

Phenylpyrrolocytosine as an Unobtrusive Base Modification for Monitoring Activity and Cellular Trafficking of siRNA

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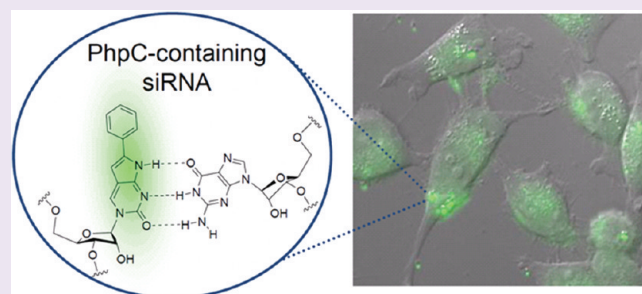
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S Supporting Information

ABSTRACT: 6-Phenylpyrrolocytosine (PhpC) is a cytosine mimic with excellent base-pairing fidelity, thermal stability, and high fluorescence. In this work, PhpC-containing small interfering RNAs (siRNAs) are shown to possess thermal stability and gene silencing activity that is virtually identical to that of natural siRNA. The emissive properties of PhpC allow the cellular trafficking of PhpC-containing siRNAs to be monitored by fluorescence microscopy. Accumulation in the cytoplasm of HeLa cells was observed using real time imaging. These findings demonstrate that PhpC-modified siRNAs retain the properties of natural siRNAs while allowing for fluorescence-based detection and monitoring, providing an ideal system for probing siRNA uptake and trafficking.



siRNAs^{1,2} are an invaluable tool for validating gene function, and massive efforts on the part of academia and industry aim to access this natural pathway for therapeutic applications.³ However, recent setbacks⁴ underscore the need to elucidate the mechanisms of RNAi. Fluorescently labeled oligonucleotides are one of the primary tools used for this purpose.^{5–10} However, bulky hydrophobic molecules can alter biological properties of oligonucleotides, including uptake,¹¹ and can complicate additional termini conjugation with lipophilic or targeting moieties.

Fluorescent nucleobase analogues are proving to be suitable alternatives to terminally conjugated dyes for a variety of biological applications.^{12–14} The 6-phenylpyrrolocytosine (PhpC) fluorescent nucleobase modification (Figure 1) has particularly favorable properties among nucleobase analogues.^{15–19} PhpC base pairs with guanine on complementary DNA or RNA strands, has excellent binding affinity and mismatch discrimination, and ranks among the most fluorescent C-analogues to date. The fluorescence change depends on the surrounding microenvironment, that is, fluorescence intensities of the PhpC nucleoside, a PhpC-containing single strand, or a PhpC-containing duplex are different.^{17,18} It was recently demonstrated that PhpC can serve as a cytosine mimic in terms of its recognition by enzymes.¹⁵ In this context, a DNA–RNA heteroduplex possessing a single PhpC substitution was used to develop a molecular-beacon type RNase H

assay that fluorometrically reports on the cleavage of the enzyme substrate.¹⁵

Many chemical modifications have been developed to improve various properties of siRNAs without compromising biological activity.^{20,21} However, the use of chemically modified nucleobases in siRNAs has attracted more limited attention,^{22–31} and to our knowledge, none bearing fluorescent properties have been exploited as probes to monitor siRNA cellular distribution. We now report that PhpC provides a suitable replacement to bulky terminal fluorophores in siRNA due to its emissive properties and minimal impact on cellular enzyme function. siRNAs incorporating PhpC modifications can be visualized by fluorescence microscopy without relying on terminal fluorophore conjugation. Furthermore, PhpC-containing siRNAs are well tolerated in RISC, showing excellent gene silencing activity, and maintain recognition by immunoreceptors. The evidence points to PhpC as an unobtrusive fluorescent label for investigating RNAi and siRNA uptake.

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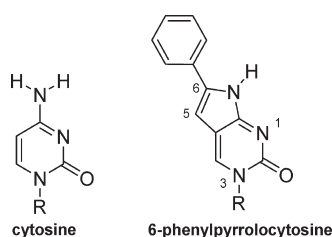


Figure 1. Cytosine and 6-phenylpyrrolocytosine (PhpC). The numbering for PhpC is indicated.

