Water-Soluble, Meso-Substituted Cationic Porphyrins—a Family of Compounds for Cellular Delivery of Oligonucleotides

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ABSTRACT

The delivery of oligonucleotides to appropriate intracellular compartments is crucial to their development as tools in gene function studies and as therapeutics. Here, we report the characterization of meso-substituted cationic porphyrins as a large class of water-soluble reagents for oligonucleotide delivery. These porphyrins form non-covalent complexes with single-stranded oligonucleotides and deliver these molecules into the nuclei of cell lines in culture. The porphyrins protect oligonucleotides from nuclease degradation, and delivery is unaffected by the presence of serum. Delivery capacity is dependent on the charge ratio and concentration of the oligonucleotide and porphyrin used to form the complex, on the chemical substituents of the oligonucleotide and on the identity of the cationic porphyrin. This class of molecules provides a versatile set of water-soluble delivery reagents that could contribute to the development of oligonucleotide drugs.

INTRODUCTION

The development of short synthetic nucleic acids, such as external guide sequences (EGSs) (7), ribozymes (12) and antisense molecules (25), as therapeutics and as reagents for use in functional genomics requires, in some circumstances, the codevelopment of effective delivery tools (8). An ubiquitous delivery reagent for use in cell culture and animal studies might protect the oligonucleotide from nuclease degradation, convey the oligonucleotide to a cellular target, promote uptake of the oligonucleotide and allow the oligonucleotide to accumulate in free form at an intracellular site of action. Delivery reagents, such as cationic liposomes (6,15), conventional liposomes (23), polymeric conjugates (14,27) and dendrimers (2), have been used for oligonucleotide delivery, and each might be useful for a given application. Lipid particulates might not be the reagents of choice for in vivo applications because they become opsonized when injected intravenously (4,16) and are rapidly recognized by the reticuloendothelial system (Reference 18 and G. Takle, unpublished data).

We have previously described an oligonucleotide delivery system that has utilized heme as a targeting ligand for hepatocytes that was designed to take advantage of the liver functioning as the major organ for porphyrin metabolism (22). In the work described here, we have extended this line of rea-

soning to include non-lipid-associated porphyrins, and we present data to show that water-soluble porphyrins containing a positive charge are effective cellular delivery reagents for single-stranded oligonucleotides.

The meso-substituted cationic porphyrins (InnoPhor™ delivery reagents) form a class of molecules that have previously been shown to interact with duplex oligonucleotides, either by external binding with or without self-stacking, presumably as a result of a charge interaction with nucleic acid phosphate and/or by partial or full intercalation depending on the identity of the porphyrin (3,9–11,19). These molecules have been used as probes to investigate the structure of nucleic acids. They are effective tumor-localizing agents (24,26), and related porphyrins have been used in the treatment and detection of a number of cancers. They have been shown to cross the nuclear membrane (9,17). We hypothesized that these features of the water-soluble, meso-substituted cationic porphyrins potentially make them ideal candidates for use in nucleic acid delivery, and the data presented here support this contention.

MATERIALS AND METHODS

Porphyrins were obtained from Porphyrin Products (Logan, UT, USA). Meso-substituted, water-soluble cationic porphyrins used in this work were meso tetra (*N*-methyl-4 pyridyl)por-

phyrin tosylate [TMPyP(4)], meso tetra (4-*N,N,N*, trimethylanilinium)porphyrin chloride (TAPP), meso tetra (4-sulfonatophenyl) porphyrin dihydrochloride (TPPS) and metal-chelated variants (Figure 1). Porphyrins were stored in the dark at room temperature, and stock solutions, stored at 4°C, were used within 24 h of preparation. The millimolar extinction coefficient for TMPyP(4) is 226 in water at 421 nm, the wavelength of the Soret band (13). Users are urged to consult the relevant Material Safety Data Sheet (MSDS) for each porphyrin before use for any handling precautions.

