

Light-activated RNA interference using double-stranded siRNA precursors modified using a remarkable regiospecificity of diazo-based photolabile groups

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ABSTRACT

Diazo-based precursors of photolabile groups have been used extensively for modifying nucleic acids, with the intention of toggling biological processes with light. These processes include transcription, translation and RNA interference. In these cases, the photolabile groups have been typically depicted as modifying the phosphate backbone of RNA and DNA. In this work we find that these diazo-based reagents in fact react very poorly with backbone phosphates. Instead, they show a remarkable specificity for terminal phosphates and very modest modification of the nucleobases. Furthermore, the photo deprotection of these terminal modifications is shown to be much more facile than nucleobase modified sites. In this study we have characterized this regiospecificity using RNA duplexes and model nucleotides, analyzed using LC/MS/MS. We have also applied this understanding of the regio-specificity to our technique of light activated RNA interference (LARI). We examined 27-mer double-stranded precursors of siRNA ('dsRNA'), and have modified them using the photo-cleavable di-methoxy nitro phenyl ethyl group (DMNPE) group. By incorporating terminal phosphates in the dsRNA, we are able to guide DMNPE to react at these terminal locations. These modified dsRNA duplexes show superior performance to our previously described DMNPE-modified siRNA, with the range of expression that can be toggled by light increasing by a factor of two.

INTRODUCTION

RNA interference is a broadly observed biological process by which gene expression is controlled with small RNAs (1–4). In addition to being a fundamental mechanism in biology, it has also proven to be a useful tool for exploring the role of gene expression in a range of phenomena (5–8). Multiple research groups have explored a variety of nucleic acid modifications to expand the utility of small interfering RNAs (9–15). In addition, a wide range of nucleic acid-based biochemical processes have been modulated using light sensitive modifications (16–22). Our and others' interest is in bringing RNA interference under the control of light by incorporating light sensitive modifications into siRNA (23–26). Controlling the spacing, timing and degree of gene expression by modulating the spacing, timing and amount of light applied to cells could be extremely beneficial for patterning of gene expression, creating a sustained siRNA delivery method, examining the fundamental kinetics of RNA interference and especially for the study of development, a process which is closely linked to the location, timing and degree of expression of key proteins (morphogens).

In our recent publication, we demonstrated that RNA interference can be controlled by light through putatively random modification of the siRNA duplex by the di-methoxy phenyl ethyl (DMNPE) photolabile protecting group (26). We presumed that this modification was principally on phosphate groups. We observed that moderate caging (one to two groups per duplex) was unable to completely abrogate RNA interference prior to irradiation. We also showed that increasing the number of protecting groups on the siRNA increased the blockage of RNA interference, but the amount of irradiation required to then deprotect such highly modified siRNA resulted in

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phototoxicity (26). In addition to this use of random modifications, we have showed that specific modification of the 5' phosphate of the antisense strand of a siRNA duplex with a photolabile group also blocks RNA interference, however, again only partially (25). This has also been explored by Nguyen *et al.* (24). Our interest is in enhancing LARI by developing a system that completely abolishes the ability of modified siRNA to silence genes in the absence of light and completely regains its activity upon irradiation with an amount of light that does not result in phototoxicity.

To develop this ideal tool, we have examined the caging of blunt end precursor RNA duplexes (dsRNA) of siRNA. This was based on the assumption that double-stranded RNA precursors of siRNA would be more sensitive to modifications as they need to be processed by both Dicer and RISC before exhibiting their activity, compared to native siRNA duplexes, which need to be processed only by RISC (27). In addition, dsRNAs have been shown to exhibit faster gene silencing, longer duration of action and increased potency compared to siRNAs. Due to the faster gene silencing observed with dsRNAs, caging dsRNAs directly could also be a potential solution to limit the lag time in RNA interference observed with caged siRNAs.

In the present work, we demonstrate that the effective modulation of RNA interference with light using dsRNA hinges critically on the use of RNA duplexes that contain terminal phosphates because of a marked difference in the reactivity of diazo-DMNPE with terminal versus internal phosphates. This differential reactivity was confirmed using both RNA duplexes and model nucleotides, characterized using mass spectrometry. The results presented here demonstrate an enhanced method for controlling RNA interference with light and also establish the importance of using duplexes with terminal phosphates for optimal modulation of RNA interference with light.

MATERIALS AND METHODS

Caging of dsRNA with diazo-DMNPE

Diazo-DMNPE was synthesized by addition of MnO₂ (10 mg, 0.115 mmol) to 4,5-dimethoxy-2-nitroacetophenone hydrazone (2.5 mg, 0.011 mmol) (Molecular Probes) in DMSO (250 μ l) followed by gentle agitation for 45 min at room temperature. This suspension was then filtered through celite to remove MnO₂ and a volume of this filtrate (25.6 μ l corresponding to 250 eq. of DMNPE to dsRNA) was gently agitated with dsRNA (50 μ l, 24.7 μ M) for 24 h at room temperature, protected from light. Unreacted caging material was removed by precipitation of caged dsRNA using ethanol and glycogen, followed by two extractions with CHCl₃.

