

High potency silencing by single-stranded boranophosphate siRNA

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ABSTRACT

In RNA interference (RNAi), double-stranded short interfering RNA (ds-siRNA) inhibits expression from complementary mRNAs. Recently, it was demonstrated that short, single-stranded antisense RNA (ss-siRNA) can also induce RNAi. While ss-siRNA may offer several advantages in both clinical and research applications, its overall poor activity compared with ds-siRNA has prevented its widespread use. In contrast to the poor gene silencing activity of native ss-siRNA, we found that the silencing activity of boranophosphate-modified ss-siRNA is comparable with that of unmodified ds-siRNA. Boranophosphate ss-siRNA has excellent maximum silencing activity and is highly effective at low concentrations. The silencing activity of boranophosphate ss-siRNA is also durable, with significant silencing up to 1 week after transfection. Thus, we have demonstrated that boranophosphate-modified ss-siRNA can silence gene expression as well as native ds-siRNA, suggesting that boranophosphate-modified ss-siRNAs should be investigated as a potential new class of therapeutic agents.

INTRODUCTION

RNA interference (RNAi) is a form of post-transcriptional gene silencing in which double-stranded RNA (dsRNA) targets complementary mRNAs for destruction [reviewed in (1–4)]. RNAi occurs in a wide variety of organisms, ranging from protozoa to mammals. There has been considerable interest in harnessing the power of RNAi to treat human diseases such as viral infections (5,6), cancer (7,8) and

sepsis (9). While questions remain about the precise mechanism of RNAi, recent work has provided a clearer understanding of the process. In the endogenous RNAi system, the effector molecule is a double-stranded short interfering RNA (ds-siRNA) ~21 bp in length with 3' dinucleotide overhangs (10). ds-siRNAs associate with Argonaute family proteins to form an RNA induced silencing complex (RISC) (11,12). One of the strands of the ds-siRNA is removed or destroyed and only a single strand remains in the mature RISC (13–16). The complex then targets mRNA complementary to the incorporated strand (13). Thus, it is the antisense strand of the ds-siRNA that targets a particular mRNA for destruction.

Several studies have shown that it is possible to bypass the dsRNA stage by introducing single-stranded antisense RNA ~21 bases long into RNAi-competent cells or cell lysates (13,17,18). These single-stranded siRNA (ss-siRNA) molecules have several advantages over the standard, ds-siRNA, particularly in the context of potential clinical applications. First, as only one RNA strand is needed, the cost and work of preparation is reduced by half. Single-stranded molecules may also avoid one of the major obstacles to therapeutic use of ds-siRNA: unintended side effects. For example, a recent study showed that both chemically and enzymatically synthesized ds-siRNA species induced interferon expression, whereas none of the ss-siRNA species provoked a detectable interferon response (19). Off-target silencing of genes with similar but non-identical sequences to the sense or antisense strand is another potential obstacle to the specificity of siRNA activity (20). By using a single strand, the risk of off-target silencing guided by the sense strand is eliminated. Finally, ss-siRNAs may circumvent the RNAi-suppressing activity of dsRNA-binding proteins (21,22).

Despite these potential advantages of ss-siRNAs, the single-stranded molecule has a significant shortcoming; ss-siRNAs do not efficiently induce RNAi. ss-siRNA must

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be used at concentrations that are 6- to 100-fold higher than that of ds-siRNA to achieve comparable levels of gene silencing (13,17,18). The reason for the poor silencing activity of the single-stranded molecules is not yet known.

To investigate whether the low level of silencing by ss-siRNAs could be improved, we sought to examine the activity of ss-siRNAs with the boranophosphate (BP) backbone modification. We have shown previously that BP-modified ds-siRNAs are more active for gene silencing than the corresponding phosphodiester- or phosphorothioate-backbone ds-siRNAs (23). In the boranophosphate (BP) linkage (Figure 1A), a non-bridging phosphodiester oxygen is replaced with an isoelectronic borane ($-\text{BH}_3^-$) moiety. BP nucleic acids maintain the ability to base pair with high specificity and affinity and BP nucleotides can be readily incorporated into DNA and RNA molecules by DNA and RNA polymerases, permitting synthesis of stereoregular boranophosphate oligonucleotides (23–28). The boranophosphate modification appears to be well tolerated in mammalian systems; BP ds-siRNAs do not induce any appreciable toxicity in HeLa cells (23). Furthermore, BP DNA dinucleotides are minimally toxic to rodents, and the degradation product of BP oligonucleotides (borate) is minimally toxic to humans (23,29,30).

In this work, we demonstrate that partial BP-modification increases the silencing activity of ss-siRNA against green fluorescent protein in HeLa cells. In our system single-stranded BP-siRNAs can silence gene expression as well as native ds-siRNA.

