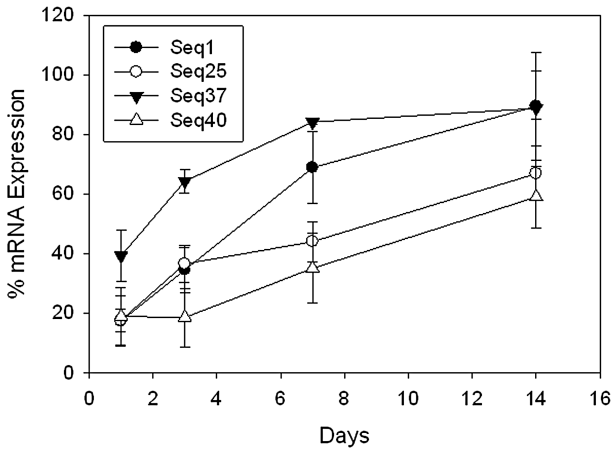

Figure 5. Comparison of the duration of activity of four Apob siRNA sequences with dT-dT overhangs. Seq1—filled circles; Seq25—open circles; Seq37—filled triangles; Seq40—open triangles.

over the course of the experiment (Seq40) and while other siRNAs lost as much as 30% of their activity per day (Seq5, 25 and 37).

Further *in vitro* characterization of these sequences included dose–response curves with the siRNAs in three of the overhang chemistries and a second *in vitro* duration assay using the LNP formulated siRNAs. We noted no consistent difference in the IC50s that depended on the guide strand chemistry (Figure 2).

We have also demonstrated that duration is a unique parameter of siRNA function that is experimentally separable from both the maximum reduction of the target mRNA and also from the potency of the siRNA. The experiments, assay conditions and analysis methods described here will allow for a much greater understanding of this critical component of siRNA action in future experiments.

The observed differences in the duration of activity of the siRNAs in our experiments appear to come from two sources. First, comparison of the four sequences with constant overhangs demonstrates a sequence dependence of duration. Second, comparison of the same sequence with different overhangs shows a clear difference between dT-dT overhangs and rN-rN or oU-oU overhangs. What is particularly striking about this observation is that there is no discernable difference in the maximum potential for silencing activity of these siRNAs. The phenomenon of differential duration of siRNAs *in vivo*

therefore appears to be independent of their ability to reduce the target mRNA.

In this study, we examined only relatively minor changes to the chemical composition of the 2-nucleotide siRNA 3′-overhangs of the guide strand. We identified differences in duration of silencing *in vivo* between the nucleotide overhang that has become the industry standard (thymidine) and 2-*O*-methyl-uridine or ribonucleotides. One interesting facet of the observation is that it is not necessarily the 2-*O*-methyl modification of the overhangs that extends duration, as the ribonucleotide overhangs showed similar performance. Indeed for one of the four tested sequences (Seq37), the siRNA with the ribonucleotide overhang actually appears to have a measurably longer duration than the siRNA with the 2-*O*-methyl-uridine overhang. Instead, it appears that the thymidine overhangs are consistently detrimental to obtaining maximum duration of silencing from the siRNAs both *in vitro* and *in vivo* and should therefore be avoided for any siRNA that is intended to be developed for therapeutic purposes or for extended action in any context.

These observations raise the critical question of how a thymidine overhang might reduce duration of siRNA activity. The presence of thymidine residues may expose the siRNA to DNAses. It is also possible that dT-dT overhangs bind less stably to the 3′ nucleotide binding pocket of Argonaute (11), reducing stability of assembled RISC complexes. Alternatively, the thymidine nucleotides may be poor substrates for a beneficial intracellular activity. For example, the HEN1 methyltransferase of Arabidopsis has been shown to have a preference for methylating the 2′OH of the 3′ nucleotide of both miRNAs and siRNAs and in fact to be incapable of methylating the 3′ nucleotide if that nucleotide is a deoxyribonucleotide, requiring a free 3′OH and 2′OH on the 3′ nucleotide (18). In addition the mouse homologue of HEN1 has been demonstrated to methylate the 3′ termini of Piwi-interacting RNAs (piRNAs) (19–22). If HEN1 methylation of the 2′OH of the 3′ ribonucleotide of an exogenously introduced siRNA stabilizes the siRNA intracellularly, then dT-dT siRNA may be more vulnerable to degradation. By using a HEN1 substrate as the 3′ nucleotide, or by using a 3′ nucleotide that is already methylated at the 2′OH position (2-*O*-methyl-uridine), we may be recapitulating the preferred state *in vivo*.

In summary, we demonstrate that it is possible to differentiate both *in vivo* and *in vitro* siRNAs that appear to have identical activities *in vitro* under conditions standard in the field. Furthermore, duration of activity appears to be affected by both the underlying sequence of the siRNAs and the chemical nature of the guide strand overhangs. Specifically, we have demonstrated that the *de facto* manufacturing standard for guide strand overhangs (thymidine nucleotides) has a detrimental effect on both *in vitro* and *in vivo* duration of action of siRNAs.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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