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RESEARCH PAPER

Enhanced Light-Activated RNA Interference Using Phosphorothioate-Based dsRNA Precursors of siRNA

Ashish Kala • Simon H. Friedman

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ABSTRACT

Purpose To improve light-activated RNA interference by incorporating phosphorothioate linkages into the dsRNA used. The rationale behind this approach is that the groups have the potential to improve nuclease stability and therefore prevent cleavage of photolabile groups from the RNA termini prior to photolysis.

Methods Photolabile groups (di-methoxy nitro phenyl ethyl or DMNPE) were incorporated into multiple double-stranded precursors of siRNA (dsRNA) that had six, two or no phosphorothioate linkages at the 3′ and 5′ ends of the strands. They were analyzed for their ability to toggle light-activated RNA interference with light and for serum stability.

Results Incorporation of phosphorothioate linkages increased serum stability of all dsRNA examined. Presence of DMNPE groups reduced overall stability of the modified RNA relative to the analogous species without DMNPE modification. DMNPE-modified dsRNA with two phosphorothioate linkages in each strand significantly improved the window of expression toggled by light.

Conclusions Incorporating phosphorothioate groups into dsRNA both stabilizes them towards degradation by serum enzymes, as well as improves them as the basis for light-activated RNA interference.

KEY WORDS light-activated gene expression · phosphorothioate groups · RNA interference · serum stability · siRNA

INTRODUCTION

Multiple approaches have been explored to bring gene expression under the control of light (1–6). The promise of such techniques is that they would allow the spacing, timing and amount of irradiation to control the spacing, timing and amount of gene expression. This would allow for biological systems to be manipulated and understood at unprecedented levels which would be of particular benefit to the study of developmental biology and targeted delivery. To this end, our group developed light-activated RNA interference, or LARI, several years ago (7–9). This technique takes advantage of the endogenous RNA interference pathway and, using siRNA or dsRNA modified with photolabile groups, allows RNA interference to be controlled with light. Subsequently, others have also attempted to bring RNA interference under the control of light using a variety of approaches (10–12).

Previously, we have modified siRNA and their dsRNA precursors with the di methoxy nitro phenyl ethyl group (DMNPE) and shown that RNA interference can be toggled with such an RNA (9). We have shown that the reactive diazo-DMNPE group does *not* modify backbone phosphates of RNA randomly as previously described, but rather very specifically modifies terminal phosphates, if they have been incorporated (8). This remarkable regiospecificity was confirmed by model studies and MS/MS analysis. It allows us to precisely install four photolabile groups per duplex by preparing the original duplex with four terminal phosphates.

Electronic Supplementary Material The online version of this article (doi:10.1007/s11095-011-0529-z) contains supplementary material, which is available to authorized users.

A. Kala • S. H. Friedman (✉)
Division of Pharmaceutical Sciences, School of Pharmacy
University of Missouri
2464 Charlotte St.
Kansas City, Missouri 64108, USA
e-mail: friedmans@umkc.edu

The rationale behind light-activated RNA interference is that modified RNA will interact poorly with the cellular machinery responsible for RNA interference (such as the RISC complex in the case of siRNA and Dicer in the case of dsRNA precursors) and thus be blocked. Irradiation will release native RNA and allow RNA interference to proceed. What we have found is that while the release of native RNA is complete upon irradiation, the initial blocking of RNA interference by the DMNPE group is incomplete, i.e. there is some knockdown of expression even prior to irradiation. This may be caused by several mechanisms: 1) the photocleavable group may not completely block the interaction of modified RNA with RISC/Dicer, 2) the group may be removed by cellular nucleases prior to irradiation. Evidence has shown that even partially nuclease-degraded siRNA/dsRNA have the ability to effect RNA interference; thus, nuclease-reacted duplexes may lose the terminal nucleotide (and hence DMNPE group) while maintaining the ability to knock down expression (13).

Our lab is exploring multiple solutions to this problem of incomplete block of RNAi prior to irradiation. One solution is to modify DMNPE itself to increase its ability to interfere with the interaction with RISC/Dicer. Another is to increase the nuclease resistance of the modified RNA to prevent the DMNPE groups from being prematurely cleaved. This latter approach is described in this work. Specifically, we have incorporated phosphorothioate linkages between the bases at the termini of the RNA to make it more difficult for exo-nucleases to remove the terminal bases and, thus, the DMNPE group. Using this modification, we have significantly improved the range of expression toggled by LARI.