MATERIALS AND METHODS

Synthesis of boranophosphate siRNAs

ss- and dsRNA molecules were synthesized as described previously (23). Briefly, DNA oligonucleotides were purchased from Qiagen: T7 promoter primer, 5'-TAATACGACTCATATAG-3'; EGFP1 templates: sense, 5'-AAGTTCACCTTGATGCCGTTCTATAGTGAGTCGTATTA-3' and antisense, 5'-AAGAACGGCATCAAGGTGAAGTATAGTGAGTCGTATTA-3'; control EGFP1 templates: sense, 5'-AAGTTCACCGTAGTTCGTTCTATAGTGAGTCGTATTA-3' and antisense, 5'-AAGAACGGAACTACGGTGAAGTATAGTGAGTCGTATTA-3'; EGFP2 templates: sense, 5'-AAGGACTTGAAGAAGTCGTGCTATAGTGAGTCGTATTA-3' and antisense, 5'-AAGCAGACTTCTTCAAGTCCTATAGTGAGTCGTATTA-3'; Control EGFP2 templates: sense, 5'-AAGGACTTTGAAGAAGCGTGCTATAGTGAGTCGTATTA-3' and antisense, 5'-AAGCAGCTTCTTCAAAGTCCTATAGTGAGTCGTATTA-3'. ssRNA molecules were synthesized using T7 RNA polymerase. To create boranophosphate modified oligonucleotides, one or more ribonucleoside 5'-(α -P-borano)triphosphates were added to the transcription reaction in place of the corresponding rNTPs. RNAs were then purified as single strands using phenol extraction and Microspin G-50 Micro Columns (Amersham) or PAGE and suspended in water. To make dsRNA, sense and antisense strands were first annealed and then purified and concentrated using Microcon YM-10 centrifugal filters (Millipore). RNA concentration was determined by UV absorbance at 260 nm.

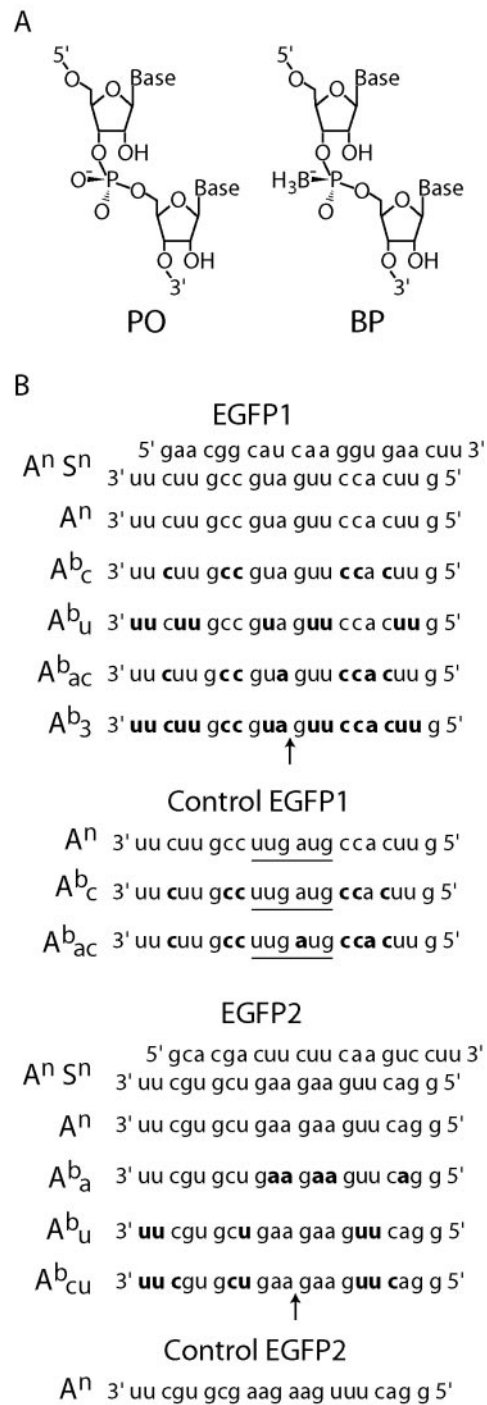


Figure 1. Sequence and structure of interfering RNA. (A) Structure of native (left) and boranophosphate (*Sp* isomer, right) ribonucleic acid backbone linkages. (B) Native and modified siRNA species. Modified nucleotides are shown in boldface. For control siRNAs, the inverted sequence is underlined. Arrows indicate the linkage opposite the expected target cleavage site. A, antisense strand; S, sense strand; b, boranophosphate; n, native; a, adenosine; c, cytosine; u, uridine; 3, adenosine, cytosine and uridine.

Cell culture and transfection

EGFP-Trex HeLa cells, which express enhanced green fluorescent protein (EGFP) under control of a tetracycline responsive promoter, were cultured as described previously (23).

Twenty-four hours after plating, cells were transfected with siRNA using Oligofectamine (Invitrogen) according to the manufacturer's instructions with 1.35 μ l Oligofectamine per well of a 24-well plate. The concentration of siRNA in the cell culture medium ranged from 3.1 to 50 nM, as described in figure legends. Twenty-four hours after siRNA transfection, cells were induced to express EGFP with 1 μ g/ml tetracycline. For the EGFP1 control sequence, the six central nucleotides of the active EGFP1 sequence were inverted. As the control sequence for EGFP2, we used an siRNA in which the eight central nucleotides inverted because the sequence with six inverted nucleotides had an unacceptable degree of homology to some human mRNAs.