Electrospray ionization mass spectrometry

dsRNA (50 μ l, 24.7 μ M) was treated with 25 μ l of 10 M ammonium acetate for 10 min at room temperature to enhance the displacement of sodium ions. The dsRNA was then precipitated using ethanol and glycogen and dissolved in water:acetonitrile (50:50, 50 μ l) containing 1%

triethylamine to make a final concentration of 24.7 pmol/ μ l. The dsRNA were then analyzed using a Q Trap mass spectrometer (ABI), operated in the negative ion mode.

Culture and transfection of cells

HeLa cells (ATCC) were plated at 70% confluency in 96-well plates (Corning), 18–20 h prior to transfection. Cells were transfected with 0.099 μ g pEGFP-C1 and 0.132 μ g pDsRed2-N1 plasmids (Clontech) and 0.19 pmoles (1.56 nM) of caged or uncaged dsRNA using lipofectamine (Invitrogen). After 6 h, the transfection mixture was replaced with 100 μ l OPTI-MEM (Gibco) and UV treated cells were exposed to UV light for 10 min using a Blak-Ray lamp (Model XX-15L, 30 watts). Cells were protected from short length UV by a WG-320 longpass filter (Edmunds Industrial Optics). Cells were further cultured in 200 μ l antibiotic free DMEM (Invitrogen) supplemented with 10% FBS. After 42 h, the cells were washed with 200 μ l phosphate buffered saline (PBS, Invitrogen) and 100 μ l PBS was added to each well. GFP and RFP expression were quantitated by fluorescence spectroscopy using a microplate reader. Normalized signals were generated by taking the GFP/RFP ratio for each experimental point, after correcting for signal in cells treated with lipofectamine alone.

Analysis of caging reaction using the model nucleotide system

Uridine mono phosphate (Sigma) and uridyl (3'-5') uridine (Sigma) were made up to a final concentration of 1 mM (400 μ l) in water. As described above, diazo-DMNPE was synthesized by gently shaking a mixture of MnO₂ (10 mg, 0.115 mmol) and 4,5-dimethoxy-2-nitroacetophenone hydrazone (2.5 mg, 0.011 mmol) (Molecular Probes) in DMSO (250 μ l) for 45 min at room temperature, filtering through celite and washing the filter pad. An amount of DMSO (143.3 μ l, 12 mM) containing 4.3 eq. of diazo-DMNPE to model nucleotides was added and formation of caged nucleotides and loss of starting material was monitored using an Agilent 1100 DAD coupled to a Q Trap LC/MS/MS System (ABI). Enhanced MS (EMS) mode was used for identifying the various species and enhanced product ion (EPI) mode was used for the MS/MS studies.

RESULTS

Caging double-stranded precursors of siRNA with diazo-DMNPE

To test the ability of caged precursor RNA duplexes of siRNA to control RNAi with light, we tested a GFP targeting dsRNA described by Rossi and coworkers (27) (Figure 1). This dsRNA was selected as it has been shown to possess excellent efficacy for the reduction in expression of the target GFP, and has also been demonstrated to be processed by Dicer, a qualification we require for potentially increased sensitivity to photo-sensitive modifications. In addition, it has also been shown to evade the

activation of RNA dependent Protein Kinase (PKR) and induction of interferons, a problem associated with the use of longer dsRNAs (27). The photolabile moiety that we used for this study was the DMNPE group that we previously used to cage siRNAs (26). The diazo reagent which can react with the dsRNA was prepared as described in our previous work by reaction of the hydrazone precursor with manganese dioxide. GFP targeting dsRNAs were caged using a 250-fold excess of diazo reagent relative to RNA duplex.

The activity of modified dsRNAs was analyzed using a high throughput gene expression system previously used by our group to analyze modified siRNAs (25,26) and by other groups for assessing the effects of modified siRNAs and shRNAs on gene expression (28–30). Briefly, we co-transfect dsRNAs with GFP and RFP expressing plasmids into HeLa cells plated in 96-well plates. This allows us to acquire multiple replicates of each experimental condition and to quantitate GFP and RFP expression in live cells with a scanning fluorescence multi-plate reader. HeLa cells were transfected with the GFP and RFP expressing plasmids and modified or unmodified dsRNA.

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5' AAGCUGACCCUGAAGUUCAUCUGCACC 3'
3' UUCGACUGGGACUUCAAGUAGUCGUGG 5'
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GFP targeting 27-mer Dicer substrate dsRNA

Figure 1. 27-mer dsRNA duplex used in this study. The bottom line represents the anti-sense strand of the duplex.

Plasmid controls were transfected with GFP and RFP plasmid only, and mock transfection controls contained neither plasmid nor dsRNA. After a transfection period of 6 h, the wells were either exposed to light for 10 min or masked. After allowing the cells to culture for an additional 42 h, the GFP and RFP signals were quantitated using microplate fluorescence. All fluorescent signals were corrected for the same signal in mock-transfected cells that were treated only with the transfection agent lipofectamine. Signals are reported as GFP expression normalized to RFP expression. In addition to serving as a control for any variation in transfection efficiency, normalizing the amount of GFP to RFP allows us to monitor for any non-specific or toxic effects of modified dsRNAs used in our study.