Table 1. siRNA Duplexes with Melting Temperatures

Duplex	Sequence ^[a]	T_m (°C) ^[b]
control siRNA	<i>sense strand</i> 5' -GCUUGAAGUCUUAAUUAAtt-3'	54.1
	<i>antisense strand</i> 3' -ggCGAACUUACAGAAAUUAUU-5'	
scrambled	5' -GCUUGAUUUCUGAAAUUAAtt-3' 3' -ggCGAACUAAAAGACUUUAAUU-5'	nd
siPhpC-1	5' -GCUUGAAGU <u>C</u> UUUAAUUAAtt-3' 3' -ggCGAACUUACAGAAAUUAUU-5'	54.6
siPhpC-2	5' -GCUUGAAGUCUUUAAUUAAtt-3' 3' -ggCGAACUU <u>C</u> AGAAAUUAUU-5'	54.6
siPhpC-3	5' -G <u>C</u> UUGAAGU <u>C</u> UUUAAUUAAtt-3' 3' -ggCGAACUUACAGAAAUUAUU-5'	55.7
siPhpC-4	5' -GCUUGAAGUCUUUAAUUAACc-3' 3' -gg <u>C</u> GAA <u>C</u> UUCAGAAAUUAUU-5'	53.0
siPhpC-5	5' -GCUUGAAGU <u>C</u> UUUAAUUAAtt-3' 3' -ggCGAACUU <u>C</u> AGAAAUUAUU-5'	55.6
siPhpC-6	5' -G <u>C</u> UUGAAGU <u>C</u> UUUAAUUAAtt-3' 3' -ggCGAACUU <u>C</u> AGAAAUUAUU-5'	nd
siPhpC-7	5' -G <u>C</u> UUGAAGU <u>C</u> UUUAAUUAAtt-3' 3' -cc <u>C</u> GAA <u>C</u> UUCAGAAAUUAUU-5'	55.2
siPhpC-8	5' -G <u>C</u> UUGAAGU <u>C</u> UUUAAUUAACc-3' 3' - <u>cc</u> <u>C</u> GAA <u>C</u> UUCAGAAAUUAUU-5'	55.1
siPhpC-8S	5' -G <u>C</u> UUGAAGU <u>C</u> UUUAAUUAACc-3' 3' -ggCGAACUUUCAGAAAUUAUU-5'	55.6
siPhpC-8A	5' -GCUUGAAGUCUUUAAUUAAtt-3' 3' - <u>cc</u> <u>C</u> GAA <u>C</u> UUCAGAAAUUAUU-5'	53.2
siPhpC-9	5' -GCUUGAAGUCUUUAAUUAACc-3' 3' - <u>cc</u> CGAACUUUCAGAAAUUAUU-5'	54.2

^a Sense strands are listed on top and antisense strands below. Legend: RNA, dna, **PhpC**, **dPhpC**. T_m values were measured at duplex concentrations of 1 μ M in buffer (10 mM sodium phosphate, pH 7.0, 50 mM NaCl). ^b T_m and IC_{50} values are the average of three independent experiments. T_m values represent the average of at least 3 independent experiments. nd, not determined.

RESULTS AND DISCUSSION

PhpC Does Not Perturb siRNA Duplex Stability. A well-characterized 21-mer siRNA sequence targeting firefly luciferase (Table 1) was utilized for these experiments.^{32–34} We replaced the cytosine at position 10 of the sense strand (passenger strand) with PhpC (siPhpC-1), as well as the nearest C residue of the antisense strand (guide strand) opposite the sense strand cleavage

site (siPhpC-2). We then added multiple PhpC inserts to either the sense or antisense strand (siPhpC-3, siPhpC-4), to both strands (siPhpC-5, siPhpC-6), or modified all the siRNA cytosines (siPhpC-7). We used a DNA analogue of PhpC (dPhpC) to modify the 3'-overhangs of duplexes siPhpC-8 and siPhpC-9, with or without additional internal modifications, respectively.

Melting temperatures (T_m) of the native and PhpC-modified siRNA duplexes were determined via UV-melting experiments and are provided in Table 1. In general, PhpC was slightly stabilizing when placed in the center of the duplex and slightly destabilizing when placed close to the 3'-terminus, consistent with previous reports.¹⁵ All changes were less than 1 °C per modification. Thus the effects of PhpC incorporation on T_m values are minimal, suggesting this modification does not perturb native RNA properties.

Number of PhpC Insertions Does Not Always Correlate with Fluorescence Intensity. PhpC is suitable for molecular biological studies since it has a high fluorescent quantum yield of 0.31¹⁵ that is red-shifted compared to most other biomolecules (λ_{max} excitation 370 nm; λ_{max} emission 465 nm).¹⁴ The fluorescence of PhpC-containing siRNA duplexes and their constituent single strands is shown in Figure 2. All strands containing at least one PhpC insertion showed fluorescence, but the intensity varied depending on sequence length and base composition.¹⁵ The incorporation of several PhpCs on the same strand can lead to an increase (antisense strand of siPhpC-2 compared to antisense strands of siPhpC-4 and siPhpC-7), a decrease (sense strand of siPhpC-3 compared to sense strand of siPhpC-8S), or no change in fluorescence intensity (sense strand of siPhpC-1 compared to sense strand of siPhpC-3). The most fluorescent single-stranded oligonucleotide in this study was the antisense strand that contained 3 PhpC insertions (siPhpC-7, Figure 2).