Flow cytometry analysis

Cells were trypsinized and fixed in 1% formaldehyde in phosphate-buffered saline, 48 h post-transfection. Cells were analyzed for EGFP expression using a FACScan (Becton-Dickinson) flow cytometer. Data were processed using CellQuest software (Becton-Dickinson).

Cellular RNA analysis

Cells were transfected in culture medium at a final siRNA concentration of 12.5 nM. EGFP1 production was induced with 1 μ g/ml tetracycline. Cells were trypsinized and total cytoplasmic RNA isolated using the RNeasy kit (Qiagen), 22 h after EGFP induction. RNA from each sample (2 μ g) was separated by formaldehyde-agarose gel electrophoresis, transferred to a positively charged nylon membrane and crosslinked by UV irradiation. The immobilized RNA was hybridized with 32 P-labeled RNA probes complementary to the EGFP coding sequence and to human β -actin (to normalize for signal). Hybridization was quantified using a Molecular Dynamics phosphorimaging system.

Quantitative RT-PCR

Cells were transfected in culture medium at a final siRNA concentration of 25 nM or mock transfected with Oligofectamine (Invitrogen) alone. Cells were trypsinized and total cytoplasmic RNA isolated using the RNeasy kit (Qiagen), 24 h after transfection. RNA from each sample (1 μ g) was used to make cDNA using the iScript kit (Bio-Rad). Quantitative PCR was performed using SYBR Green I (Invitrogen), Platinum *Taq* (Invitrogen), 1.5 mM $MgCl_2$ and primers at a concentration of 0.2 μ M. PCR was performed on a Roche Lightcycler and data were analyzed using Lightcycler software. The following primers were used: human Ago2, 5'-TGGCTGTG-CCTTGTAACACGCT-3' and 5'-CGCGTCCGAAGGCTGCTCTA-3'; human GAPDH, 5'-CATGTTTCGTCATGGGTGTG-AACCA-3' and 5'-AGTGATGGCATGGACTGTGGTCAT-3'.

RNase susceptibility analysis

EGFP1 and EGFP2 RNAs (10 pmol) were incubated with 0.5 and 2 ng RNase A (Roche), respectively, in 5 μ l RNase reaction buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA and 150 mM NaCl) at 37°C for specified lengths of time. The RNase reactions were stopped by adding 2 vol of the gel-loading buffer (10 M urea, 50 mM EDTA, 0.1 bromophenol blue and 0.1% xylene cyanol), and loaded on a

4% agarose gel. After SYBR Gold (Molecular Probes) staining, the gels were photographed under UV illumination and analyzed using Fluorescent Image Analytical Software (Hitachi Software Engineering Co. Ltd).

RESULTS

ds-siRNAs and several species of ss-siRNAs, all targeting EGFP, were synthesized as described previously (23) (Figure 1). Briefly, all RNA species were synthesized using T7 RNA polymerase and purified as single strands. To create BP-modified siRNAs, one or more native ribonucleoside triphosphate was replaced by its BP counterpart in the transcription reaction.

Silencing activity of native and BP ss-siRNA

Native and BP ss-siRNAs targeted to EGFP were tested for silencing activity in HeLa cells that express EGFP under control of a tetracycline-inducible promoter. For these studies, cells were transfected with ds- or ss-siRNA, induced to express EGFP, and then, after 24 h, analyzed by flow cytometry to measure the level of EGFP protein expression. The fluorescence of cells transfected with siRNA was compared with the fluorescence of mock-transfected cells (treated with Oligofectamine alone) to determine the percent inhibition of EGFP expression. To control for non-specific effects of the RNA, we tested control sequence ss-siRNAs with the central 6 or 8 nt inverted (Figure 1B). None of the control ss-siRNAs silenced EGFP expression (Figure 2).

Initially, EGFP1 sequence ss-siRNAs were tested at a concentration of 25 nM because this is the concentration at which silencing by native ds-siRNA approaches a maximum, thereby allowing comparison of the peak silencing activity of

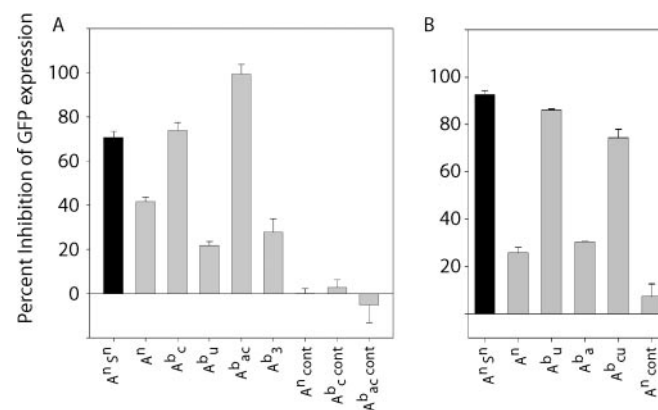


Figure 2. Position specific effects of boranophosphate modifications on silencing activity of ss-siRNAs. (A) Percent inhibition of EGFP fluorescence in cells treated with native or boranophosphate EGFP1 siRNA at 25 nM. To account for variations in transfection efficiency, the results of each individual experiment were normalized to results for native siRNA-treated cells in that experiment. Error bars represent the standard error. (B) Percent inhibition of EGFP fluorescence in cells treated with native or boranophosphate modified EGFP2 siRNA at 12.5 nM. Error bars represent the standard error. ds-siRNA is represented by closed bars. ss-siRNAs are represented by gray bars. A, anti-sense strand; S, sense strand; b, boranophosphate; n, native; a, adenosine; c, cytidine; u, uridine; 3, adenosine, cytidine and uridine; cont, inverted control sequence.