MATERIALS AND METHODS

PS dsRNA Caging with DMNPE-diazo

MnO_2 (10 mg, 0.115 mmol) was added to a solution of 4,5-dimethoxy-2-nitroacetophenone hydrazone (2.5 mg, 0.011 mmol) (Molecular Probes) in DMSO (250 ml). After agitation at room temperature for 45 min, the suspension was filtered through Celite (Molecular Probes) to remove MnO_2 from the activated caging compound. A volume of this filtrate (25.6 µl, corresponding to 250 eq. of DMNPE to PS dsRNA) was gently agitated with PS dsRNA (50 µl, 24.7 uM) at room temperature for 24 h, protected from light. The caged PS dsRNA was treated with 37.8 µl of 10 M ammonium acetate for 10 min at room temperature, followed by precipitation with ethanol in the presence of glycogen.

HPLC Analysis

Caged PS dsRNA duplexes were purified via reverse phase HPLC. The analysis was carried out using a Varian Microsorb C8 column (250×4.6 mm). Elution was done at a flow rate of 1 ml/min. Solvent-A contained pH 7, 0.1 M triethylammonium acetate buffer. Solvent-B was pH 7, 0.1 M triethylammonium acetate buffer in 50% acetonitrile. The gradient used was 0% B to 10% B over 15 min, increased to 40% B at 25 min and to 100% B at 50 min and then maintained at 100% B until 60 min. Precipitated caged PS dsRNA were dissolved in water (90 µl), followed by their injections in HPLC. Collected fractions were dried using Savant speed vac system. Furthermore, dried fractions were redissolved in water, and appropriate dilution was made for analytical runs.

Electrospray Ionization Mass Spectrometry

Purified caged PS dsRNA was dissolved in 100 µl of a 50:50 water-acetonitrile mixture containing 1% triethylamine to make a concentration which ranged from 3–5 µM. Analysis was carried out in the negative ion mode using Q Trap mass spectrometer (ABI).

Culture and Transfection of Cells

HeLa cells were obtained from American Type Culture Collection (ATCC). Cells were plated at 70% confluency in a 96-well plate (Corning) 18 h prior to transfection. The medium in each well was replaced with a mixture containing 0.099 ug pEGFP-C1 plasmid, 0.132 µg pDsRed2-N1 plasmid (Clontech), 0.19 pmoles of caged or uncaged dsRNA (1.56 nM) and 1.125-µl lipofectamine (Invitrogen) in 120 µl OPTI-MEM (Gibco). Six hours after transfection the transfection mixture was removed, and cells were rinsed with 150 µl of OPTI-MEM. One hundred µl OPTI-MEM was then added to each well, and "irradiated" cells were exposed to light for ten minutes from a UV lamp (Blak-Ray Lamp, Model XX-15 L, 30 W) at a distance of 10 cm from the lamp. A WG-320 longpass filter (Edmunds Industrial Optics) was used to protect the cells from short-length UV. OPTI-MEM was then replaced with 200 µl antibiotic-free DMEM (Invitrogen) supplemented with 10% FBS. Cells were incubated for 42 h followed by washing with 200 µl of phosphate-buffered saline (PBS, Invitrogen). One hundred µl PBS was then added to each well. GFP and RFP expression were quantitated using a microplate reader.

Serum Stability of PS dsRNA

One hundred and seventy pmol of RNA duplexes (2 µl of 85 µM solution) were incubated at 37°C in 55 µl of 80%

FBS in DMEM (Gibco). Aliquots of 3.4 µl (containing 10 pmol of PS dsRNA) were collected at different time points and frozen at −80°C. One µl of 6X gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water) was added to the aliquots. Samples were run on 15% non-denaturing polyacrylamide gels in TBE buffer followed by GelStar (Lonza) staining. Gel reading was performed using Gel Doc 2000 (Bio-Rad). Digitized images of the gels were analyzed in photoshop for band signal. These signals were corrected for background signal and plotted using KaleidaGraph software.