When the two 3' dT overhangs were replaced with dPhpC, there was a substantial fluorescence decrease (comparing siPhpC-8 with siPhpC-7, single strands or duplexes). This may be caused by excimer formation or some other self-quenching mechanism by PhpC. Guanines can reduce the quantum yield of fluorophores in a distance-dependent manner through an electron transfer mechanism.^{35,36} However, a homologue of the antisense strand of siPhpC-7 and siPhpC-8 with two deoxyguanosine overhangs instead of dT or dPhpC showed only a 12% reduction in fluorescence intensity. Thus, electron transfer by guanine does not appear to play a dominant role in the quenching of PhpC in long oligonucleotides. In addition, we noted that the quenching effect is not limited to neighboring PhpC residues, as the two dPhpC overhangs in the sense strand of siPhpC-8S caused a decrease in fluorescence from a distance of 10 nucleotides.

The fluorescence of PhpC-containing oligonucleotides is generally quenched when hybridized to complementary RNA or DNA.^{15,18,19} With the exception of overhang-modified siPhpC-8 and siPhpC-9, PhpC-modified siRNA duplexes exhibited lower fluorescence emission than the sum of their individual single-stranded components (Figure 2, green bars). Curiously, the intrastrand PhpC quenching phenomenon was not observed between PhpC residues on different strands in the same duplex. In other words, the amount of quenching upon duplex formation of a PhpC-containing oligonucleotide does not vary considerably whether the complementary strand is native RNA or RNA containing PhpC.

PhpC-Modified siRNAs Silence Gene Expression. PhpC was evaluated as a nucleobase modification for RNAi. Remarkably, all siRNA duplexes with single or multiple insertions of

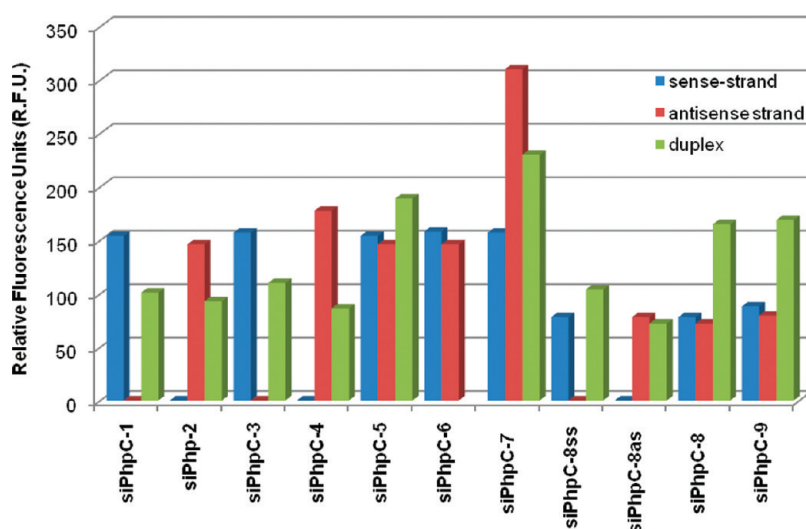


Figure 2. Fluorescence intensity of PhpC-modified siRNAs does not follow a simple correlation with number of PhpC modifications from comparing duplexes (green) to constituent strands (sense strands in blue and antisense strands in red). Samples were measured at a concentration of 1 μ M in 10 mM phosphate buffer (pH 7.0) and 50 mM NaCl at 25 $^{\circ}$ C, $\lambda_{\text{ex}} = 360$ nm, $\lambda_{\text{em}} = 465$ nm. The data represent the average of at least two independent measurements.

