G-Quadruplex DNA

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Guanidinium-Modified Phthalocyanines as High-Affinity G-Quadruplex Fluorescent Probes and Transcriptional Regulators**

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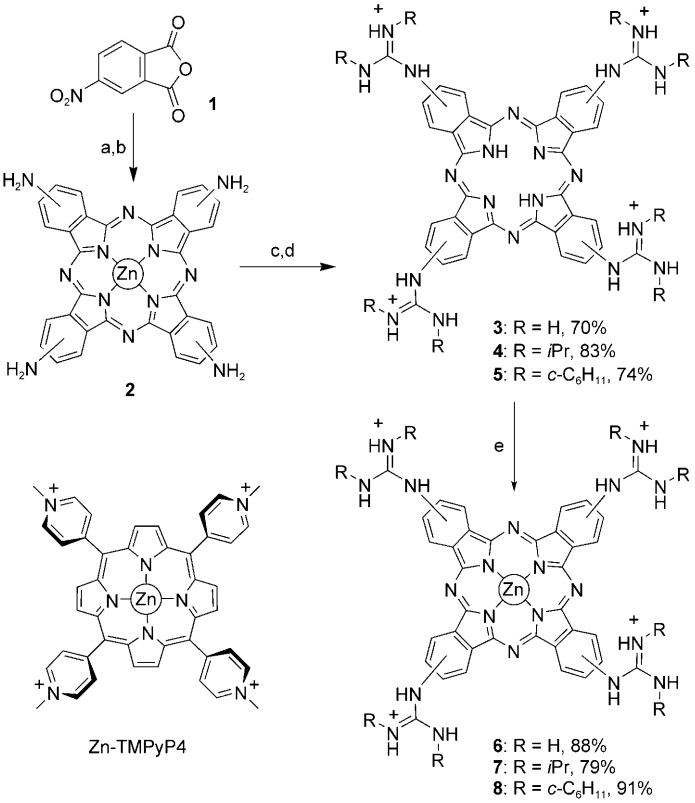
G-quadruplex structures are among the most interesting and best-characterized DNA folding motifs.[1] DNA sequences that can form stable G-quadruplexes in vitro have been implicated in a wide range of functions in vivo, including the regulation of telomere stability,[2] the regulation of promoters,[3] and viral integration and recombination.[4] Many groups have developed small-molecule ligands for G-quadruplexes because of their potential to inhibit cancer growth by disrupting telomere and/or promoter activities.[5] A much more limited selection of G-quadruplex ligands with useful fluorescence properties has been reported.[6] Despite such notable progress, G-quadruplex ligands exhibiting truly high affinity ($K_d \leq 2$ nM) and high specificity (>5000-fold lower affinity to duplex DNA) have remained elusive.[5,6] Such potent and selective binding might be needed to effectively compete with cellular proteins that can bind G-rich DNA and RNA with K_d values in the mid-pM range.[7]

We are interested in high-affinity G-quadruplex ligands with dual functions: they should exhibit both "turn on" photoluminescence and the ability to regulate gene expression.[3,6] These orthogonal readouts might be used in concert to probe potential relationships between G-quadruplex structure and function in vivo. We are exploring this new concept through the design, synthesis, and evaluation of a new family of porphyrazine derivatives where simultaneous variation of the metal center and guanidinium group can modulate the DNA specificity, cellular uptake, and photophysical properties of the phthalocyanine scaffold.

Structure-selective G-quadruplex ligands often have extensive shape and charge complementarity with the stacked G-tetrads that constitute G-quadruplex DNA.[5] For example, pyridinium- and ammonium-containing porphyrazine derivatives exhibit enhanced G-quadruplex specificity relative to the widely studied, yet nonselective ligand 5,10,15,20-tetrakis(*N*-methyl-4-pyridyl)porphine (TMPyP4).[8,9] These molecules bind to G-quadruplex DNA with modest affinities (K_d = 100–200 nM), but no information regarding their cellular uptake, luminescence, or transcriptional regulation has been reported.[8]

We are interested in cationic phthalocyanines with guanidinium groups because the cellular uptake and RNA/DNA affinity of guanidinium-containing molecules are better than the analogous ammonium-containing compounds.[10] We therefore synthesized a small family of guanidiniophthalocyanines (GPcs) by treating tetraaminozinc phthalocyanine **2** with various carbodiimides in an ionic liquid (pyridine/pyridine hydrochloride) at 120°C (Scheme 1).[11] Under these conditions, zinc was removed from both the starting materials and products to furnish the metal-free GPcs **3**–**5** (Scheme 1). These reactions revealed a novel method for the synthesis of metal-free phthalocyanines,[11] but we were



Scheme 1. Synthesis and structures of guanidiniozinc phthalocyanines **6**–**8**.[13,15] Reagents and conditions: a) zinc chloride (0.25 equiv), ammonium molybdate (0.1 mol%), nitrobenzene, 185°C, 4 h, 98%; b) sodium sulfide (12 equiv), DMF, 60°C, 1.5 h, 75%; c) a carbodiimide (20–50 equiv), pyridine/pyridinium chloride, 120°C, 20 h; d) trifluoroacetic acid (TFA)/water; e) zinc chloride, sodium acetate/acetic acid, 120°C, 1–4 h, TFA/water. The trifluoroacetate counterions of compounds **3**–**8** were exchanged for chloride for solubility tests and biological studies.