the different siRNAs. Similar to earlier studies (13,17,18), we found that native ss-siRNA could inhibit EGFP expression, but was significantly less active than native ds-siRNA (Figure 2A). When BP-modified siRNAs were studied, we found that ss-siRNA with BP-modified cytidine residues (A_c^b) silenced EGFP expression with the same efficiency as the unmodified ds-siRNA. Furthermore, an anti-EGFP ss-siRNA with modified adenosine and cytidine residues was significantly more active than native ds-siRNA (99% versus 70% reduction in EGFP expression, respectively).

While BP modification of cytidine and adenosine residues significantly enhanced silencing activity of ss-siRNAs, ss-siRNAs that included modified uridines (A_u^b and A_3^b) were notably less active than unmodified ss-siRNA. Therefore, we examined why these species had reduced EGFP silencing activity. We have demonstrated previously in ds-siRNAs that a high proportion of BP residues in the center of the antisense strand reduces siRNA silencing activity (23). As there are four uridine residues among the central six nucleotides (those that surround the predicted target cleavage site, Figure 1B) in the EGFP1 sequence, the reduced silencing activities of A_u^b and A_3^b were consistent with the pattern established previously with ds-siRNAs, and suggest that modification of the central bases of ss-siRNAs reduces silencing activity in a manner analogous to the reduction seen with modification of the central bases of double-stranded BP-siRNAs.

While we suspected that extensive modification of central nucleotides caused the reduction in siRNA silencing, the loss of activity in ss-siRNAs with modified uridines could also have been due to modification of the two residues on the 3' end, or simply due to the presence of modified uridines. To further examine the relationship between modification sites and ss-siRNA activity, a different anti-EGFP sequence (EGFP2) was tested. This sequence has two uridines at the 3' end; however, none of the six central nucleotides are uridines. Instead, there are four adenines in this region (Figure 1B). Once again, the native ss-siRNA had a much lower level of silencing activity than native ds-siRNA (Figure 2B). However, BP-modification of the uridine residues (all peripheral) caused a dramatic increase in ss-siRNA activity, almost restoring it to the level of ds-siRNA. In contrast, when adenosine residues were modified in the EGFP2 molecule (resulting in modification of 3 of the central 6 nt), activity remained at a low level. These results demonstrate that BP-modified uridine residues and 3' overhangs are compatible with ss-siRNA activity and provide additional support for the hypothesis that multiple central BP modifications interfere with efficient silencing.

The overall pattern of increased activity with peripheral modification and reduced activity with central modification was consistent for both anti-EGFP sequences. However, in contrast to the results for EGFP1 ss-siRNAs, increasing the degree of peripheral BP modification in EGFP2 ss-siRNAs caused a slight decrease in silencing activity (Figure 2B, A_u^b and A_{cu}^b).

Dose response of native and BP ss-siRNA

We determined silencing dose response curves for several anti-EGFP1 siRNAs (Figure 3). As we had observed when

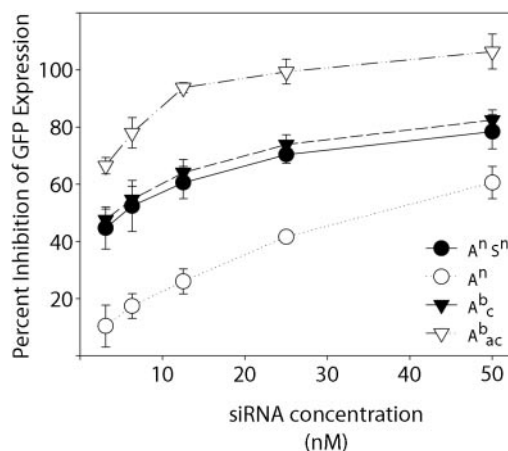


Figure 3. Effect of boranophosphate modification on silencing dose response of ss-siRNAs. Silencing of EGFP fluorescence in cells treated with native or boranophosphate EGFP1 siRNA over a range of concentrations. (A) The activity of each siRNA at 50 nM is normalized to an arbitrary value of 100. (B) Raw dose response data. Error bars represent the standard error. $A^n S^n$ is represented by closed circles, A^n by open circles, A_c^b by closed triangles and A_{ac}^b by open triangles. A, antisense strand; S, sense strand; b, boranophosphate; n, native; a, adenosine; c, cytidine.

a single siRNA concentration was used, the double-stranded native $A^n S^n$ siRNA exhibited moderate silencing activity. $A^n S^n$ induced an ~80% reduction in EGFP fluorescence at 50 nM siRNA and had half maximal silencing activity in the range of 2 nM. In contrast, A^n silences EGFP expression only modestly, inducing only ~60% silencing at 50 mM with half maximal silencing at ~15 nM. When BP cytidine residues are introduced to the EGFP1 ss-siRNA, its activity improves such that the dose response curve of A_c^b is almost identical to that of $A^n S^n$. When modified cytidine and adenosine residues are used, silencing activity of the ss-siRNA becomes highly active; A_{ac}^b induces close to 100% EGFP silencing at 25 nM, with half-maximal silencing induced at ~1 nM siRNA.