The results are summarized in Figure 2A. Contrary to our expectations that caged dsRNAs might be more sensitive to modifications, diazo-DMNPE reacted dsRNAs exhibited an insignificant loss of RNA interference activity. Equally surprisingly, the caged dsRNAs were unable to regain the small amount of blocked activity upon exposure to non-toxic levels of light. Both of these results appeared contrary to our experience with caged siRNAs. We have previously shown that caging with the above mentioned conditions results in a 3% caging efficiency (approximately one to two DMNPE groups incorporated on average per siRNA duplex) (26). To ascertain that the dsRNAs used in this study followed a similar caging behavior and determine whether a change in the caging efficiency when using dsRNAs was responsible for

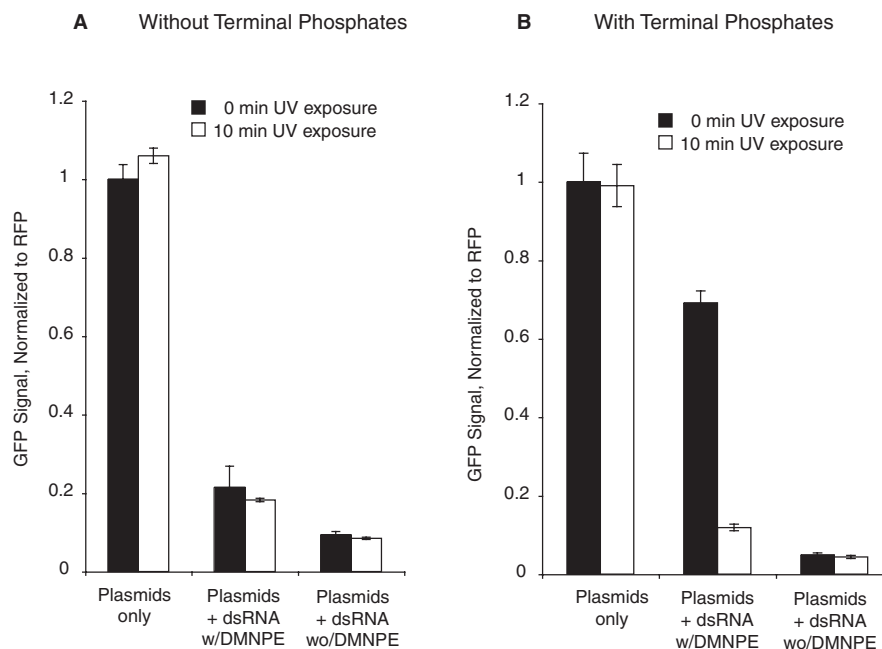


Figure 2. Effect of DMNPE-modified dsRNA on GFP expression with and without irradiation. (A) dsRNA without terminal phosphates. (B) dsRNA with four terminal phosphates (3' and 5' on both strands). HeLa cells were co-transfected with GFP and RFP expressing plasmids and GFP targeting dsRNA, modified or unmodified with the DMNPE group as indicated. Dark bars represent normalized GFP signal in cells that were not irradiated. Light bars represent normalized GFP signal in cells irradiated for 10 min. For each set of experimental conditions, the GFP/RFP ratio was normalized to the same ratio in the non-irradiated, plasmid-only sample. Five experimental points are averaged for each value and the standard error indicated.

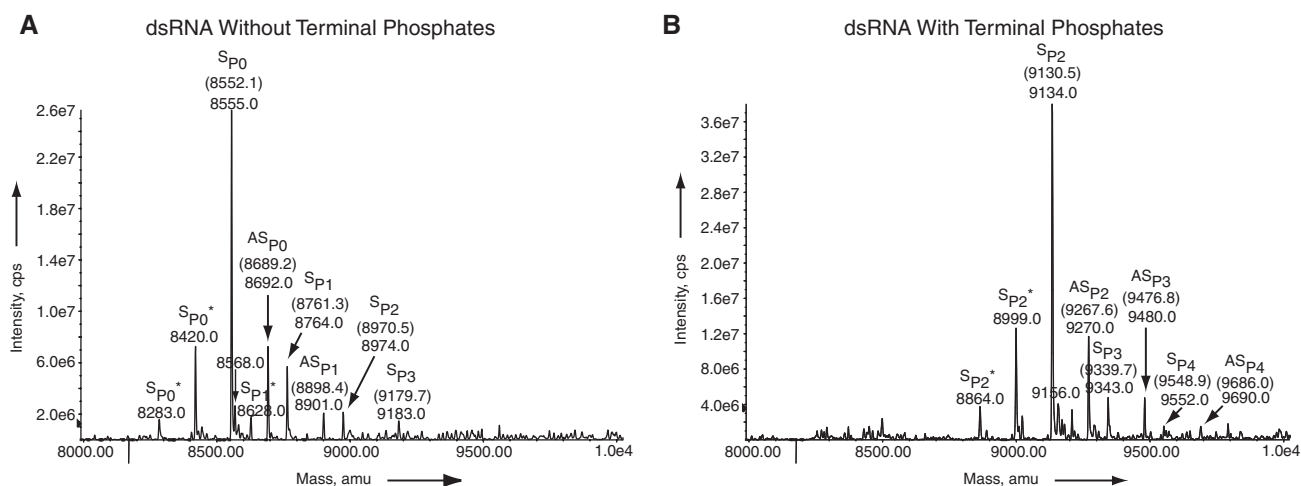


Figure 3. ESI mass spectrum of dsRNA after reaction with DMNPE. (A) dsRNA without terminal phosphates. (B) dsRNA with four terminal phosphates (3' and 5' on both strands). Peaks are annotated to indicate the strand (AS = antisense; S = sense) and the number of photolabile groups (P0 = none, P1 = one group, etc.). Below this annotation is the expected m/z ratio in parentheses and the observed m/z ratio. An asterisk indicates a species resulting from depurination. cps = counts per second.

the lack of modulation of modified dsRNAs with light, we decided to further characterize the modified dsRNAs used in this study by ESI MS.