RESULTS AND DISCUSSION

Modification and Characterization of Oligonucleotides

We examined two different phosphorothioate-containing dsRNAs: one with the final phosphodiester bonds at the 3′ and 5′ ends of each strand replaced with a phosphorothioate bond, designated PS2, and one with the final three phosphodiester bonds at the 3′ and 5′ ends replaced with phosphorothioate bonds, designated PS6. These were then compared with the same sequence with normal phosphodiester bonds, designated PS0 (Fig. 1). All species had 3′ and 5′ terminal phosphates to guide reaction of the diazo-DMNPE with the termini. Briefly, dsRNAs were reacted with excess diazo-DMNPE overnight. The modification reaction is self limiting because of the significantly higher rate of reaction of terminal phosphates than internal phosphates or nucleobases mentioned above and described by our group previously (8). The major product of the reaction is a duplex with one DMNPE group on each of four terminal phosphates. The nucleic acids were precipitated from solution and purified by HPLC.

The analytical chromatograms of the purified PS2 sample at 260 nm (nucleotide lambda max) and 346 nm (DMNPE lambda max) are shown in Fig. 2. Using these chromatograms, and the relative extinction values of the DMNPE chromophore and nucleobase chromophore, we calculated the mole ratio of the DMNPE groups to dsRNA duplex. The 346 nm integration and DMNPE 346 nm extinction coefficient were used directly to assess the moles of DMNPE present. The 260 nm chromatogram was used to assess the moles of RNA, after correction of the 260 nm component due to the DMNPE group (assessed by using the extinction ratios at 260/346 and the 346 nm integration value). Using this approach, we determined a ratio of DMNPE groups to dsRNA of 4.27. This is consistent with our expectation that one group is incorporated per terminal phosphate.

This ratio was confirmed by electrospray mass spectrometry (Fig. 3). Samples were run as previously described (14). Ammonium acetate was used to precipitate the purified duplexes, and they were infused at a concentration of 5 µM. Raw spectra, which contain multiple peaks representing different charge states of a given specie, were deconvoluted to give the final spectrum. The deconvoluted spectrum of purified, DMNPE-modified PS2 sample is shown in Fig. 3. This spectrum primarily shows sense and antisense strands with two DMNPE groups incorporated. We typically see separated strands in the ESI-MS of small duplexes (8,14). The expected mass (in parenthesis) and the observed mass (listed below in the spectrum) are within 20 amu of each other. More importantly, the "delta-delta" or difference between the absolute errors in mass in different species is 0.0. The spectrum of PS6 also shows two modifications per strand, with an absolute error in mass of ~7 amu and a delta-delta of 1.1 amu. Both spectra also show −135 depurination peaks, mass spectrum artifacts that we and others observe in duplexes, modified and unmodified.

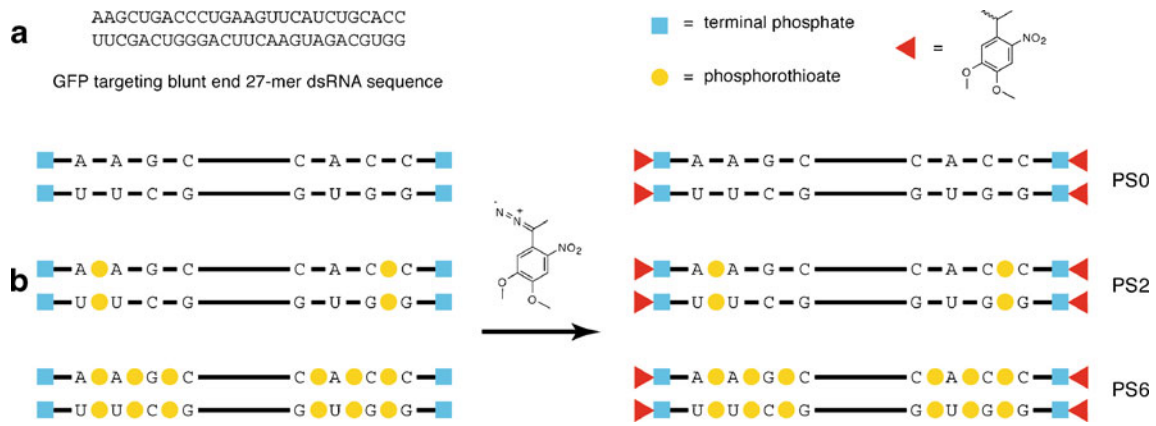

Fig. 1 (**a**) 27-mer blunt end dsRNA sequence used to target GFP. (**b**) Normal and phosphorothioate-containing duplexes used in this study and their modification with diazo-DMNPE.