Oligonucleotides

Oligonucleotides were synthesized and purified as described previously (22). Underlining indicates phosphorothioate linkages, uppercase indicates 2' O-methyl substituted nucleotides, and lowercase indicates unmodified ribonucleotides. Oligonucleotide A: AUGAUAGAAGGUUCGAAUCCUU-CACGCCGC. Oligonucleotide B: agcgaugaagguucgaauccuucccaggac. Oligonucleotide C: FITC-GAGGAAACGCCGCt (t indicates a 3'3' inverted t residue). Oligonucleotide D: GAGGAAACGCCGC. Oligonucleotide E: GAGGAAACGCCGC. Oligonucleotides were ³²P-labeled at their 5' ends using T4 polynucleotide kinase, and labeled oligonucleotides were purified

from unincorporated radioactivity by Sephadex® G-25 minispin-column chromatography (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Interaction of Porphyrin with Oligonucleotide

Porphyrin solutions were made up in sterile 150 mM NaCl as 10 mg/mL stock solutions and diluted as indicated. Oligonucleotides were dissolved in sterile 150 mM NaCl as 6 mg/mL stock solutions and diluted as indicated. To form complexes, dilutions of porphyrin and oligonucleotide were made in 150 mM NaCl, and equal volumes were mixed and allowed to stand at room temperature for 15 min.

An estimate of the stoichiometry of binding in the complex was made by measuring the bathochromatic shift in the Soret peak of the porphyrin upon addition of oligonucleotide. Complexes containing 1.75 nmol (8.8 µM) TMPyP(4) and 0–1.0 nmol (0–5 µM) Oligonucleotide A were prepared in 200 µL 150 mM NaCl and allowed to form at room temperature for 15 min. Absorbance spectra (350–450 nm) were obtained using a Cary 1E UV-Visible Spectrophotometer (Varian, Palo Alto, CA, USA), and the shift in the wavelength of the Soret peak was plotted against amount of oligonucleotide added.

Protection of 0.5 nmol Oligonucleotide B from nuclease degradation in

human serum following complexing with 22 nmol TMPyP(4) was carried out as described previously (22).

Cellular Delivery of Oligonucleotides by Cationic Porphyrins

HepG₂ 2.2.15 cells (21) were maintained as described previously (22). This cell line was used in all experiments. Cells were seeded at 10⁴ cells/100-µL well in 96-well plates. One, five and ten µg (0.1, 0.5 and 1.0 nmol giving 2.5, 12.5 and 25 µM, respectively, in the complex) Oligonucleotide A, containing trace ³²P-labeled Oligonucleotide A, were complexed with 0–73 nmol (0–1834 µM) TMPyP(4) in 40 µL 150 mM NaCl and delivered to wells in replicates of six. This resulted in concentrations of oligonucleotide in the culture medium of 0.718, 3.57 and 7.14 µM with 0–524 µM TMPyP(4). After 4 h, the cells were washed three times with phosphate-buffered saline (PBS) and treated with lysis buffer (22). Radioactivity in cell lysates was determined using a Model 1900TR Liquid Scintillation Analyzer (Packard Instrument, Meriden, CT, USA) and nucleic acids prepared for analysis by 15% polyacrylamide gel electrophoresis (PAGE).

Intracellular Distribution of Oligonucleotide Following Delivery with Cationic Porphyrins

Two methods were used: (i) cell fractionation and (ii) fluorescence microscopy.

Cell fractionation. Cells were seeded onto 96-well plates at 10⁴/100-µL well. Complexes containing ³²P-labeled Oligonucleotide A (1.0 nmol; 7.14 µM in the culture medium) and 0–100 µg porphyrin (resulting in 0–524 µM TMPyP(4) final concentration in the culture medium, 0–722 µM for TAPP) were added in triplicate to the wells. After 4 h, the cells were washed three times with PBS and lysed in Nonidet® P-40 buffer (5). Nucleus and cytoplasm-enriched fractions were separated (5), and the radioactivity associated with each fraction was determined.

The reproducibility of the fractionation technique was investigated by mixing cytoplasm-enriched fractions

Figure 1. Structure of meso-substituted cationic porphyrins used in this study. (I) TMPyP(4); (II) TAPP; (III) TPPS.