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interested in making zinc-containing GPcs because of reports of luminescent zinc-containing phthalocyanines with good cellular uptake.[12] The metal-free GPcs **3**–**5** were therefore heated with zinc chloride to generate the corresponding guanidiniozinc phthalocyanines **6**–**8** (Scheme 1). These products were characterized by ^{1}H NMR spectroscopy, high-resolution mass spectrometry, and analytical HPLC.[13] Compounds **6**–**8** appear to be single regioisomers, consistent with a previous report that the zinc-templated cyclotetramerization of **1** exhibits regioselectivity.[14,15] Unexpectedly, only the tetrakis(diisopropylguanidinio)zinc phthalocyanine "Zn-DIGP" (**7**) and its metal-free analogue **4** exhibited good solubility in water and were therefore studied further.

To probe the G-quadruplex affinity of Zn-DIGP (**7**) we used two direct and complementary fluorescence-based methods with DNA derived from the human telomeric repeat ("Htelo") and from the c-Myc oncogenic promoter ("c-Myc").[16] To probe the specificity of these interactions, we also evaluated an unfolded variant of Htelo ("Htelo-Mut"), and the C-rich complement strands of c-Myc ("c-Myc-C") and Htelo ("Htelo-C") which can form i-motif structures.[17]

To determine the stoichiometries of Zn-DIGP/oligonucleotide binding, the absorbance spectrum of 1 μM Zn-DIGP was monitored as a function of DNA concentration (Figure 1 A and Figure S4 in the Supporting Information).[13] These experiments were conducted using Zn-DIGP concentrations much higher than the K_d values for G-quadruplex binding; we determined stoichiometries of 2:1 for Zn-DIGP/c-Myc, 1:1 for Zn-DIGP/Htelo, and 4:1 for Zn-DIGP/Htelo-Mut.[13]

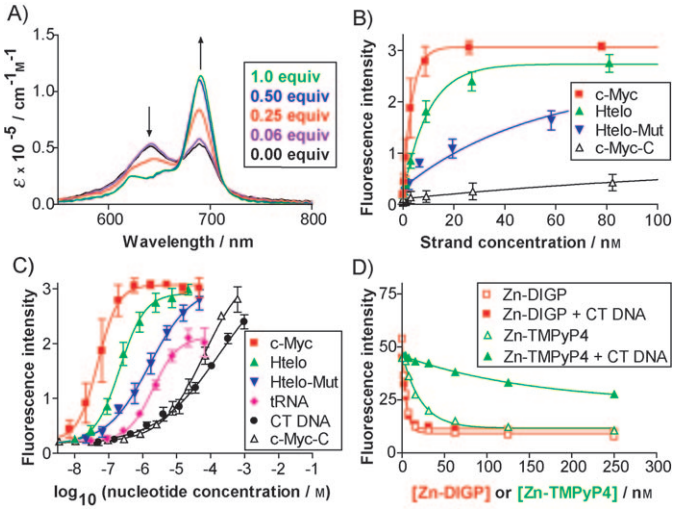

Figure 1. A) Absorbance spectra of a 1 μM solution of Zn-DIGP upon titration with 0–1 equiv of a prefolded 22-mer quadruplex DNA derived from the c-Myc promoter.[16] B, C) Fluorescence intensities of 10 nM solutions of Zn-DIGP ($\lambda_{ex.}$ = 620 nm, $\lambda_{em.}$ = 705 nm) upon addition of nucleic acids.[13] D) Fluorescence quenching of 10 nM solutions of the 5′-fluorescein end-labeled c-Myc DNA ($\lambda_{ex.}$ = 495 nm, $\lambda_{em.}$ = 520 nm) upon titration with Zn-DIGP or Zn-TMPyP4 in the presence or absence of 220 μM of CT DNA (nucleotide concentration). All samples were prepared and analyzed in a "TKE" buffer consisting of 50 mM Tris-HCl (pH 7.4), 150 mM KCl, and 0.5 mM EDTA. Error bars indicate standard deviations.