Kinetics of silencing by native and BP ss-siRNA

Next, we examined the effect of BP modification on ss-siRNA silencing over time. HeLa cells were transfected with siRNA, induced to express EGFP after 5 h or after 1, 3, 5 or 7 days and fixed 24 h after induction. To assess the duration of silencing relative to each interfering RNA's activity, the level of silencing after 1 day was normalized to 100. While the activity of all RNA species starts to decline after 3 days, the activity of native ss-siRNA (A^n) declines much more quickly than that of native ds-siRNA ($A^n S^n$) or BP ss-siRNAs (A_c^b , A_{ac}^b) (Figure 4). It should be noted that the raw data reveal that, consistent with our other results, native ss-siRNA was significantly less active than native ds-siRNA and that modified ss-siRNAs were as active (A_c^b) or more active (A_{ac}^b) than native ds-siRNA at all times tested (data not shown). Notably, the raw data also reveal that A_{ac}^b still reduced EGFP expression by ~50% 1 week after transfection. Finally, while other investigators have reported that ss-siRNA achieves maximum silencing more quickly than ds-siRNA (18), we did not observe such a pattern.

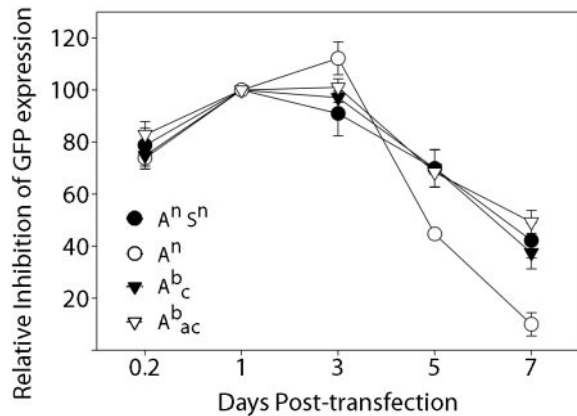


Figure 4. Effect of BP modification on the duration of ss-siRNA silencing. Percent inhibition of EGFP fluorescence over time in cells treated with native ds-siRNA or native/BP ss-siRNA (all EGFP1 sequence) at 25 nM. To examine the relative change in activity over time, the degree of silencing at 1 day after transfection for each siRNA was set at an arbitrary value of 100. Error bars represent the standard error. AⁿSⁿ is represented by closed circles, Aⁿ by open circles, A_c^b by closed triangles and A_{ac}^b by open triangles. A, antisense strand; S, sense strand; b, boranophosphate; n, native; a, adenosine; c, cytidine.

BP ss-siRNA silences by reducing target mRNA levels

siRNAs inhibit protein expression primarily by inducing cleavage of target mRNA. However, it has also been shown that some RNA species, known as miRNAs, can silence genes by inhibiting translation of their targets (31). Previous work suggests that the presence of a base mismatch or a bulge when the siRNA pairs with its target can cause it to function through the miRNA pathway rather than by the RNAi mechanism (32). Because miRNAs have been shown to function as single strands (33), we investigated whether BP ss-siRNAs might act by inhibiting translation of target mRNA. The effects of BP ss-siRNA and native ds-siRNA on EGFP mRNA levels were examined by northern blot and were normalized to β -actin expression. Similar to ds-siRNA, BP-modified ss-siRNA caused a reduction in the level of EGFP mRNA (Figure 5A). To compare the relative change in protein and mRNA levels, samples of the cells used for northern analysis were analyzed by flow cytometry. If the BP ss-siRNA inhibited translation in addition to reducing mRNA levels, it should cause a relatively greater protein inhibition than mRNA inhibition, compared with unmodified ds-siRNA. Instead, for both native ds-siRNA and BP ss-siRNA, the percent inhibition of protein expression closely matches the percent inhibition of mRNA expression (Figure 5B). Furthermore, the ratios of the reductions in protein levels to the reductions in mRNA levels are the same for BP ss-siRNA and native ds-siRNA. We therefore conclude that modified ss-siRNAs inhibit protein expression primarily by reducing EGFP mRNA levels, rather than by inhibiting translation.