Characterization of modified dsRNAs using ESI-MS

The ESI mass spectrum of the reacted dsRNA indicated that the dsRNAs were composed primarily of unmodified sense and antisense strands. (Figure 3A) From this we concluded that the low level of caging was responsible for the inability of dsRNAs to lose activity upon reaction with DMNPE.

We compared these results with our previous results obtained with modified siRNA to determine the reasons for the difference in caging observed between the siRNA and the dsRNA (26). On close inspection of the ESI mass spectrum of the modified siRNA reported in our earlier work, we observed that the antisense strand was predominantly modified with a single DMNPE group whereas the sense strand was predominantly unmodified. Upon examination of the chemical composition of the two strands of siRNA, we could identify only one significant chemical difference: The presence of a terminal phosphate on the 5' end of the antisense strand that was not present on the sense strand. This suggested to us that terminal phosphates reacted more efficiently with diazo-DMNPE than internal phosphates. We concluded that the lack of terminal phosphates on the dsRNA duplex might be the key reason for the limited caging exhibited by the dsRNA compared to siRNA.

To examine this hypothesis, we then tested dsRNA which had phosphate groups incorporated on all four terminals. They were caged as described above using a 250-fold excess of diazo reagent relative to RNA duplex. The ESI mass spectrum acquired with this sample shows that the major species for each strand has two modifications (Figure 3B). This stands in marked contrast to the mass spectrum of the un-phosphorylated dsRNA after

reaction with diazo-DMNPE, in which the major species were unreacted sense and anti-sense strands.

Confirming difference in reactivity using model nucleotide studies

To further support our conclusion that the lack of terminal phosphates was responsible for the inability of dsRNAs to be modulated effectively with light, we analyzed the differences in caging behavior of terminal and internal phosphates using a model system consisting of uridine mono phosphate (UMP) and uridyl (3'-5') uridine (UpU). Since UMP contains a single terminal phosphate and UpU contains a single internal phosphate, this represents an ideal system to compare the differences in rates of caging between internal and terminal phosphates (Figure 4). We reacted these nucleotides with a 4-fold excess of diazo-DMNPE to total phosphates (internal and terminal), the same ratio of diazo-DMNPE to total phosphates we use for caging RNA duplexes.

Using LC-MS we found that UMP was completely modified after approximately 16 h, whereas UpU exhibited <20% reaction during this same period of time (Figure 4). The kinetics of these reactions were analyzed by HPLC, using a ratio of the area represented by unmodified model nucleotide to the area represented by the summation of all the species representing modified and unmodified nucleotides. This gave the proportion of starting material (UMP or UpU) as a function of time. There were some more highly modified species present at very low concentrations (<5%), which were not included in our analysis. As expected from our results with caging dsRNAs, UMP was completely converted to its modified form within a few hours, whereas UpU was converted to its modified form incompletely and at a very slow rate compared to UMP (Figure 5). The reaction of UpU leveled off at 20% reaction, likely due to consumption of diazo-DMNPE via a competing reaction with water. To support these observations we have also done a