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Table 1. Charge Ratio of Oligonucleotide and Porphyrin in the Complex and Resulting Percentage Cell-Association of Oligonucleotide

µg oligonucleotide	nmol -ve	µg TMP			
		3	10	30	100
		nmol +ve			
		7.3	24	73	243
Charge ratio (porphyrin +/oligonucleotide -)					
10	29	0.25 (0.40)	0.82 (8.97)	2.51 (47.40)	8.38 (39.19)
5	14.5	0.5 (0.58)	1.65 (27.73)	5.44 (54.95)	16.76 (49.94)
1	2.9	2.51 (9.94)	8.25 (34.93)	25.10 (48.03)	83.80 (47.45)

Numbers in parentheses indicate the percentage of the added Oligonucleotide A that was cell-associated for each combination (Figure 4B).

containing ³²P-labeled oligonucleotide with unlabeled nucleus-enriched fractions and vice-versa following delivery of oligonucleotide with porphyrin or with cationic lipid DOTAP:DOPE (dioleoyl trimethylammonium propane: dioleoyl phosphatidyl ethanolamine; Avanti Polar Lipids, Alabaster, AL, USA). Less than 10% crossover of oligonucleotide between fractions was observed using this protocol.

Fluorescence microscopy. Cells were plated onto round glass coverslips in 6-well plates (2 mL vol) and treated with complexes containing 10 nmol (36 µM in the culture medium) porphyrin, 73 nmol (9.25 µM) Oligonucleotide C for 4 h as described previously (22). Controls were: porphyrin alone (36 µM), porphyrin plus nonfluorescent Oligonucleotide E and Oligonucleotide C alone. Cells were viewed using a 520–560-nm barrier filter to remove any possible background fluorescence contributed by the porphyrin.

Effect of Chemistry of Oligonucleotide on Delivery

All-phosphorothioate 13-mer Oligonucleotide D and all-2' O-methyl Oligonucleotide E, both having the same sequence, were labeled using ³²P as above, and 0.5 nmol (8.3 µM final concentration in the culture medium) of each were delivered to 2.2.15 cells using 0–22 nmol (0–157 µM final concentration) TMPyP(4) as described

above. Cell-associated radioactivity in the nucleus and cytoplasm-enriched fractions were determined as described above.

RESULTS

Chemically modified, single-stranded RNA oligonucleotides (EGSs) (7) interacted with TMPyP(4) as demonstrated by a bathochromatic shift in the Soret peak of the porphyrin from 421 nm with no oligonucleotide to 440 nm at saturation (Figure 2) (3). Similar results were

Figure 2. 1.76 nmol TMPyP(4) was complexed with 0–1.0 nmol Oligonucleotide A in 200 µL 150 mM NaCl, and the absorbance spectrum from 350–450 nm was obtained for each complex. The wavelength of the Soret peak for each complex was plotted against the amount of Oligonucleotide A in the complex, and the point at which no further increase in the wavelength of the Soret band occurred was taken as the point of saturation.

obtained for the porphyrin TAPP, in which the shift in the Soret peak was from 415–428 nm (data not shown).

By titrating the nucleic acid against TMPyP(4) and following the wavelength of the shifting Soret band to its maximum, an estimate of the charge ratio of interaction between Oligonucleotide A and porphyrin at saturation was determined to be 0.603. In this case, 0.4 nmol oligonucleotide complexed with 1.75 nmol porphyrin. Then, taking 29 negative charges per oligonucleotide molecule and 4 positive charges per porphyrin, this calculates as 11.6 nmol oligonucleotide negative charges complexing with 7 nmol porphyrin positive charges, hence a charge ratio of 0.603.

The presence of TMPyP(4) in a complex protected oligoribonucleotides from degradation by the nuclease activity present in 50% human serum (Figure 3) with >75% of the all-RNA oligonucleotide remaining full-length after 20 h.