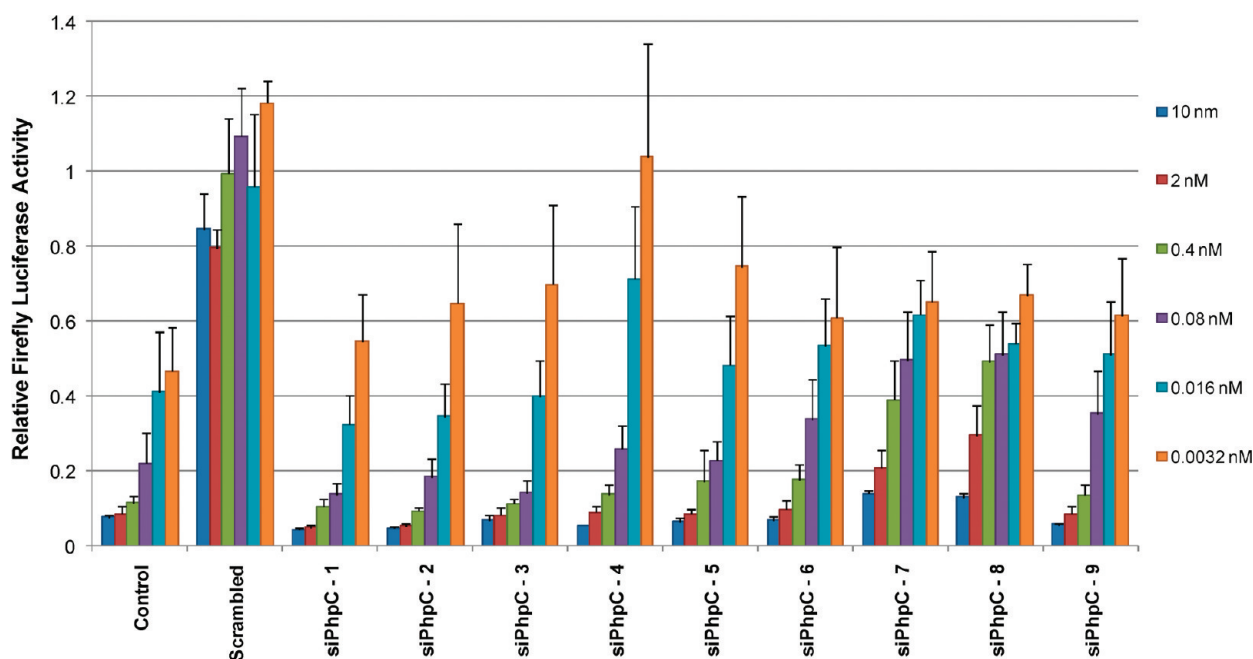


Figure 3. siRNAs containing PhpC silence the firefly luciferase gene in HeLa cells at subnanomolar concentrations. Data represent the average of at least two independent experiments performed on separate days, each run in duplicate. Error bars show standard deviations.

PhpC in either or both strands were potent inhibitors of luciferase activity with subnanomolar IC_{50} values (Figure 3). PhpC placed beside or opposite the scissile phosphate of the sense strand showed little change in gene silencing activity, suggesting that this modification does not impair Ago2 catalysis. Replacing the two 3' deoxynucleotide overhangs on each strand with dPhpC was also well tolerated (siPhpC-8 and siPhpC-9). Structural data have shown that substrate binding to Ago2 is influenced by base composition at the 5'-terminus of the antisense strand,³⁷ and our data confirm that the position of PhpC within the duplex also affects silencing potency. When two

PhpCs were inserted close to the 3'-end of the antisense strand (siPhpC-4), gene silencing efficiency was lost at the lowest siRNA concentration. Replacing all internal C residues with PhpC (siPhpC-7 and siPhpC-8) also significantly reduced the gene silencing efficiency compared to that of the natural siRNA. Studies from another group on 5-propynyluridine-modified siRNAs, which like PhpC-siRNAs contain a bulky group extending into the major groove,²⁷ suggested that steric clashes with Ago2 can decrease gene silencing. Recently, a study of modified adenosine with groups extending in the minor groove of RNA suggested that certain positions in the siRNA are more prone to

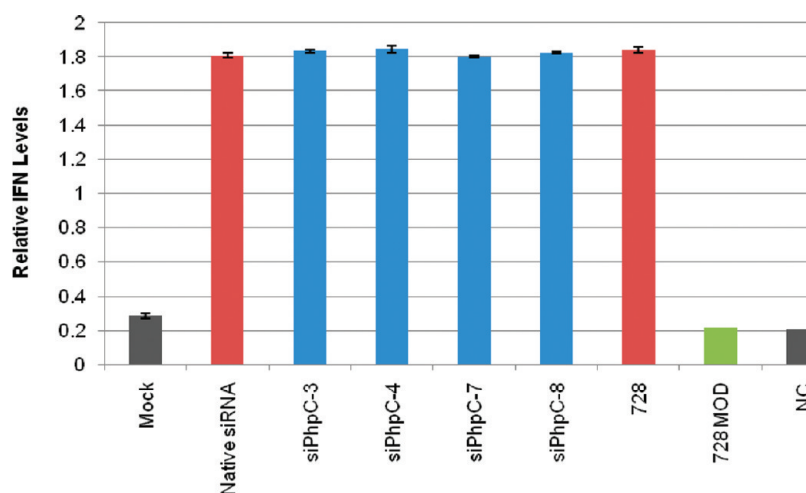


Figure 4. siRNAs containing PhpC show identical immunostimulatory activity compared to that of native siRNAs. A nonimmunomodulatory siRNA containing 2'F-RNA and 2'F-ANA modifications (728 Mod) was used as a positive control, along with the corresponding native siRNA for this sequence (728). The “Mock” control contains the transfecting agent without siRNAs. The negative control cells (NC) contain no IFN from PMBC cells.

clash with protein residues of Ago2.³¹ Nevertheless, these previous studies and our current work show that bulky nucleobase modifications such as PhpC can be tolerated in siRNAs, depending on RNA sequence and the position of the modified nucleotide. Future studies should also address how well PhpC is tolerated in the seed region of the antisense strand, since the sequence used in the current study did not contain any C residues in this important region.