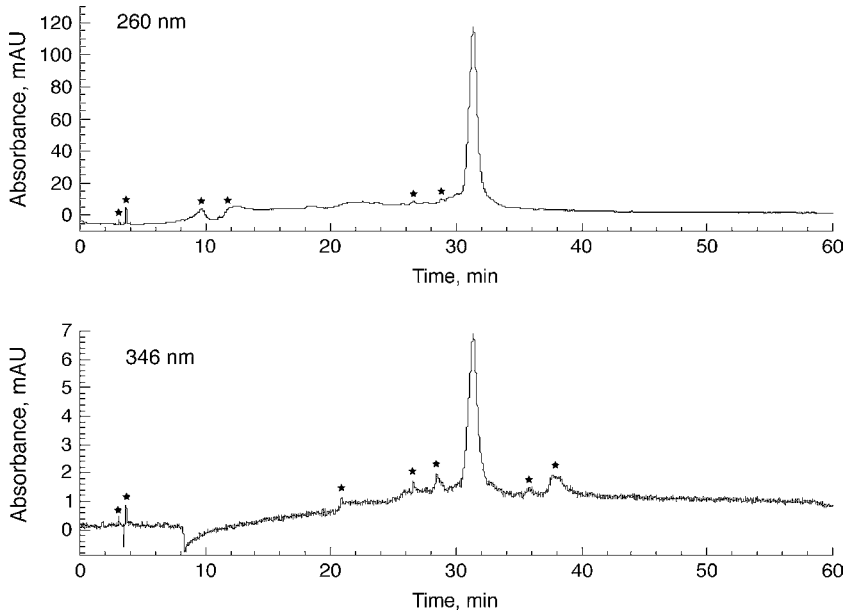

Fig. 2 Chromatograms of purified PS2 at 260 nm and 346 nm, the lambda max values of the RNA and DMNPE, respectively. Starred peaks are also found in blank runs. Integrations indicate a ratio of 4.27 DMNPE groups per RNA duplex.

These spectra are consistent with the mole ratio determinations from HPLC data described above and further confirm the regiospecificity of the diazo-DMNPE reaction.

Biological Assessment of Modified Duplexes

We then tested the effectiveness of these duplexes to toggle gene expression with light using our standard test system (9). HeLa cells were plated at 70% confluency in 96-well plates and allowed to culture for 18 h. Duplexes at a concentration of 1.56nM and plasmids expressing GFP and RFP were then cotransfected into these cells. After 6 h of transfection, medium was removed, the cells rinsed, and fresh medium added. Cells were then either irradiated or not with a Blak-Ray fluorescent bulb having an emission maximum of 360 nm. All light was filtered using a 320 nm long pass filter to prevent phototoxicity. Cells were then returned for culturing for an additional 42 h.

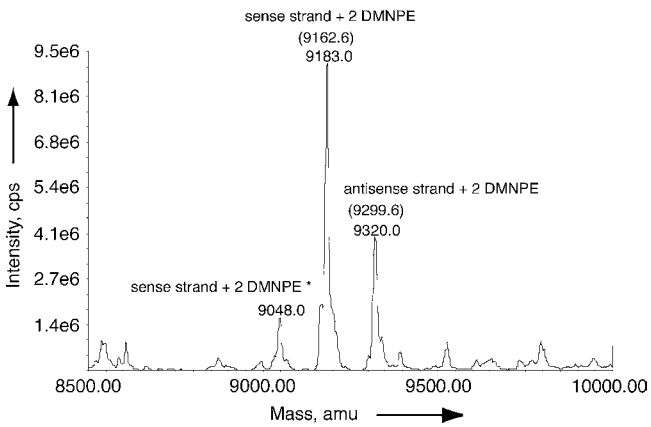

Fig. 3 Deconvoluted mass spectrum of purified tetra-modified PS2 showing both sense and anti-sense strands with two modifications each. Starred peaks are depurination products.

