Bioorganic & Medicinal Chemistry 18 (2010) 1111–1116

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Cationic N-confused porphyrin derivative as a better molecule scaffold for G-quadruplex recognition

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ARTICLE INFO

Article history:
Received 13 November 2009
Revised 15 December 2009
Accepted 16 December 2009
Available online 24 December 2009

Keywords:
Cationic N-confused
Porphyrin
G-quadruplex

ABSTRACT

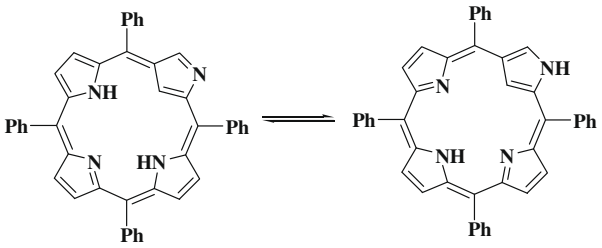
One N-confused porphyrin derivative was prepared and its first observation that it could stabilize G-quadruplex and possessed high selectivity over duplex DNA was made, furthermore, it was also proved to possess the capacity to induce a structural transition from the antiparallel to the mixed-type hybrid G-quadruplex structure.

1. Introduction

It has been reported by numerous groups that guanine-rich nucleic acid sequences are able to form G-quadruplex structures via Hoogsteen base pair.[1–3] A large number of putative sequences in many biologically pivotal genomic regions that might form quadruplex structures have been identified, such as the chromosomal telomeres,[4] the promoters of oncogenes,[5–9] and the immunoglobulin switch region.[10] Among these reports, small molecules targeting the G-quadruplex have aroused a significant amount of interest due to their potential biological and clinical applications in drug design and disease diagnosis.[11–29] Therefore, we have more opportunities to design specific molecules that specifically recognize and stabilize G-rich sequences.

The human telomeric quadruplex has been extensively investigated, and it has been reported that the formation of G-quadruplex structures will lead to the inhibition of telomerase activity and interfere with telomere extension.[30,31] Moreover, such structures, if stabilized, may lead to tumor cell death. Several convincing reports have indicated that quadruplex motifs are particularly enriched in the promoter regions of genes.[5–9] This suggests that G-quadruplex formation may be involved in the regulation of gene expression.[32–35] The promoter regions of genes such as c-myc and bcl-2 have an inherent conformational G-quadruplex structure mode. For instance, the c-myc promoter region has a parallel mode[7] while the bcl-2 promoter region has a mixed parallel/antiparallel mode.[8] Thus, the development of new G-quadruplex ligands, especially the sequence and mode selective ligands, is a promising approach for the development of cancer chemotherapeutics and for cancer diagnosis.

N-Confused porphyrin (NCP) has a nitrogen atom pointing outward and a carbon atom located in its internal core. In addition, NCP contains a confused pyrrole ring connected to the *meso*-carbons at the α and β positions.[36–38] The inner and outer nitrogen atoms can undergo NH-tautomerism generating two types of tautomers (Scheme 1).[38] Because NCP has an additional external functional site for protonation or coordination, this molecule garners particular interest to a variety of researchers. However, few reports have been published in regard to the medicinal or biological applications of NCP.[39]



Scheme 1. NH-tautomerism of NCP.

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doi:10.1016/j.bmc.2009.12.049

Herein, we report the synthesis of a water soluble cationic NCP derivative and investigate its interaction with a G-quadruplex structure. The basis of our design preserves the planar macrocyclic ring skeleton to enable interactions with G-quartets. The cationic side chains of this molecule are introduced to enhance the water solubility and binding ability of NCP via grooves of DNA in addition to the negatively charged sugar–phosphate backbone. For the first time, we have identified an NCP derivative that has sufficient sequence and mode selective binding to G-quadruplex structures as evaluated by CD spectroscopy, a Taq polymerase stop assay, SPR experiments, CD melting curves, and FRET melting curves coupled with competitor and qualitative molecular modeling (Scheme 2).