PhpC Modifications Do Not Perturb Immunostimulation. A major side effect of administering siRNAs is their recognition by toll-like receptors (TLRs) and other components of the innate immune system. This triggers immune responses leading to the release of cytokines.^{38–40} Tools designed to probe and monitor cellular siRNA processes should not alter their recognition by TLRs. Chemical modification of siRNAs can reduce immunostimulatory effects, presumably by interfering with their recognition by TLRs, but only a few studies have examined the immunostimulatory properties of nucleobase-modified siRNAs.^{31,41,42} We wondered if PhpC would perturb the recognition of siRNAs by immune proteins. Treatment of human peripheral blood mononuclear cells (PMBCs) with PhpC-modified siRNA led to an immune response similar to that of unmodified siRNA with the same sequence (Figure 4). An siRNA containing chemical modifications previously shown to reduce immunostimulation³⁴ did not induce any measurable cytokine release (728 MOD, Figure 4). These results suggest that PhpC is a good mimic for natural RNA in terms of its recognition by immune receptors and demonstrate the merits of PhpC as an unobtrusive fluorescent label.

PhpC Allows Monitoring of the Localization of siRNAs in Cultured Cells. It would be advantageous if an innocuous chemical modification could also serve as a fluorescent reporter to monitor siRNA uptake and localization. This would facilitate microscopy studies representing the true behavior of native siRNAs unaffected by fluorophore conjugates. To this end, we followed the uptake of PhpC-containing siRNAs by fluorescence microscopy.

We first attempted to observe the fluorescence of an siRNA containing only one PhpC incorporation (siPhpC-1); however, this required high exposure times (0.4 s, Supplementary Figure S1). Cells containing no fluorescent oligonucleotides also showed

some background fluorescence at these exposure times likely resulting from NADH and other fluorescent molecules.⁴³

To increase the signal over background fluorescence, we used the siRNA containing 9 PhpC incorporations (siPhpC-8). Very clear fluorescent images of siPhpC-8 were acquired using much lower exposure times (36 ms), and essentially no background fluorescence was observed in the cells containing the unmodified control siRNA under these imaging conditions (Figure 5, row C vs row A). siPhpC-8 shows accumulation into punctate structures inside the cell (Figure 5, row C and D). These structures may be attributed to the siRNA/Lipofectamine 2000 particles.⁴⁴ The nucleus is clearly delineated using the DRAQ5 stain, showing accumulation of siPhpC-8 in the cytoplasm, the location of siRNA-mediated mRNA degradation. This distribution is consistent with studies by others on the localization of siRNAs using traditional fluorescent tags.^{6,7} PhpC allows monitoring of duplexes containing PhpC in only one of the constituent strands (siPhpC-8S and siPhpC-8A, Supplementary Figure S1). Together, these results indicate that PhpC allows monitoring of the localization of siRNAs in cultured cells. The best results were obtained when using multiple (>4) PhpC incorporations to amplify the signal since PhpC fluorescence can be additive given the proper sequence context.

We then proceeded to monitor the trafficking of siPhpC-8 in live HeLa cells in real time (Figure 6). In order to minimize photobleaching and any phototoxic effects of the ultraviolet light on the cells, time-lapse experiments were conducted with the incident light power reduced to 5% (50% lamp power and a 10% neutral density filter) and longer exposure times.⁴⁵ After 15 min post-transfection, we observed the appearance of some bright structures on the periphery of cells, presumably siPhpC-8-Lipofectamine 2000 particles, and little intracellular fluorescence. After 90 min, the peripherally associated fluorescence subsided and the fluorescence within the cells intensified from 90 to 180 min post-transfection. We attribute this to the release of siPhpC-8 into the cytoplasm, which corresponds well with the post-transfection time at which gene silencing can be observed when delivering siRNA into HeLa cells using Lipofectamine 2000 as the transfecting agent.⁴⁶

Conclusion. This work establishes that PhpC is a useful tool for monitoring the biodistribution of siRNAs. Insertion of PhpC