Oligoribonucleotides complexed with TMPyP(4) were delivered to 2.2.15 hepatoma cells (Figure 4A), and significant amounts of full-length oligonucleotide remained in the cell-associated fraction after 4 h (Figure 4C). In this experiment, a range of TMPyP(4) amounts were used to give a series of complexes with different net charges. Complexes with net positive charge were more effective at delivering greater amounts of oligonucleotide

to cells. However, the concentration of oligonucleotide and porphyrin in the complex was also important in determining the level of delivery (Table 1). For example, a complex having a net charge of +2.51, comprised of 2.5 μM oligonucleotide with 55 μM TMPyP(4) [resulting in a concentration of 0.718 μM oligonucleotide and 15.7 μM TMPyP(4) in the culture medium] delivered 10% of the total dose added to the cells (Figure 4B). A complex with the same net charge formed from 25 μM oligonucleotide and 550 μM TMPyP(4) [7.18 μM oligonucleotide and 157 μM TMPyP(4) in the culture medium] delivered 50%. The amount of material delivered to the cells reached saturation of about 50% of the added dose at 550 μM TMPyP(4) with 2.5, 12.5 and 25 μM oligonucleotide in the complex. These results indicated that both the net charge of complex and the concentration of components influenced the high-level delivery. Taking 0.1 nmol to be an underestimate of the potential amount of oligonucleotide delivered by TMPyP(4) to 3×10^4 cells, this then calculates to 2.0×10^9

oligonucleotide molecules per cell.

Cationic porphyrin-mediated delivery of oligonucleotides was observed in a variety of cell types in culture, including primary hepatocytes, promyelocytes (NB4), human glioblastoma cells (U-87 MG), human neuroblastoma cells (SK-N-SH), human colon adenocarcinoma cells (CaCo-2), Chinese hamster ovary cells (CHO), Green monkey kidney cells (Vero), mouse kidney cells (LLCMK2), fetal rat liver cells (FNRL), DU 145 prostate cancer cells and T24 bladder cancer cells (Reference 1 and data not shown). Using the same complexes that gave the high-level delivery in the previous experiments (12.5 μM oligonucleotide with 550 μM porphyrin), we demonstrated that the presence or absence of serum did not affect the delivery of oligonucleotides (data not shown).

Complexes composed of 4.9 μM TMPyP(4) with 0.7 μM oligonucleotide delivered daily to 2.2.15 cells for 10 consecutive days were not toxic, as determined by the tetrazolium assay for mitochondrial dehydrogenase activity. TMPyP(4) delivered alone to 2.2.15

Figure 3. Protection of all-RNA Oligonucleotide B from human serum nuclease degradation by complexing with TMPyP(4). M, full-length marker; 30, 30 min; 20, 20 h.

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cells at concentrations $>300 \mu\text{M}$ for 4 h showed no toxicity. However, concentrations of TMPyP(4) $>20 \mu\text{M}$ delivered for 24 h brought about a $>50\%$ re-

duction in formazan product. We used a 4-h delivery time for all of the experiments reported here.

The meso substituent on the porphyrin was important for the delivery to hepatoma cells, as is the identity of the chelated ion with axial ligands (Figure 5). The negatively charged, water-soluble TPPS interacted with the nucleic acid, as evidenced by changes in the absorbance spectrum, but demonstrated little cellular delivery (Figure 5). TAPP delivered lower amounts of oligonucleotide than TMPyP(4). The manganese (III) and cobalt (III) chelates of TMPyP(4) delivered less oligonucleotide compared with the zinc (II),

copper (II), iron (III), magnesium (II), nickel (II) chelates and TMPyP(4) alone. This reduction in the delivery capacity is probably not a result of ineffective complex formation, because those porphyrins delivering low levels of oligonucleotide were able to complex with the oligonucleotide, as determined by the Soret peak shifts (data not shown).

TMPyP(4) was able to deliver oligonucleotides with different chemical modifications to cells (Figure 6), however the ratio of porphyrin to oligonucleotide giving the highest level of delivery varied depending on the chemistry of the oligonucleotide, independent of the sequence. The all-phosphorothioate oligonucleotide was delivered to greater levels than the all-2' O-methyl oligonucleotide when the charge ratio of the complex was lower (Figure 6B).

The intracellular distribution of fluorescein isothiocyanate (FITC)-labeled oligoribonucleotide delivered with TMPyP(4) was determined in vitro (Figure 7). Within 4 h, all 2.2.15 cells were labeled with fluorescence apparent in the cytoplasm and concentrated in the nucleus. FITC oligoribonucleotide delivered with the Fe(III) chelate of TMPyP(4) was also concentrated in the nucleus (Figure 7C). However, there appeared to be a specific intranuclear site of accumulation of the oligonucleotide using this porphyrin.