The GFP and RFP produced over this time period were determined using a microplate reader. Five replicates of each assessed condition allow for narrow error bars. All of the tested duplexes are based on the same sequence known to target GFP expression and not RFP expression. RFP expression is used as an internal control. All signals are corrected for the autofluorescent signal found in untreated cells. The raw GFP/RFP signal ratios for a given experimental point are then expressed as a proportion to that same ratio as found in unirradiated cells treated with plasmids alone. This allows for a determination of specific knockdown of the target GFP.

The results of our first comparison between dsRNA without phosphorothioate groups (PS0) and dsRNA with two phosphorothioate groups per strand (PS2) are shown in Fig. 4. Dark bars and light bars indicate GFP expression in cells that were masked or irradiated respectively. The DMNPE-modified PS0 sample gives a toggling of expression similar with what we have previously observed. The DMNPE-modified PS2 sample produces a window of expression that is significantly wider, due to an increase in GFP expression in the absence of light, and to a decrease in GFP expression following irradiation. We repeated this set of experiments to confirm this increased window of expression and found virtually identical pattern of increase (seen in Supplementary Material). The average increase in the expression window of these two experiments is 40%, a significant improvement. The absolute values of expression can vary from experiment to experiment, which is why we assess the improvement in this window relative to a previously characterized duplex.

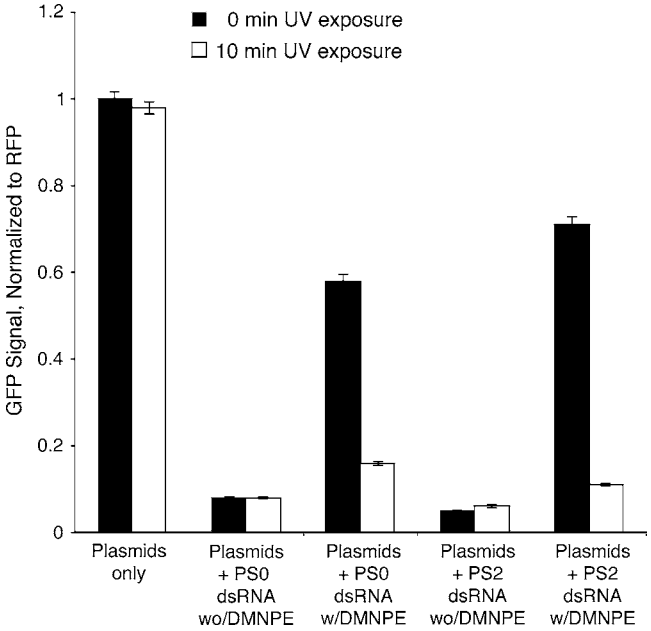

Fig. 4 Comparison of light-activated RNA interference using PS2 and PS0 duplexes. Dark bars indicate signals from unirradiated cells, light bars from cells irradiated for ten minutes.

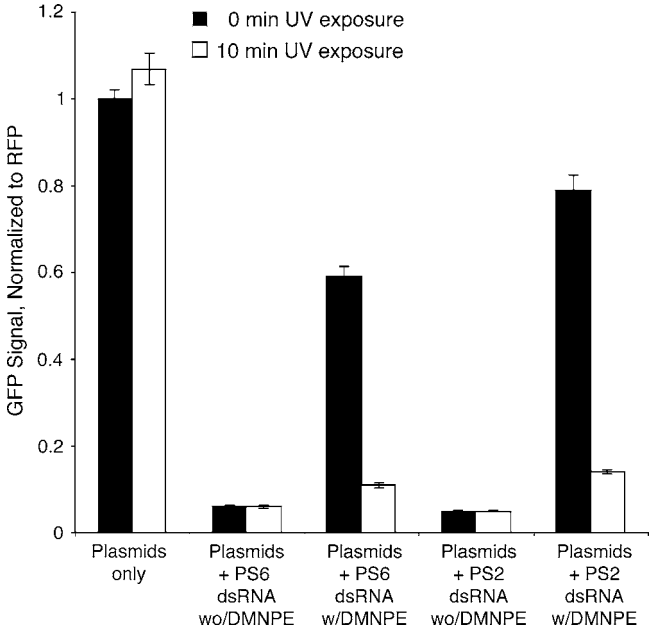

Fig. 5 Comparison of light-activated RNA interference using PS2 and PS6 duplexes. Dark bars indicate signals from unirradiated cells, light bars from cells irradiated for ten minutes.