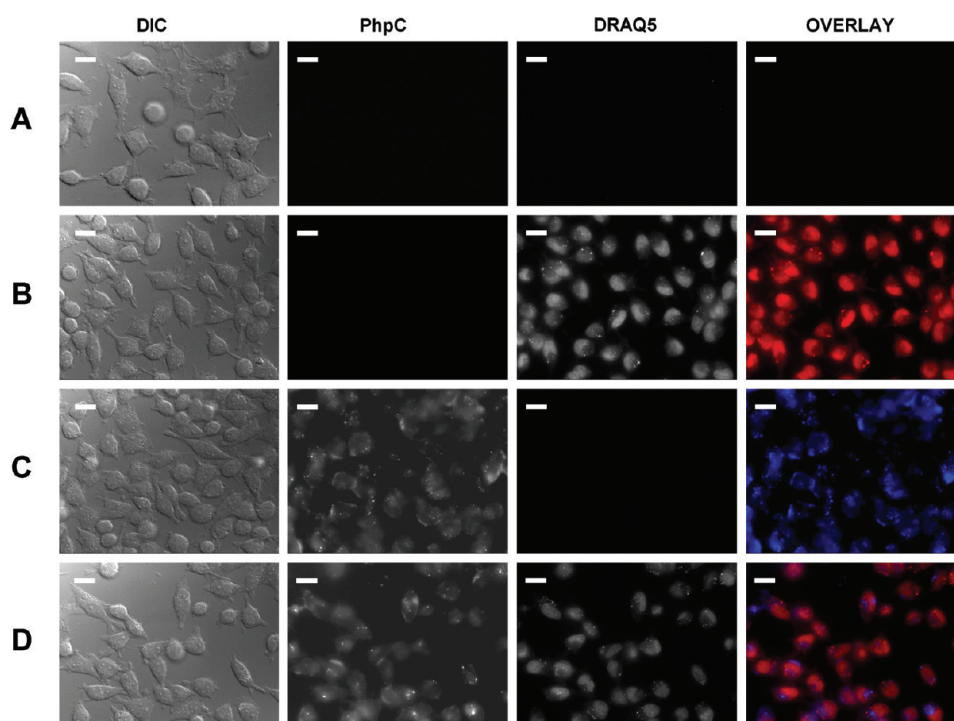


Figure 5. siRNA containing 9 PhpC insertions (siPhpC-8) shows cellular localization in HeLa cells by fluorescence microscopy. Cells were stained with DRAQ5 to identify the nuclei, and images were acquired 6 h post-transfection with siPhpC-8 and the unmodified control siRNA. Images were collected with differential interference contrast (DIC) optics to show overall cell morphology, using the DAPI filter set for PhpC and a Cy5 filter set for DRAQ5. The overlay images show siPhpC-8 in blue and DRAQ5 in red. (Row A) HeLa cells transfected with control siRNA without DRAQ5 staining. (Row B) HeLa cells transfected with control siRNA and stained with DRAQ5. (Row C) HeLa cells transfected with siPhpC-8 without DRAQ5 staining. (Row D) HeLa cells transfected with siPhpC-8 and stained with DRAQ5. Images were processed using MetaMorph software (Molecular Devices, Inc.) to adjust brightness, contrast, and gamma. These parameters were adjusted to the same levels for all images within the figure. Scale bar is 20 μm .

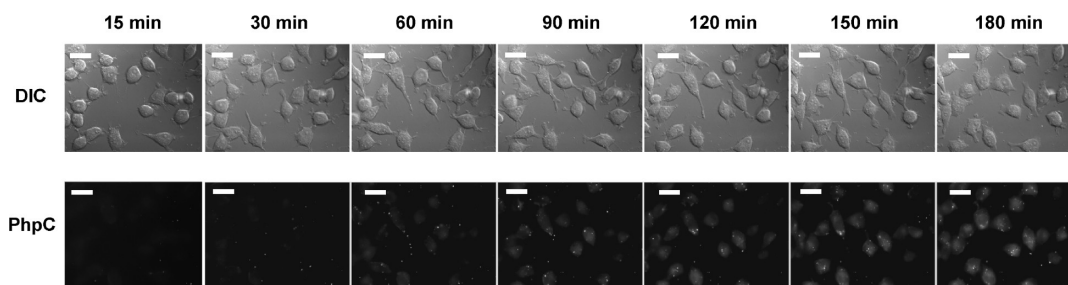


Figure 6. Following the accumulation of a PhpC-modified siRNA in cells in real time. DIC and siPhpC-8 images were collected as a time-lapse image series showing the accumulation of siPhpC-8 inside HeLa cells over time. Images were processed using MetaMorph software to adjust brightness, contrast, and gamma. These parameters were adjusted to the same levels for all images within the figure. Scale bar is 20 μm .

did not markedly decrease the gene silencing activity of siRNAs in HeLa cells, even though previous work suggests that base modifications that protrude in the major groove of RNA can diminish binding to RISC.²⁷ PhpC-modified siRNAs are active whether the PhpC modification is placed in the sense strand, antisense strand, beside the scissile phosphate, or at the 3'-termini. These encouraging results warrant further RNAi studies with PhpC-modified siRNAs.