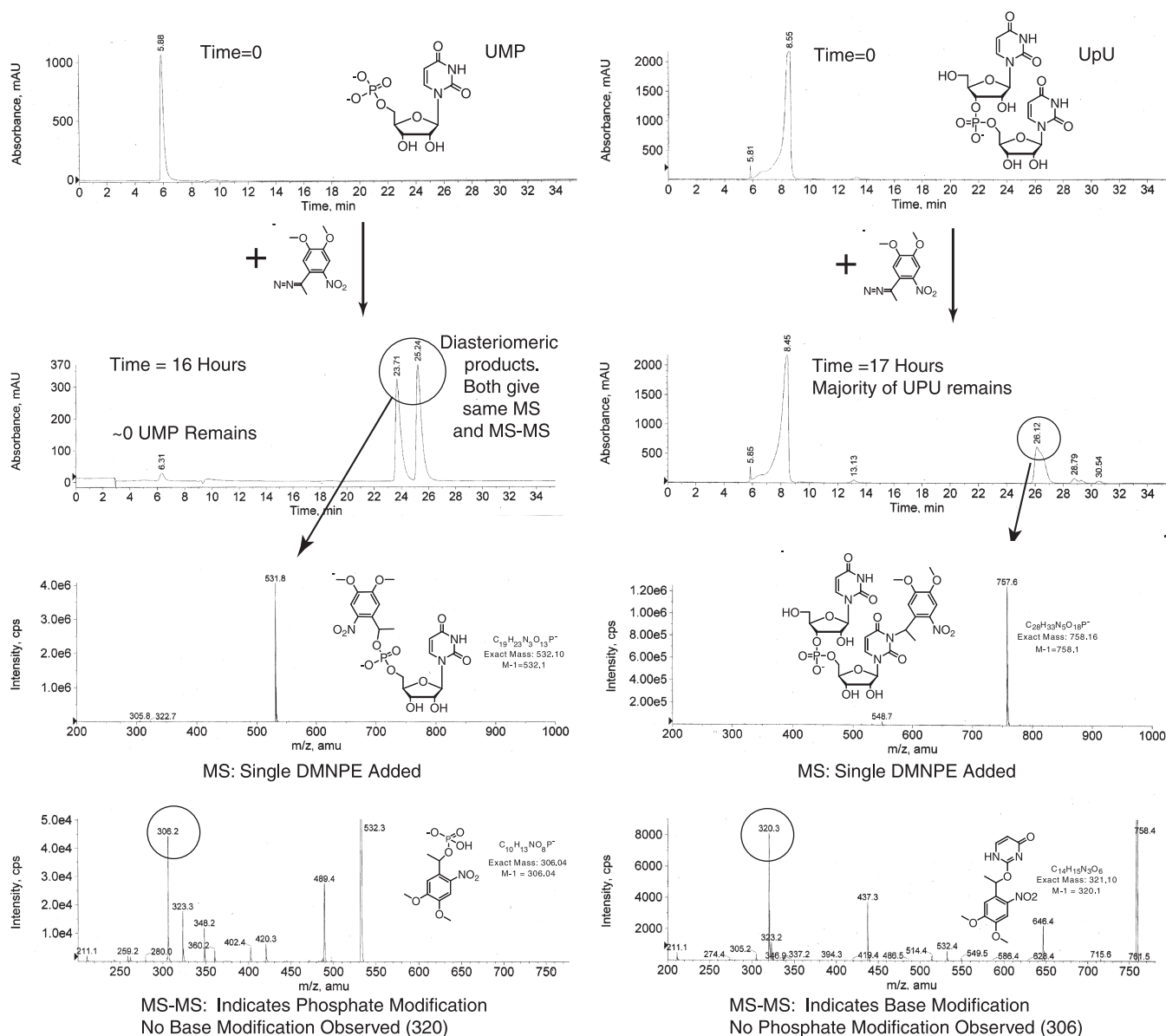


Figure 4. Characterization of differential reactivity of DMNPE toward UMP (left) and UpU (right). First row depicts the HPLC of unreacted nucleotide. The second row depicts the HPLC of reacted UMP and UpU after 16 and 17 h, respectively. The third row depicts the mass spectrum of the major product species formed in this time. The fourth row depicts the MS/MS of this species, as well as a species consistent with the spectrum.

competition experiment, in which equal concentrations of UMP and UpU compete for diazo-DMNPE in the same reaction vessel. After 16 h of reaction, 88.4% of the observed DMNPE mono-adducts were to UMP, with the remaining 11.6% to UpU. These results are summarized in supplemental data, and show that, as before, UMP is more reactive towards DMNPE than is UpU. These combined model nucleotide studies support that the lack of terminal phosphates in dsRNA was indeed responsible for its insignificant caging.

Determining site of diazo-DMNPE modification using LC/MS/MS studies

We also conducted MS/MS studies to determine diazo-DMNPE's site of reaction on UMP and UpU (Figure 4).

The MS–MS spectrum of the modified UMP contained peaks corresponding to a modification on the phosphate (calculated MW 306.19, observed MW 306.2), but no peaks corresponding to a modification on the nucleobase (calculated MW 320.1). Conversely, the MS–MS spectrum of the modified UpU contained peaks corresponding to a modification on the nucleobase (calculated MW 320.1 observed MW 320.3), but no peaks indicating a modification on the phosphate (calculated MW 306.19). In Figure 4, the imide nitrogen is shown as the site of modification on the nucleobase, but the nucleobase potentially could be modified elsewhere, including the carbonyl oxygens of the nucleobase. These studies indicate that the diazo-DMNPE reacted with the terminal phosphate of UMP but not to the internal phosphate of UpU, where instead it was bound to the nucleobase.

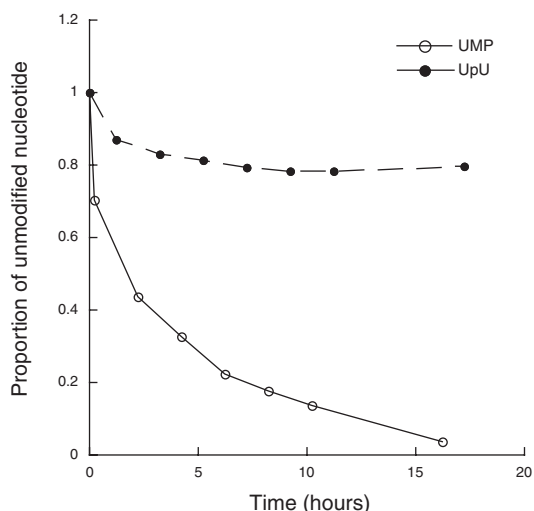


Figure 5. Kinetics of UMP and UpU modification with DMNPE. UMP and UpU were reacted with DMNPE and the proportion of unmodified nucleotide was determined by integration of the HPLC chromatogram. Open circles represent proportion of unmodified UMP and solid circles represent proportion of unmodified UpU.