The success of the use of phosphorothioate linkages to increase the block of RNA interference prior to irradiation suggested that additional phosphorothioate linkages could increase this effect. We therefore prepared DMNPE-modified GFP targeting dsRNA with the final three phosphodiester linkages in the 5′ and 3′ ends of each strand being replaced by phosphorothioates (e.g. duplex PS6). In addition, the duplexes were capped with phosphates on each of the four termini. These were reacted with diazo-DMNPE, purified, and analyzed as described above for duplex PS2. The major specie produced was shown as before to be the duplex modified with four DMNPE groups. This was confirmed by an analysis of the mole ratio of DMNPE groups to duplex (found to be 4.15) as well as from the mass spectrum. Chromatograms and the mass spectrum for duplex PS6 are found in Supplementary Material.

The biological assessment of duplex PS6 was performed identically to that described for PS2 above. However, instead of comparing PS6 with PS0, it was compared with PS2 to determine if it improved relative to that duplex. Interestingly, PS6 did not perform as well as PS2 and had a larger knockdown of GFP expression prior to irradiation (Fig. 5). Again, multiple repetitions of this experiment reproduced this difference (see Supplementary Material).

Analysis of Serum Stability

In an attempt to understand the trend we observed in the LARI expression window, we then examined the serum stability of all the species analyzed. This included PS0, PS2 and PS6, all with and without DMNPE modification. We used a modified approach of that used by multiple groups (15–17). The RNA species were individually incubated in 80% fetal bovine serum over a period of 90 h. At regular intervals, aliquots were sampled, flash frozen and saved for analysis. At the termination of the experiment, the samples were thawed, mixed with loading buffer and run on 15% native polyacrylamide gels. Gels were stained with GelStar and visualized using a Bio-Rad Gel Doc 2000, and individual bands were quantitated using Photoshop. The ratio of band intensity *versus* untreated RNA was plotted as a function of time and a first-order fit performed. Each species was examined in two separate experiments. A representative gel for PS6 and its corresponding fit are shown in Figs. 6 and 7, respectively. Gels and plots for PS0 and PS2 as well as repetitions using all three species are included in Supplementary Material. We observed that the products of degradation do not appear to stain to the same degree as the initial duplexes. This may be due to less efficient staining of shorter lengths of nucleic acid. Our kinetic analysis therefore was fit to the loss of starting material.

A bar graph depicting the relative first-order constants for degradation of all six species analyzed is shown in Fig. 8. This represents the average value for two repetitions of the study. As might have been anticipated, there is a trend with both DMNPE-modified and native duplexes, with increasing numbers of phosphorothioate linkages leading to greater stability. What is surprising is that for each duplex, the DMNPE-modified version is moderately less stable