BP ss-siRNA activity requires Argonaute2 expression

Having determined that BP ss-siRNA silence protein expression by mRNA cleavage, we further examined the process of silencing by these molecules. Previous work has suggested that native ss-siRNA act through the RISC to induce RNAi (13,17,18). However, in all of these studies,

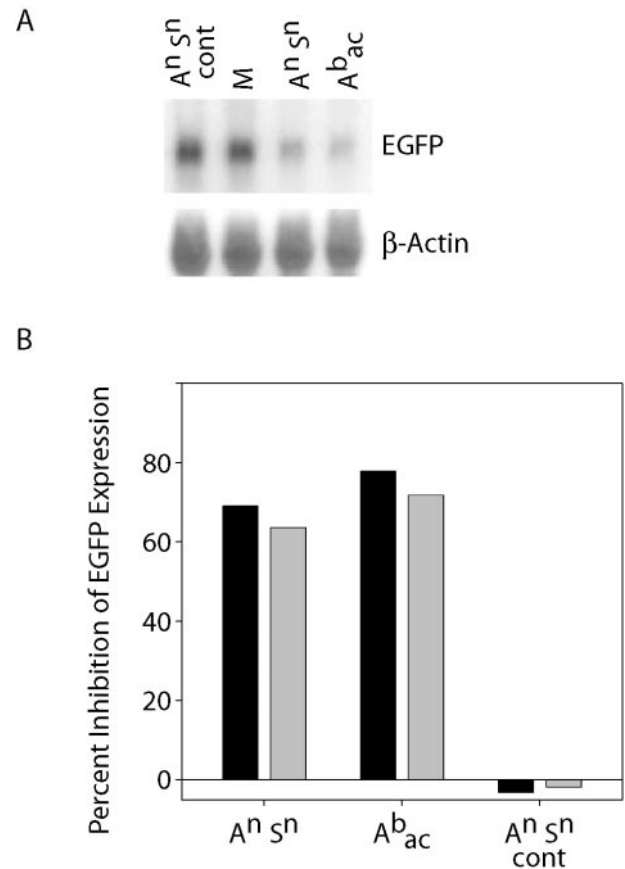


Figure 5. Effect of single-stranded BP siRNA on EGFP mRNA and protein levels. (A) Northern blot of total cytoplasmic RNA from mock transfected cells (M) or cells transfected with EGFP1 siRNA. (B) Percent reduction in mRNA (closed bars) and protein (gray bars) levels as determined by northern and FACS analysis. Samples of cells from the same population were used for both analyses. EGFP mRNA levels were normalized to β -actin expression. A, antisense strand; S, sense strand; n, native; b, boranophosphate; a, adenosine; c, cytidine; cont, EGFP1 control sequence.

the concentrations of ss-siRNA required to achieve gene silencing comparable with that induced by ds-siRNA were 6- to 100-fold higher. Therefore, it is important to determine whether the highly efficient silencing observed with BP ss-siRNA is due to RISC-related activity or to engagement of a different gene silencing mechanism, such as induction of degradation by a double-stranded RNase (34). To determine the RISC-dependence of BP ssRNA silencing, we examined the effect of siRNAs that have been shown to knock-down expression of Argonaute2 (Ago2), a key component of the RISC (35). For these studies, EGFP-HeLa cells were transfected with Ago2 siRNA or with control, non-silencing siRNA. The cells were transfected with native ds-siRNA (which is known to interact with the RISC) or BP ss-siRNA targeted to EGFP, 24 h later. Cells were induced to express EGFP 24 h after the second transfection and analyzed by flow cytometry 24 h after induction. It has been demonstrated previously that when RISC is disrupted by knocking-down Ago2 expression, silencing by ds-siRNA is eliminated (35). If BP ss-siRNA acts through a RISC-dependent process, its silencing activity should be also dramatically reduced when Ago2 is silenced.

The siRNA targeted to Ago2 caused a notable reduction in Ago2 expression as measured by quantitative RT-PCR (Figure 6A). We found that when Ago2 expression was suppressed, the ability of native ds-siRNA to silence EGFP expression was reduced dramatically, compared with cells initially transfected with control siRNA (Figure 6B). The silencing activity of BP ss-siRNA was abrogated to a similar extent by Ago2 silencing, indicating that BP ss-siRNA acts primarily through a RISC-dependent process (Figure 6B). To ensure that inhibiting Ago2 expression does not, by itself, affect EGFP expression, we also examined cells transfected with Ago2 siRNA, followed by control siRNA. The Ago2

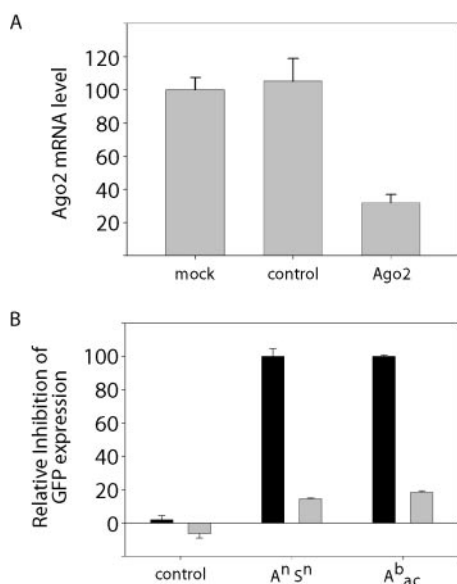


Figure 6. Silencing by boranophosphate ss-siRNA is Ago2-dependent. (A) Cells were mock-transfected with Oligofectamine alone (mock) or transfected with control ds-siRNA (control) or ds-siRNA targeted to Ago2 (Ago2). Ago2 mRNA levels were measured by quantitative RT-PCR and normalized to GAPDH mRNA levels. (B) Cells were transfected with control ds-siRNA (closed bars) or ds-siRNA targeted to Ago2 (gray bars). Twenty-four hours later, cells were retransfected with control ds-siRNA, EGFP1 native ds-siRNA (AⁿSⁿ) or EGFP1 BP ss-siRNA (A^b_{ac}). EGFP fluorescence was measured by flow cytometry. Data were normalized with silencing by anti-EGFP siRNAs after initial transfection with control siRNA set at an arbitrary value of 100. Error bars represent standard error.