Determining kinetics of photo-deprotection of diazo-DMNPE modified dsRNAs

We then compared the rates of deprotection of the diazo-DMNPE-modified phosphorylated dsRNA (containing phosphates on all four ends) with the rates of deprotection of diazo-DMNPE-modified unphosphorylated dsRNA (lacking terminal phosphates) by using ESI-MS. To analyze the rates of deprotection, solutions of modified dsRNA were irradiated in a setup identical to that used for photo-deprotection in live cells, except that there were no cells or media present. To quantitate the different species in a given sample, we used a mass spectrometry based method that we have reported in our earlier work (25). We have validated this method in our previous work by showing that the mass spectrometer signal integration can be used to quantitate the amounts of structurally related oligonucleotides in the presence of a standard oligonucleotide (25). To analyze the rates of deprotection, samples were irradiated for different lengths of time and analyzed by ESI-MS. We examined the ratio of the summation of the signal of all modified species to the summation of the signal for all species, modified and unmodified (Figure 6).

We found that the diazo-DMNPE-modified phosphorylated dsRNA has a greater degree of modification prior to irradiation than does the DMNPE-modified unphosphorylated dsRNA. This is completely consistent with our previously described mass spectrometry studies with duplexes and model nucleotides. In addition, the diazo-DMNPE-modified phosphorylated dsRNA is deprotected more quickly than diazo-DMNPE-modified unphosphorylated dsRNA. This data helps explain some of our original work, in which we had demonstrated that we could increase the amount of modification on siRNA by using higher equivalents of diazo-DMNPE, but were not able to deprotect it efficiently with non-toxic levels

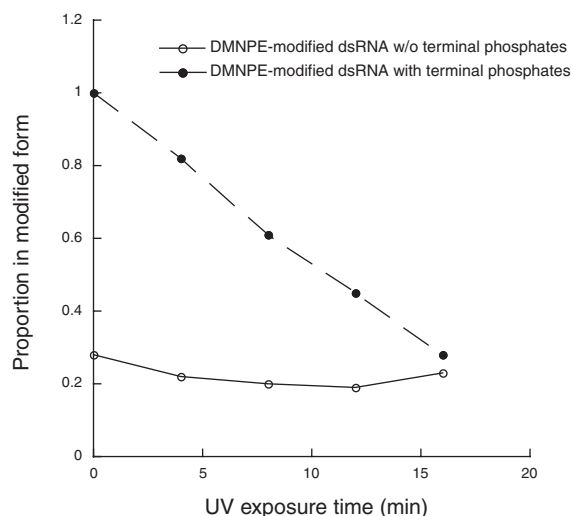


Figure 6. Deprotection kinetics of DMNPE-modified dsRNA. dsRNA duplexes, modified with DMNPE, were exposed to UV and the amount of dsRNA in modified or unmodified form was followed using an ESI-MS assay. The proportion of dsRNA in modified form was obtained by taking a ratio of the amount of dsRNA containing any level of modification to the total dsRNA content in that sample. Solid circles represent this value determined for dsRNA containing phosphates on all four ends. Open circles represent this value for dsRNA without any terminal phosphates. Phosphorylated dsRNA is both more highly modified by DMNPE, and more rapidly deprotected by irradiation.

of light (26). Our photo-deprotection studies using ESI-MS suggest that the modification on the terminal phosphate is removed with lower levels of light but the additional groups on the backbone phosphates or on the nucleobases are deprotected at a comparatively slower rate leading to incomplete deprotection with non-toxic levels of light. Normalized data can mask the toxicity of the irradiation, as cells that die reduce both their GFP and RFP expression. In our setup, a 10 min exposure is the maximum that we can achieve while not affecting the absolute level of GFP or RFP expression significantly in control samples. We use any changes in this absolute level of GFP and RFP expression in controls as one indicator of phototoxicity.

Examining modulation with light using phosphorylated dsRNAs modified with diazo-DMNPE

Having demonstrated that we can achieve a significant degree of caging by using dsRNAs containing terminal phosphates, we analyzed the ability of these modified dsRNAs to modulate gene expression by light using the gene expression system described above. Diazo-DMNPE-modified dsRNAs containing terminal phosphates on all four ends provided a significant improvement in the ability to control RNAi with light when compared with our previously described light activated siRNA (Figure 2B). The ability of modified dsRNAs to induce RNA interference is strongly attenuated prior to irradiation and is fully restored after irradiation, leading to a 6-fold decrease in gene expression. This represents a substantial