Fractionation of the cells into a nuclear pellet and a post-nuclear (cytoplasmic) supernatant, using nonionic detergents and low-speed centrifugation, was used for analysis of the partitioning of oligonucleotides within cells. These cell-fractionation data complement the data obtained from the fluorescence micrographs, indicating that up to 80% of the delivered oligonucleotide became localized in the nuclear-enriched pellet (Figure 8A). In contrast, when cationic liposomes were used as the delivery reagent, approximately 30% of the delivered material was nuclear (22). The extent of nuclear localization was dependent on the nature of the amine, the quarternary amine (TAPP) showing less nuclear delivery than pyridinium ion [TMPyP(4)]. Full-length oligonucleotide was recovered from the nuclear fraction (Figure 8B).

Figure 4. (A) Delivery of Oligonucleotide A complexed with TMPyP(4) to 2.2.15 hepatoma cells. Micrograms of oligonucleotide associated with 3×10^4 cells per well following delivery of 1.0 nmol (■), 0.5 nmol (◆) and 0.1 nmol (●) oligonucleotide complexed with 0–73 nmol TMPyP(4). Means ± 1 standard deviation (SD) of data from 6 wells. (B) Percent of oligonucleotide delivered that became cell-associated. (C) Lysates were pooled, and Oligonucleotide A recovered from the cell lysates from the 0.5-nmol oligonucleotide experiment was separated on a 15% polyacrylamide gel and visualized on a PhosphorImager® (Molecular Dynamics, Sunnyvale, CA, USA). Lane markers refer to the μg of TMPyP(4) used for delivery (0–100 μg corresponding to 0–73 nmol). M, full-length oligonucleotide marker.

Figure 5. Influence of porphyrin and chelated metal atom on oligonucleotide delivery. (A) Oligonucleotide A (0.5 nmol) complexed with 0–200 μg (0–147 nmol) TMPyP(4) (■), 0–200 μg (0–228 nmol) Fe (III) TMPyP(4) (●) and 0–200 μg (0–202 nmol) TAPP (◆). Means ± 1 SD of data from 6 wells (3×10^4 cells/well). (B) 0.1 nmol Oligonucleotide A: 22 nmol porphyrin complex were formed with TMPyP(4) metal chelates indicated and compared with the same complex comprising unchelated TMPyP(4) (T), TAPP (A) or TPPS (labeled SO3). DOTAP: DOPE (3.6 nmol) was used for comparison of a cationic lipid (D). Means ± 1 SD from 6 wells.

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DISCUSSION

The water-soluble cationic porphyrins provide a large, versatile, easy-to-use group of water-soluble reagents that efficiently deliver oligonucleotides to cell nuclei. One of the salient features of this group of molecules is that there are abundant chemical substitutions that can be made on the porphyrin macrocycle that might allow design of better reagents for a particular oligonucleotide or cell-type applications. From the data described here, we suggest that chemical modification of the oligonucleotide and the identity of the cationic porphyrin are important determinants in specifying uptake and distribution within 2.2.15 cells. There are significant differences in the capacity of two closely related porphyrins [TMPyP(4) and TAPP] to deliver oligonucleotides to 2.2.15 cells.

Numerous reports have described

Figure 6. Chemical substitution of oligonucleotide determines level of cellular delivery by TMPyP(4). An all 2' O-methyl substituted 13-mer (Oligonucleotide E) (A) and an all phosphorothioated 2' O-methyl substituted 13-mer (Oligonucleotide D) (B) with the same sequence were complexed with TMPyP(4) and delivered to 2.2.15 cells. Cells were fractionated into nucleus-enriched (■) and cytoplasm-enriched (◆) samples, and oligonucleotide present in each fraction was determined as in Materials and Methods. Means ± 1 SD of data from 6 wells.