Zn-DIGP exhibits highly desirable "turn-on" fluorescence upon binding nucleic acids. Upon saturation with DNA or RNA, a 200-fold or larger increase in photoluminescence from Zn-DIGP was observed (Figure 1 B, C and Figure S4 in the Supporting Information).[13] The quantum yields of the resulting complexes in water were 0.06 ± 0.02 according to comparisons with both Cy5 and cresyl violet as fluorescent standards.[18] These modest quantum yields are compensated by large molar extinction coefficients for absorbance of Zn-DIGP complexes ($\varepsilon = 30000$–$130000\ cm^{-1}M^{-1}$; Figure 1 A).

Fluorescence data collected using 10 nM Zn-DIGP (Figure 1 B) were analyzed using an independent 2:1 binding model,[9a] and revealed an apparent equilibrium dissociation constant (K_d) for the Zn-DIGP/c-Myc complex of ≤ 2 nM for each binding site. A limit must be reported since the probe concentration used for direct detection (10 nM) was much higher than the K_d values, and self-association of Zn-DIGP might cause an underestimated DNA binding affinity for all reported binding interactions. Using a 1:1 binding model, an apparent K_d value of (6 ± 4) nM was estimated for Htelo, while the mutated construct Htelo-Mut bound with an apparent K_d value of (60 ± 10) nM (Figure 1 B). G-quadruplexes derived from the c-kit, VEGF, and insulin promoters also bound to Zn-DIGP with K_d values of approximately 10^{-8} M, while the C-rich sequences c-Myc-C and Htelo-C bound to Zn-DIGP with about 1000-fold lower affinities (Figure 1 B, C).[16]

To determine the G-quadruplex specificity of Zn-DIGP relative to heterogeneous nucleic acids derived from cells, the fluorescence intensities of 10 nM solutions of Zn-DIGP were measured upon titration of a mixture of tRNA or calf thymus (CT) DNA.[19] Highly selective G-quadruplex binding was revealed by comparing these binding isotherms on a logarithmic scale of nucleotide concentration (Figure 1 C). This plot allows for direct comparisons between oligomeric and polymeric nucleic acids of differing lengths and reflects both the number of binding sites and the relative affinities. With the assumption that the size and frequency of binding sites (in nucleotides) are roughly similar,[19] these data indicate that Zn-DIGP binds to the c-Myc quadruplex with at least 100- and 5000-fold higher affinity than to tRNA and CT DNA, respectively (Figure 1 C).

The impressive G-quadruplex affinity and specificity of Zn-DIGP was confirmed by monitoring the fluorescence quenching of a 5′-fluorescein-labeled c-Myc DNA upon titration of Zn-DIGP in the presence or absence of a 1000-fold nucleotide excess of CT DNA (Figure 1 D). As a control, the known cationic porphyrin Zn-TMPyP4 (Scheme 1) was also evaluated. Consistent with the promiscuous binding of Zn-TMPyP4 to nucleic acids,[9b] a 10-fold loss in the apparent binding affinity of Zn-TMPyP4 was observed in the presence of competitor CT DNA (Figure 1 D), while a high apparent affinity ($K_d \leq 2$ nM) between Zn-DIGP and c-Myc was measured even in the presence of a 1000-fold excess of CT DNA (Figure 1 D).

Sensitive detection of Zn-DIGP can be accomplished using standard imaging systems compatible with Cy5 as revealed by both wide-field and confocal fluorescence microscopy experiments. As a result of its profluorescent proper-

ties, Zn-DIGP can simply be added, incubated, and imaged; no washing of the cells was required, even when media is used that is supplemented with 10% FCS (fetal calf serum). Internalization of Zn-DIGP was observed in all living and fixed cells evaluated so far, including HeLa, MCF7, B16F10, SH-SY5Y, *E. coli* BL-21, and SK-Mel-28.[13] Under all conditions and with all cell lines tested, little, if any nonspecific staining of duplex DNA was observed according to co-staining experiments with Hoechst 33342 (Figure 2A–C and Figure S7 in the Supporting Information).[13]

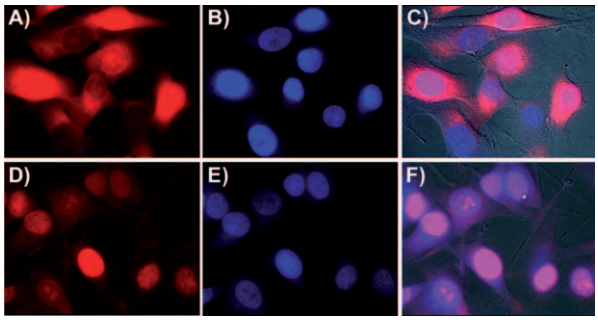

Figure 2. Fixed SK-Mel-28 cells stained with 3 μM Zn-DIGP (**7**) or DIGP (**4**) and 8 μM Hoechst 33342. A) Zn-DIGP fluorescence ($\lambda_{ex.}$ = 620 nm, $\lambda_{em.}$ = 700 nm). Similar staining patterns were also observed in cells lacking Hoechst 33342 (see Figure S7 in the Supporting Information).[13] B) Hoechst 33342 fluorescence ($\lambda_{ex.}$ = 360 nm, $\lambda_{em.}$ = 470 nm). C) Overlay of (A) and (B) with white-light absorbance. D) DIGP fluorescence. E) Hoechst 33342 fluorescence. F) Overlay of (D) and (E) with white-light absorbance. Uptake in living cells was also observed (see Figures S5 and S6 in the Supporting Information).[13] Color balance, brightness, and contrast have been optimized uniformly in each image.