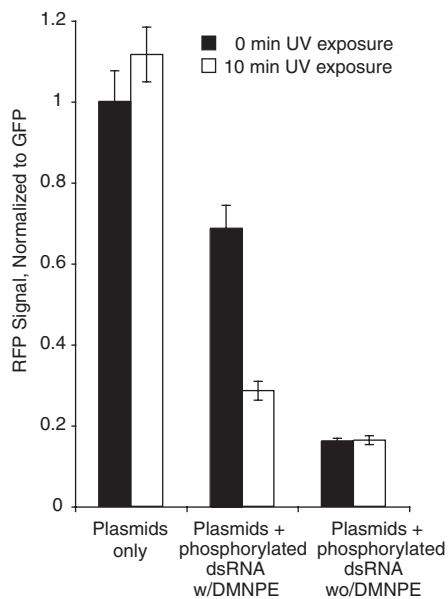


Figure 7. Effect of terminally phosphorylated RFP targeting dsRNA, modified with DMNPE on RFP expression with and without irradiation. HeLa cells were co-transfected with RFP and GFP expressing plasmids and RFP targeting dsRNA, modified or unmodified with the DMNPE group as indicated. Dark bars represent normalized RFP signal in cells that were not irradiated. Light bars represent normalized GFP signal in cells irradiated for 10 min. For each set of experimental conditions, the RFP/GFP ratio was normalized to the same ratio in the nonirradiated, plasmid-only sample. Five experimental points are averaged for each value and the standard error indicated.

improvement over the 2-fold decrease obtained both with 'random' modifications of siRNA and the specific modification of the 5' phosphate of the anti-sense strand of siRNA with a variety of photo-cleavable groups (25,26). These results also stand in stark contrast to those observed when unphosphorylated dsRNA were protected and tested, as described above. To further support our results that caging dsRNAs containing phosphates on all ends leads to an improvement in the modulation of RNAi with light, we also examined the activity of terminally phosphorylated RFP targeting dsRNAs, modified with the DMNPE group. Figure 7 shows the results of this experiment.

The diazo-DMNPE modified RFP targeting dsRNAs also show an improvement in the ability to control RNAi with light, compared to the light activated RFP targeting siRNAs (2.5-fold versus 2-fold change in gene expression) (26). The 2.5-fold change, observed with RFP targeting RNAs, and the 6-fold change, observed with GFP targeting RNAs, defines the typical range of modulation we observe upon repetition of these experiments. We have further demonstrated that the window of modulation with light can be shifted by using different concentrations of modified dsRNA (data not shown). It is likely that the presence of the DMNPE groups on the termini of the dsRNA block RNA interference by preventing the duplex from effectively interacting with Dicer.

Finally, we have also examined the effect of purification on the light activation of RNA interference using DMNPE-modified RNAs. When purified DMNPE-modified dsRNA was examined, it gave similar results to the unpurified samples used to generate Figure 2 (see Supplementary Data). In addition, we tested purified siRNA modified with DMNPE groups on four terminal phosphates. This species showed a similarly improved window of toggling relative to our originally tested siRNA which had only a single terminal phosphate, and a single DMNPE modification (see Supplementary Data). It may be that the improvement we observe is due to the increased number of modifications that can be cleaved with non-toxic amounts of light, as opposed to the specific RNA species (siRNA versus dsRNA).

DISCUSSION

The aim of our work is to create a system that allows the ability to toggle RNA interference with light. Previously described approaches to control RNAi with light i.e. random modification of siRNA and specific blocking of the 5' anti-sense phosphate with photolabile moieties, allow modulation of RNA interference with light, but do not allow complete switching from 100% gene expression to complete RNA interference upon exposure to light. We anticipated that modifying dsRNA as opposed to siRNA might be the solution to this problem as dsRNAs could be more sensitive to modifications as they need to be processed by both Dicer and RISC before exhibiting their activity.

The work that we describe in this paper shows that caging native dsRNA lacking terminal phosphates is not sufficient to effectively modulate RNA interference with light. Characterization of the modified dsRNA lacking terminal phosphates revealed that the caging reaction resulted in very low levels of modification. Although the low caging efficiency observed when caging dsRNA lacking terminal phosphates appears contrary to our experience with modifying siRNA, it in fact, supports our results obtained with caging siRNA. Both the GFP and RFP targeting siRNA that we had used in our initial experiments contained a phosphate on the 5' end of the antisense strand (26). A close examination of the ESI mass spectrum of the GFP targeting siRNA reveals that the anti-sense strand contains about one modification per strand and the sense strand was predominantly unmodified. This suggests that the terminal phosphate, which is a phosphate mono-ester, and as such chemically distinct from the internal phosphates, which are all phosphate di-esters, reacts at a greater rate with diazo-DMNPE.

To provide further evidence that there is a difference in the rate of caging between dsRNAs lacking and containing terminal phosphates, we conducted caging experiments using a model system consisting of UMP and UpU as they contain only a terminal phosphate and internal phosphate, respectively. Analyzing the modification of these species, allowed us to directly compare the rates of caging between the two species, as these model nucleotides contain only a terminal and internal phosphate. We had