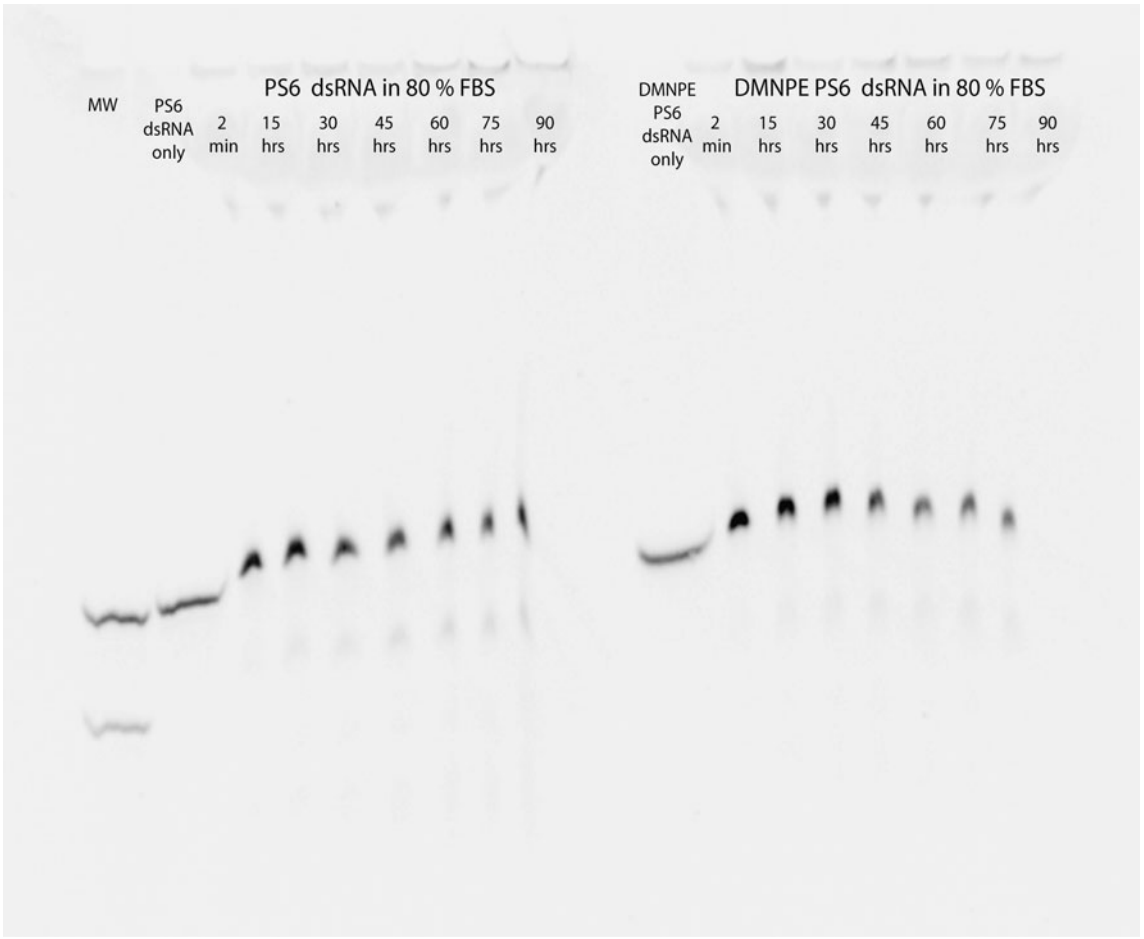

Fig. 6 Gel analysis of serum stability of PS6 phosphorotioate siRNA, with and without DMNPE groups attached.

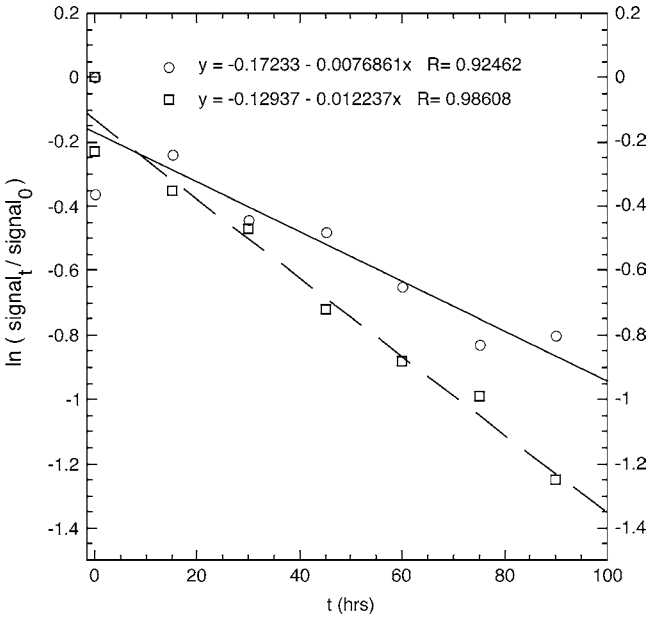

Fig. 7 Plot of quantitated gel analysis, with first-order fit indicated. Plot indicates loss of starting material. Circles are uncaged dsRNA. Squares are DMNPE caged dsRNA.