Beyond use in siRNAs alone, the microscopy images of siPhpC-8 (Figures 5 and 6) demonstrate how PhpC can serve as a tool to study cellular delivery and trafficking of oligonucleotides in general. A variety of uptake mechanisms exist for oligonucleotides, depending on how they are delivered and the chemistry of

the oligonucleotide.⁴⁷ Because the fluorescence of PhpC-modified siRNAs does not depend on a lipophilic conjugate, these siRNAs can be used with greater confidence in the exploration of the mechanism of all of these types of uptake and trafficking. Most importantly, PhpC-siRNAs have gene silencing potency and immunostimulatory properties closely mimicking those of natural siRNAs. Thus PhpC provides a powerful and unobtrusive tool for investigating the biology of oligonucleotides.

METHODS

Chemicals and solvents were ACS grade or higher and were purchased from Sigma-Aldrich or Thermo-Fisher. Anhydrous pyridine was

obtained by distilling over calcium hydride. All reagents used for oligonucleotide synthesis, including 2'-deoxyribonucleotide and 2'-TBDMS ribonucleotide phosphoramidites, were purchased from Chemgenes Corp. PhpC ribonucleoside was synthesized according to previously described methods.¹⁵ Autoclaved Milli-Q water treated with diethylpyrocarbonate (DEPC)⁴⁸ was used to manipulate RNA and prepare aqueous buffers.

Synthesis and Purification of Oligonucleotides. Solid-phase synthesis of oligonucleotides was carried out on an Applied Biosystems 3400 DNA synthesizer using standard protocols.⁴⁹ Cleavage from the solid-support was carried out in a 3:1 mixture of NH₄OH/EtOH at RT for 48 h, and removal of the 2'-O-silyl (TBDMS) protecting groups was achieved by treatment with distilled triethylammonium trihydrofluoride for 48 h. The crude, deprotected oligonucleotides were precipitated with 3 M sodium acetate and ice-cold butanol and then quantitated by their UV absorbance at 260 nm on a Cary-300 UV-vis spectrophotometer (Varian Inc.). Crude products were then analyzed and purified by denaturing PAGE (7 M urea) and visualized by UV shadowing at 254 nm for nonfluorescent oligonucleotides and 365 nm for fluorescent oligonucleotides. Full length products were excised from the gels using a sterile surgical blade and eluted in DEPC-treated water. The eluted products were desalted by size-exclusion chromatography on G-25 Sephadex (GE Healthcare).

Thermal Denaturation Curves. For UV-vis thermal denaturation experiments, nucleotides and oligonucleotides were analyzed in 1 mL solutions at 1 μ M concentrations in a buffer of 10 mM sodium phosphate at pH 7.0 and 50 mM NaCl. Double-stranded oligonucleotides were annealed in equimolar amounts by heating to 95 °C and then slowly cooling to RT on a heating block. Thermal denaturation experiments were performed on a Cary-300 UV-vis spectrophotometer equipped with a 6 \times 6 cell changer and a Peltier temperature controller. Samples were heated from 10 to 95 °C at a rate of 0.5 °C min⁻¹, and the change in absorbance was measured every 1.0 °C. The melting temperature (T_m) values were obtained by the baseline (alpha) method and defined as the point when the mole fraction of duplex was equal to 0.5. These values represent the averages of at least three independent experiments.

Fluorescence Measurements. Fluorescence intensity measurements were obtained on a Cary Eclipse Spectrophotometer equipped with a multicell Peltier temperature controller and automated polarization accessories. Measurements were carried out in 1 cm \times 1 cm quartz cells in 2 mL volumes at RT in 1 μ M concentrations in a buffer of 10 mM sodium phosphate at pH 7.0 and 50 mM NaCl. Excitation and emission bandwidths were set at 5 nm for all experiments. Emission spectra were obtained by exciting at 360 nm and monitoring the emission from 400 to 600 nm.