Compared to Zn-DIGP, the metal-free guanidinio phthalocyanine DIGP (**4**) exhibited similar G-quadruplex affinity, lower specificity (Figure S4 in the Supporting Information),[13] and more intense nuclear staining (Figure 2D–F). Depending on the cell type, application method, and incubation conditions, both DIGP (**4**) and Zn-DIGP (**7**) exhibited variable cellular localization. In living cells, these compounds were mostly contained in trafficking vesicles and perinuclear organelles,[13] while in fixed and dying cells intense staining of the nuclei and nucleoli was observed (Figure 2D–F). Similar trends were reported for guanidine-containing transporter constructs.[20]

Previous studies reported that when a dose of TMPyP4 on the order of its EC_{50} for cytotoxicity was added to cell cultures (100 μM),[21] it suppressed c-Myc promoter activity by a factor of 2 or more.[22] Given the superior G-quadruplex affinity and specificity of Zn-DIGP, we added a much lower dose (1 μM) and used quantitative real-time polymerase chain reaction (qRT-PCR) to assess c-Myc promoter activity in a neuroblastoma cell line (SH-SY5Y) known to overexpress c-Myc.[23] Despite the lack of Zn-DIGP cytotoxicity at this dose (EC_{50} > 80 μM),[13] a time-dependent decrease in c-Myc expression up to threefold was observed (Figure 3).

In summary, Zn-DIGP is the first example of a high-affinity G-quadruplex ligand exhibiting both "turn-on" luminescence and the ability to knock-down RNA expression. With a $K_d \leq 2$ nM the interaction between Zn-DIGP and c-Myc G-quadruplex DNA is the strongest binding interaction between a G-quadruplex structure and a small molecule reported to date.[5] The fluorescence properties of Zn-DIGP facilitate direct binding assays in vitro and its imaging in vivo. The cellular localization of Zn-DIGP was markedly different than probes for duplex DNA, and at relatively low doses (1 μM) it caused a rapid threefold knock-down of c-Myc mRNA. The exact mechanism responsible for this knock-down is still under investigation, but our results are consistent with quadruplex-mediated promoter deactivation.[3,22] Direct fluorescence imaging of the putative Zn-DIGP/c-Myc promoter complex in cells is not feasible as c-Myc is present as either a single or low-copy number gene. The future application of Zn-DIGP or other dual-function G-quadruplex ligands to probe tandemly repeated genes with G-quadruplex-containing promoters is a highly attractive avenue to further evaluate the relationships between G-quadruplex structures and transcriptional control.

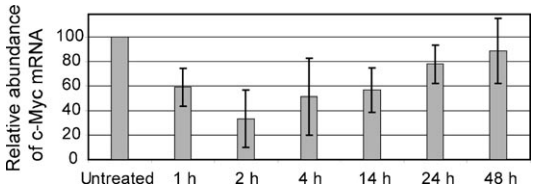

Figure 3. Relative abundance of c-Myc mRNA extracted from SH-SY5Y neuroblastoma cells treated with 1 μM Zn-DIGP for 1–48 h. qRT-PCR was used to quantify mRNA relative to the housekeeping genes β-actin, SDHA, and GAPDH. Two or more independent trials of three samples each were used to generate averages and standard deviations plotted above. Relative values among the housekeeping genes remained nearly constant.[13]

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[13] See the Supporting Information for details regarding the synthesis and characterization of **6**–**8**, as well as results for live-cell imaging, confocal microscopy, cytotoxicity, absorption and emission spectra of Zn-DIGP, titrations, qRT-PCR data, and procedures for all experiments.

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[15] As all available analytical data are consistent with both the C_4- and D_2-symmetric regioisomers, exact structures are not specified.

[16] Oligonucleotides used: $TGA(G)_3T(G)_4A(G)_3T(G)_4AA$ (c-Myc),$GTTA(GGGTTA)_4GG$ (Htelo), $GTTA(GAGTTA)_4GG$ (Htelo-Mut). Similar results were obtained for both the 22-mer c-Myc, and a longer wild-type c-Myc DNA ($(G)_4A(G)_3T(G)_4A(G)_3T(G)_4AA(G)_2T(G)_4$). "tRNA" was a mixture of mature and pre-tRNAs of type X-SA.

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