siRNA did not appreciably affect EGFP expression; mean fluorescence for mock transfected and Ago2 transfected cells were 537.1 and 536.1, respectively (in arbitrary units). The combination of the northern blot and the Ago2 knock-down data suggests that BP ss-siRNA acts primarily through the standard RNAi pathway used by ds-siRNA.

BP-modification has variable effects on ss-siRNA nuclease resistance

Having demonstrated that the BP modification dramatically improves the silencing activity of ss-siRNAs, we sought to examine the reason for this change in activity. Given the susceptibility of ssRNA to RNases and the enhanced nuclease stability of BP-modified dsRNA (23), we initially speculated that the enhanced silencing activity of BP ss-siRNAs was at least partially due to their greater resistance to RNase degradation. To test this hypothesis, we examined the susceptibility of native and BP ss-siRNAs to degradation by RNase A. As expected, unmodified ss-siRNA was very susceptible to nuclease digestion while ds-siRNA was relatively resistant to degradation (Figure 7). Boranophosphate modification did enhance nuclease resistance of some ss-siRNA species. However, BP modification did not result in a consistent improvement in nuclease stability, nor did resistance to RNase degradation correlate with greater silencing activity. Notably, the most active siRNA we tested, the EGFP1 ss-siRNA with modified adenosine and cytosine residues, was degraded at essentially the same rate as the native ss-siRNA. This result suggests that nuclease resistance may not be the sole reason for the enhanced silencing activity of BP ss-siRNAs.

DISCUSSION

ssRNA can efficiently induce RNAi

While testing the silencing activity of native and boranophosphate modified ss- and ds-siRNAs, we discovered that BP ss-siRNA can be a highly effective gene silencing tool. As seen in several previous studies (13,17,18), we found that the gene silencing activity of unmodified ss-siRNA was substantially less than that of ds-siRNA. In contrast, we found that the most effective BP-modified ss-siRNA species (EGFP1 A^b_{ac}, with modified adenosine and cytidine residues)

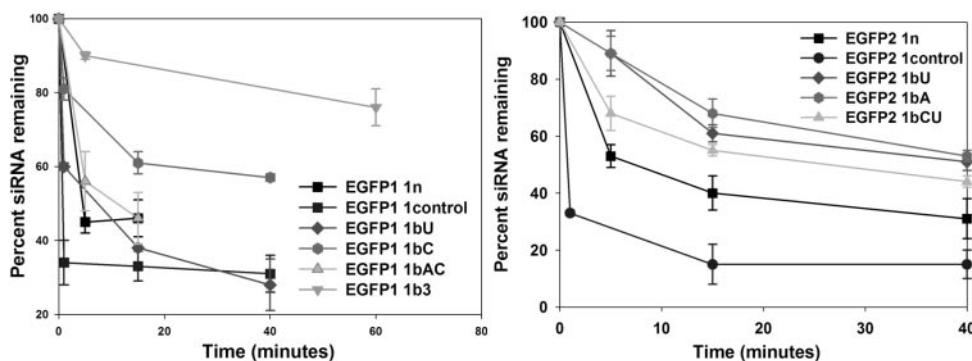


Figure 7. Nuclease stability of native and boranophosphate RNA. ss-siRNAs were incubated with RNase A for the times indicated and then assessed for degradation by agarose gel electrophoresis. b, boranophosphate; n, native; A, adenosine; C, cytosine; U, uridine; 3, adenosine, cytosine and uridine.

is even more active than native ds-siRNA, implying that ss-siRNA can be efficiently incorporated into the RISC (Figures 2 and 3). The maximum activity of A_{ac}^b is also greater than that of any BP-modified ds-siRNA that we have examined (data not shown) (23). To our knowledge, this is the first demonstration that the silencing activity of ssRNA can match or exceed that of dsRNA. Also, given our findings that BP ss-siRNA silencing is due to a reduction in mRNA levels and is Argonaute2-dependent, it seems likely that BP ss-siRNA acts through the standard RNAi pathway.

Structure-activity relationships in BP ss-siRNA

We found that position effects of BP modifications followed the same general rule we discerned when testing BP-modified dsRNA (23): peripheral BP modification is well tolerated, while central modifications cause a significant loss of activity. However, the magnitude of the benefit of peripheral BP modification was greater when applied to single-stranded molecules. At 25 nM, peripheral modification of ss-siRNA caused up to a 2.4-fold increase in silencing activity (Figure 2, A_{ac}^b), compared with a maximum increase in ds-siRNA activity of 1.3-fold (23).