siRNA Gene Silencing Assays. HeLaX1/5 cells were grown as previously described.³² The day prior to transfection, 0.5 \times 10⁵ cells were plated in each well of a 24-well plate. The next day, the cells were incubated with increasing amounts of siRNAs premixed with 1 μ L Lipofectamine 2000 reagent (Invitrogen). For the siRNA titrations, each siRNA was diluted into dilution buffer (30 mM HEPES-KOH, pH 7.4, 100 mM KOAc, 2 mM MgOAc₂) and mixed with 200 ng of plasmid pCI-hRL-con expressing the *Renilla* luciferase. Twenty-four hours after transfection, the cells were lysed in hypotonic lysis buffer (15 mM K₃PO₄, 1 mM EDTA, 1% Triton, 2 mM NaF, 1 mg mL⁻¹ BSA, 1 mM DTT, 100 mM NaCl, 4 μ g mL⁻¹ aprotinin, 2 μ g mL⁻¹ leupeptin and 2 μ g mL⁻¹ pepstatin), and the firefly and *Renilla* light units were determined using a Fluostar Optima 96-well plate bioluminescence reader (BMG Labtech) as described previously.⁵⁰ The luciferase counts were normalized to the protein concentration of the cell lysate as determined by the DC protein assay (BioRad). Cotransfecting the siRNAs and the plasmid pCI-hRL-con expressing the *Renilla* luciferase mRNA⁵¹ in the same cell line showed no difference in expression of this reporter,

demonstrating the specificity of the RNAi effects (data not shown). A scrambled native RNA duplex with identical base composition and no silencing activity was used as a negative control, and an unmodified siRNA duplex was used as a positive control.

Immunostimulation Assays in Human Peripheral Blood Mononuclear Cells. Peripheral blood mononuclear cells (PBMCs) were transfected using Lipofectamine 2000 at a final siRNA concentration of 80 nM. Briefly, 0.6 \times 10⁶ cells were suspended in 400 μ L of culture medium and transferred into a 24-well plate. For each siRNA, 2 μ L of 20 μ M siRNA was diluted in 50 μ L of OptiMEM, mixed with 1 μ L of Lipofectamine 2000 diluted in 50 μ L of OptiMEM, and then incubated at RT. After 20 min, siRNA/Lipofectamine complexes were added to the cells and were incubated for 24 h at 37 °C. After that time 20 μ L of supernatant was taken from each well and transferred to a 96-well plate, 180 μ L of HEK-Blue cells in HEK-Blue detection medium was added, and cells were left in the incubator overnight. In response to IFN production, HEK-Blue cells release soluble alkaline phosphatase. Thus the next day the colored product of the alkaline phosphatase and HEK-Blue detection medium reaction is quantitated spectrophotometrically at 650 nm. Results are presented as relative IFN units after background subtraction. The Mock contains HEK-Blue cells with supernatant from PBMC cells exposed to Lipofectamine alone. The negative control (NC) contains HEK-Blue cells in HEK-Blue detection medium only (no supernatant from PBMC cells). We also included a fully modified siRNA known to reduce immunostimulation (728 MOD)³⁴ as an additional control in our experiments. The siRNA 728 MOD has the following sequence in the sense strand: 5'-AAA UCG CUG AUU UGU GUA G-3', and the antisense strand: 5'-CUA CAC AAA UCA GCG AUU U-3', where 2'-deoxy-2'-fluoro ribonucleotides (2'-F-RNA) are **BOLD** and 2'-deoxy-2'-fluoroarabinonucleotides (2'-F-ANA) are underlined.³⁴ The duplex marked simply "728" is the analogous sequence with unmodified RNA.

Fluorescence Microscopy. HeLa X1/5 cells were grown in 35 mm glass bottom culture dishes (World Precision Instruments). The next day, the native control siRNA or PhpC-containing siRNAs were mixed with 1 μ L of Lipofectamine 2000 (Invitrogen) and incubated for 30 min. The cells were transfected with the siRNA/Lipofectamine 2000 mixture to a final concentration of 1 μ M oligonucleotide. After 4 h, DRAQ5 (Biostatus Limited) was added to the cell medium in a 1/10,000 fold dilution and incubated for 15–30 min, after which time the medium was replaced with DRAQ5-free medium. The cells were observed at 37 °C in the presence of 5% CO₂ under a Zeiss Axiovert 200 M wide field microscope, equipped with a stage mounted Chamlide CO₂ incubation chamber (Quorum Technologies) using a 63X 1.4 NA oil immersion objective lens. Excitation was from a 200 mW mercury HBO light source attenuated to 10% intensity using neutral density filters. Selection of UV excitation light and blue emission light was done using the DAPI filter set to image PhpC (λ_{exc} = 355 nm and λ_{em} = 460) and red excitation light and IR emission light using the Cy5 filter set to image the DRAQ5 nuclear stain (λ_{exc} = 620 nm and λ_{em} = 700 nm). Images were captured with Zeiss AxioVision software using a Zeiss AxioCam HRm charge coupled device (CCD) camera. Time-lapsed images were observed prior to adding the DRAQ5 stain.

■ ASSOCIATED CONTENT

S Supporting Information. Mass spectral data of oligonucleotides and additional fluorescence microscopy images. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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