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the interaction of porphyrins and metalloporphyrins with nucleic acids (3,9–11,19). At high concentrations, porphyrins can aggregate in the presence of nucleic acids, and the degree of aggregation might influence cellular delivery. Titration studies showed that at saturation, there is a slight excess of oligonucleotide in the complex (the net charge ratio of the complex is 0.603), indicating that there might be cross-linking of oligonucleotides by individual porphyrin molecules. It is interesting to note that duplex molecules of different sizes are delivered poorly by cationic porphyrins (G. Takle, unpublished data). Single-stranded oligomers might form loose aggregates held together by shared porphyrin molecules more easily than their double-stranded counterparts. However, it would be possible to link adjacent externally facing phosphate backbones on duplex

molecules. Porphyrins might intercalate into structured regions of oligonucleotides.

Our data indicate that the nature of the metal axial ligand is important in determining the delivery capacity, and the reason for this is unclear. The possibility exists that differences in delivery capacity might be related to the ability of the porphyrin to release bound oligonucleotide once inside the cell. Preliminary electron microscopic studies have shown that porphyrin/oligonucleotide complexes are taken up into early endosomes, but that the complex dissociates rapidly and is not observed in more distal vesicular structures. Cells observed by fluorescence microscopy show very little oligonucleotide sequestered into the cytoplasmic vesicular structures, in contrast to the micrographs observed using DOTAP:DOPE (22), and the oligonucleotide appears concentrated

in the nucleus, probably in free form.

Many different cell types take up oligonucleotides delivered with TMPyP(4), ranging from neuronal cells to primary hepatocytes. There does not appear to be a specific receptor-mediated event during uptake, because it was not possible to compete out uptake with excess free porphyrin (data not shown). Preliminary studies have shown that the uptake of complexes to be temperature-dependent, but was not completely abrogated at 4°C, and that reagents that inhibit endocytosis [sodium azide plus 2-deoxyglucose (20)] reduced, but did not completely inhibit uptake.

Some cationic lipid reagents show a reduced delivery capacity in the presence of serum [e.g., LIPOFECTIN® (Life Technologies, Gaithersburg, MD, USA); Reference 6], while others are resistant to serum (15). TMPyP(4) was able to protect an all-RNA oligonucleotide in the presence of serum and was unaffected by the presence of serum in cellular uptake studies. This resistance to serum

Figure 7. Intracellular distribution FITC-labeled Oligonucleotide C in 2.2.15 cells following delivery with TMPyP(4) or Fe (III) TMPyP(4). (A) 9.25 μ M Oligonucleotide C: 36 μ M TMPyP(4). 4 h delivery. (B) Same as (A) but different field showing group of cells. (C) 9.25 μ M Oligonucleotide C: 36 μ M Fe (III) TMPyP(4). (D) 9.25 μ M Oligonucleotide C alone. (E) 36 μ M TMPyP(4) alone. (F) 9.25 μ M Oligonucleotide E: 36 μ M TMPyP(4).

Figure 8. (A) Intracellular distribution of Oligonucleotide A delivered with 0–100 μ g (0–73 nmol) TMPyP(4) or 0–100 μ g (0–101 nmol) TAPP as determined by cell fractionation. TMPyP(4), nucleus-enriched fraction (■), cytoplasm-enriched fraction (●). TAPP, nucleus (◆), cytoplasm (*). Means $\pm$ 1 SD of data obtained from 3 wells. (B) Oligonucleotide recovered from cell fractions from TMPyP(4) wells [lane markers refer to μ g TMPyP(4)] was pooled and analyzed by gel electrophoresis and compared with 0.5 nmol oligonucleotide delivered with 3.6 nmol DOTAP:DOPE (L).

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suggests that the porphyrins show promise for use in in vivo studies.

Furthermore, porphyrins have been used extensively for tumor therapy and are well-characterized tumor localizers (24). Therefore, this class of molecules might be important for delivering oligonucleotides to tumors. Also, because hepatocytes are the main cell type responsible for porphyrin metabolism, the porphyrins could direct material to this cell type. Taken together, our data suggest that this completely new class of delivery molecules might be of use in the development of single-stranded nucleic acid-based reagents for functional genomics and therapeutic applications. In this regard, specific down-regulation of protein kinase C α transcripts and polypeptide by antisense phosphorothioate deoxynucleotides delivered by TMPyP(4) has been demonstrated (1).

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