Because peripheral modifications dramatically increase ss-siRNA silencing activity, we initially hypothesized that the positive attributes conferred by central modifications would offset their inhibitory effects, such that even central modifications would enhance activity. Instead, ss-siRNA species with central modifications have activity levels that are as low as or lower than those of native ss-siRNA (Figure 2). Apparently, the unfavorable effects of central modification are still significant in single-stranded molecules. The poor activity of centrally modified ds-siRNA and ss-siRNA may reflect a disruption of the enzymatic activity of the RISC, which cleaves the target directly across from the center of the interfering RNA (36). Previous studies have demonstrated that central sequence mismatches or ribose modifications inhibit ds-siRNA activity (36,37), although the regions that are sensitive to such changes appears to be more extensive than the area that is sensitive to BP modification.

Mechanisms of enhanced activity in boranophosphate ss-siRNA

We initially hypothesized that BP-modified ss-siRNAs would be more nuclease resistant than native ss-siRNA and that this would be a major reason for their enhanced silencing activity. However, we found that BP modification did not consistently result in greater resistance to RNase A digestion for the sequences tested here. Indeed, the BP ss-siRNA species with the greatest silencing activity (EGFP1 A_{ac}^b) was degraded at essentially the same rate as native ss-siRNA (Figure 7). It is still possible that BP modification could confer greater stability within the cell or in the culture medium; RNase A resistance may not directly correlate with resistance to other nucleases. However, the absence of an observable increase in RNase stability with BP modification suggests that the enhanced activity of BP ss-siRNAs may not be solely due to improved nuclease resistance. This hypothesis is supported by other work showing that modifications that confer nuclease resistance do not necessarily enhance the activity of ds- or ss-siRNAs (37,38).

Instead, we suspect that BP ss-siRNAs have other advantageous properties that confer efficient silencing activity. Borane substitution of a non-bridging oxygen results in changes in size, charge, polarity and stereochemistry (39). It can be imagined that the presence of a borane group instead of oxygen in the phosphate backbone of RNA would change their interaction with other molecules, e.g. mRNA target and RISC protein components. Indeed, we have found recently that BP modification raises the melting temperature of siRNAs, suggesting that a modified siRNA might have a greater affinity for the target mRNA than a native siRNA (40). Another characteristic of BP siRNA that may enhance its silencing activity is its tendency to make an A-form helix (40). The results of several different studies suggest that efficient cleavage of the target RNA requires the A-form structure between the guide strand of the ds-siRNA and the target (37,41,42). Investigation of the interactions between BP ss-siRNA and RISC should facilitate mechanistic understanding of RNAi and may suggest generally applicable strategies for enhancing siRNA activity.

Therapeutic potential of boranophosphate ss-siRNAs

Our examination of silencing by boranophosphate-modified ss-siRNA suggests that BP ss-siRNA could have significant therapeutic potential. The high maximum activity, potency at low concentrations and duration of silencing by EGFP1 A_{ac}^b suggest that relatively low and infrequent dosing of BP ss-siRNA could be effective, even in rapidly dividing cells. Indeed, by accounting for dilution by cell division and comparing the activity over time to dose response curves, it appears that essentially all of the decline in activity can be accounted for by this dilution. Therefore, we expect that silencing would last even longer in non-dividing cells or when the siRNA blocks cell growth (7).

In addition to its excellent silencing activity, BP ss-siRNA has advantages unique to ssRNA: simplified synthesis and no risk of off-target silencing by the sense strand. Furthermore, the smaller size and reduced negative charge of ss-siRNA may facilitate delivery into cells. We also believe that ssRNA is less likely to induce an interferon response than dsRNA. Most examinations of interferon induction by siRNAs have shown that ss-siRNAs do not provoke an interferon response (19). However, a recent publication reported that ssRNA synthesized by phage polymerases (the method used here) can induce interferon expression due to the presence of 5' triphosphate groups (43). In the context of potential clinical applications, this should not pose a significant problem because the terminal phosphates can be easily removed by calf intestinal alkaline phosphatase treatment (43). Because control sequence siRNAs (also synthesized by T7 polymerase) did not show any silencing activity (Figure 2), we do not believe that our analysis of silencing activity was affected by the presence of 5' triphosphate groups on the siRNAs we tested.

The use of single-stranded molecules may be particularly desirable in specific situations. For example, BP ss-siRNAs may permit the use of sequences whose sense strand sequence would be nearly complementary to other (off-target) human genes. In addition, the ability to use single-stranded molecules should enhance silencing by sequences that would

otherwise have poor activity due to incorporation of the inactive sense strand into the RISC [it should be noted that other strategies to favor incorporation of the active strand have also been developed (44)]. This relaxation of sequence requirements is likely to be especially important when there are relatively few potential sequences, such as when the target is a particular splice variant or mRNA with a specific point mutation. ss-siRNAs are also likely to be useful in treating viral infections because many human viruses express dsRNA-binding proteins, presumably to evade the host cell interferon response (21,22).

CONCLUSION

We have shown that boranophosphate modified ss-siRNA efficiently induces silencing at high and low concentrations and for extended periods of time. This finding is significant, because it demonstrates that, when appropriately modified, ss-siRNA can induce RNAi at least as effectively as ds-siRNA. It also introduces a new category of molecules for potential use as RNAi-based therapeutics.

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Conflict of interest statement. None declared.

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