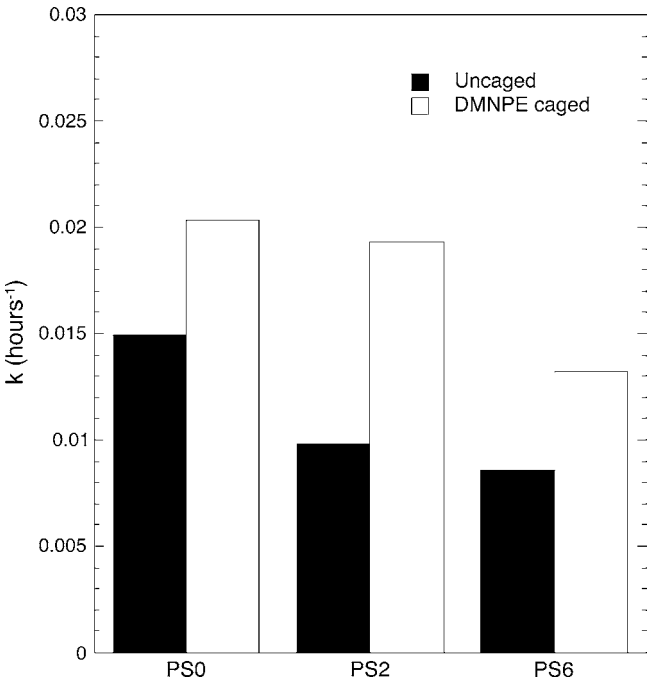

Fig. 8 Comparison of different duplex stabilities. First-order rate constant, k, indicated.

towards degradation, although with all the species, significant amounts of undegraded duplex remains even after 90 h.

CONCLUSIONS

The aim of this work was to increase the window of gene expression toggled by light using the technique we have established, namely light-activated RNA interference (LARI). To do this, we turned to non-native RNA, which incorporated phosphorothioate groups. The motivating rationale was that these groups would help prevent premature loss of the photolabile DMNPE group caused by nucleases (as opposed to light).

We took advantage of the remarkable regiospecificity that we discovered that causes diazo-DMNPE to preferentially react with terminal, as opposed to internal, phosphates in nucleic acids. We observed a similar pattern of reactivity with phosphorothioate-modified RNA, which resulted in our efficient preparation of dsRNA containing four DMNPE groups per duplex. This corresponds to the four terminal phosphates incorporated into the ends of the RNA. Thus, we have further demonstrated the generality of this approach.

We then showed that by incorporating just two phosphorothioate groups per strand, we can consistently increase this window of toggled expression by 40%. This increase is mostly attributable to the increase in GFP expression prior to irradiation. This can also be described as being a suppression of RNA interference prior to irradiation. This improved block of RNA interference through the use of PS2 is consistent with the original rationale, namely that the knockdown of expression prior to irradiation is due to loss of the DMNPE groups due to nuclease action on the strands. The incorporated phosphorothioate groups may help prevent this action and thus maintain the integrity of the DMNPE group, which blocks RNAi until irradiation takes place.

Why then was increasing the number of phosphorothioate groups in PS6 counterproductive, resulting in a narrowing of the expression window relative to PS2 (and similar to PS0)? This is a surprising result, in particular because one of the new phosphorothioate bonds is one that is cleaved by Dicer, and one might expect the block of RNAi to be higher prior to irradiation, due to increased retention of the DMNPE group. We can speculate that the increased number of PS groups in PS6 (6 per strand, 12 per duplex) actually increases the affinity of the duplex for Dicer. Phosphorothioate groups are known to enhance the non-specific affinity of nucleic acids for polymerases and nucleases, and this increased affinity could have overcome the blocking ability of the DMNPE group, thus allowing for Dicer to process the duplex into an active specie.

In examining the serum stability of these modified and unmodified duplexes we saw two broad trends. First, we observed that the presence of phosphorothioates tended to reduce the rate of degradation of the duplexes. Second, and less expected, we found that uniformly, the presence of the DMNPE group seemed to *increase* the rate of degradation of the duplexes, relative to the same specie without the DMNPE group. One interpretation of this is that the DMNPE group actually enhances the interaction of the modified RNA with nucleases, thus leading to more efficient degradation. In any case, the amount of modified RNA that remains in our biological experiments has proven to be sufficient to allow full knockdown after irradiation.

The increased range of gene expression toggled by light provided by the judicious use of phosphorothioate groups described in this work increases the utility of LARI for the examination of biological systems. Because LARI is built on the process of RNA interference, it benefits from the strengths of this fundamental mechanism. By bringing RNA interference under the control of light, disciplines such as developmental biology, which are closely linked with the spacing, timing and degree of gene expression, will be able to benefit from LARI's ability to use light to manipulate these same dimensions of gene expression.

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