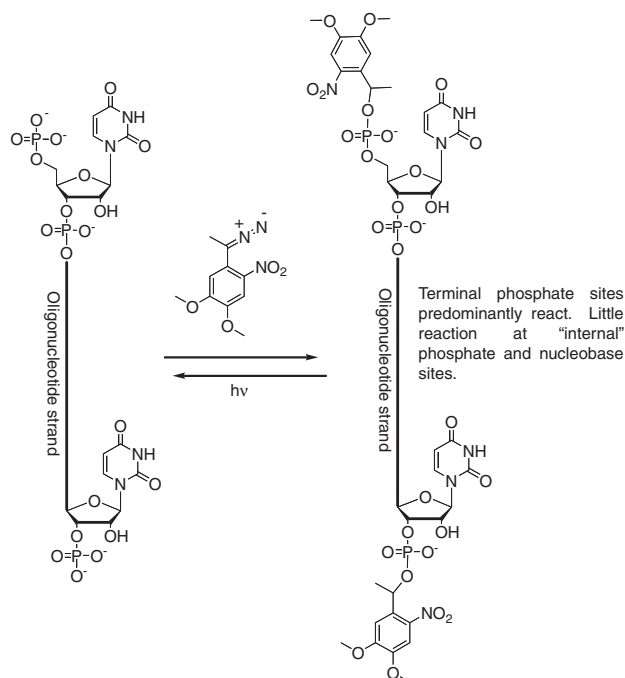


Figure 8. Schematic depiction of oligonucleotide site reactivity towards DMNPE.

assumed that the DMNPE group would bind to the internal phosphate of the UpU. However, our MS/MS studies demonstrate that the DMNPE group is chiefly bound to the nucleobase on the UpU. This suggests that the low amounts of caging that we observe with oligonucleotides lacking a terminal phosphate could be modifications on the nucleobases, rather than on the internal phosphate as we had initially assumed. Other groups attempting to cage nucleic acids with protecting groups containing a diazo functionality have also assumed that the protecting group reacts with the internal phosphate of the DNA or RNA without a detailed characterization of the modified oligonucleotides (31–33). Based on our studies, it is possible that modifications on sites other than the internal phosphate could have contributed significantly to the overall caging of these oligonucleotides.

The model studies are by their nature an approximation of the reactivity of these sites in duplexes. The simple model nucleotide system can never match the same proportions of internal/terminal phosphates and nucleobases present in the actual oligonucleotide. In addition, nucleobases in duplexes may have different reactivity when compared with unpaired nucleobases. As such the model nucleotides provide an estimate of the differential reactivity of these different sites. Given these caveats, our model studies are completely consistent with and corroborate our observations of modification in duplexes.

We have demonstrated diazo-DMNPE to have regioselectivity for terminal versus internal phosphates, and in addition to have chemoselectivity for these terminal phosphates versus nucleobases. Figure 8 describes our new understanding of diazo-DMNPE's reactivity and its subsequent effect on LARI. Our studies indicate that addition of photo-protecting groups to internal phosphates and

nucleobases contributes very little to the overall caging reaction of RNA duplexes. This paradigm is further supported by the results obtained with the characterization of modified dsRNA containing all four phosphates by ESI-MS (Figure 3). If the terminal phosphate is completely modified by diazo-DMNPE and the internal phosphates or nucleobases exhibit very little reactivity towards the caging group, we should observe approximately two modifications on both the sense and anti-sense strands as each strand contains two terminal phosphates. This is precisely what we observe. These results strongly support our conclusion that the presence of a terminal phosphate is necessary for achieving a significant amount of reaction using the caging conditions described in our experiments, and subsequent modulation of RNA interference with light.

We have also studied the rates of photo-deprotection using an ESI-MS assay that we have validated in our previous work (25). These studies demonstrate that the rate of deprotection of modified dsRNA containing terminal phosphates is greater than the rate of deprotection of modified dsRNA lacking a terminal phosphate. This suggests that there is a difference in the rate of deprotection between modified nucleobases and terminal phosphates. We are thoroughly investigating these differences to find other sites on the dsRNA that we can modify and deprotect without inducing phototoxicity. Several groups have incorporated photolabile groups in nucleic acids to gain control over the hybridization, activity or folding of nucleic acids with light (32,34–40). A thorough study of the differences in rates of deprotection from different sites on the nucleic acid with non-toxic levels of irradiation will help in the extension of these proof-of-principle studies to in vivo applications.

Finally, we use the information about the significantly increased reactivity of terminal phosphates towards photolabile diazo-DMNPE group to improve LARI through the use of photo-protected 27-mer double-stranded RNA precursors. These yield a 2.5–6-fold decrease in gene expression upon irradiation, compared with the 2-fold decrease found with siRNA containing a single terminal phosphate. We have shown that this effectiveness is predicated on the presence of terminal phosphates on the four termini of the dsRNA duplex. The presence of DMNPE modified terminal phosphates prior to irradiation presumably blocks the interaction of the modified dsRNA with Dicer, although not completely. Irradiation releases fully native dsRNA, which can then undergo processing by Dicer, followed by RISC, resulting ultimately in RNA interference. The incomplete block of RNA interference prior to irradiation may be due to different mechanisms. For example, it may be due to tolerance of the modifications by Dicer or other RNAi cellular machinery, which would reduce, but not block, the modified RNA's activity. A second mechanism may be cellular degradative processes that remove the photo-cleavable groups in the absence of light. We are examining both of these potential mechanisms as a route to improve the overall efficacy of LARI.

The results presented here deepen our understanding of the caging of nucleic acids with photo-protecting groups, describe an improved method for controlling

RNA interference with light and open up new avenues for further enhancing LARI into an effective tool for biological analysis. We are currently utilizing the knowledge gained about the differences in reactivity and deprotection rates of modifications on different sites of the dsRNA to develop new reagents that are able to completely block RNA interference prior to irradiation and thus allow for a complete switch between full gene expression, and full RNA interference.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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