2. Results and discussion

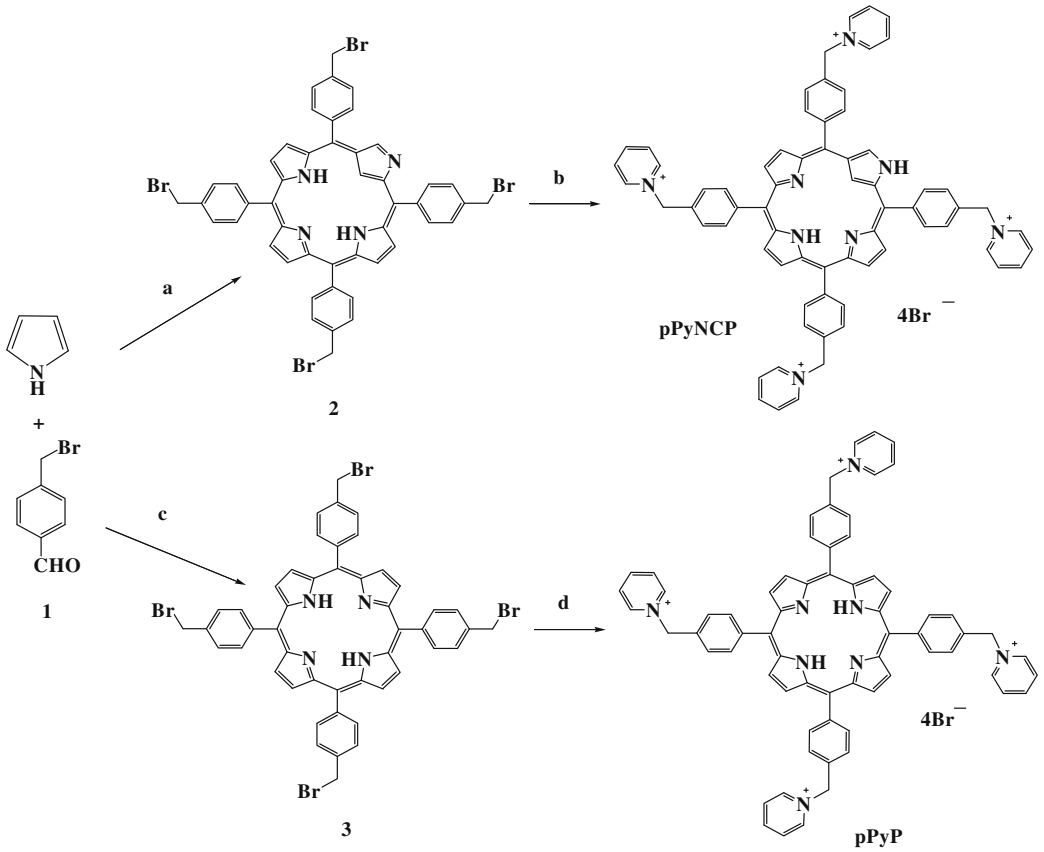
2.1. Stabilization of ligands to G-quadruplex

The ligand's ability to stabilize a G-quadruplex structure was investigated by the analysis of CD melting curves using the G4C oligomer (sequence 5′-CATGGTGGTTTGGGTTAGGGTTAGGGTTAGG GTTACCAC-3′). The results indicated that pPyNCP was able to induce the stabilization of the G-quadruplex in a physiologically relevant K^+ buffer. The ΔT_m values increased sharply with increasing concentrations of pPyNCP, approximately reaching its maxima at $r = 5$ (r = [pPyNCP]/[G4C], Table 1, Figs. S1–S3 of Supplementary data). The binding affinity of pPyNCP is similar to the well-investigated G-quadruplex stabilizer TMPyP4[40–42] and has stronger affinity than the pPyP (5,10,15,20-tetrakis(α-pyridinio-*p*-tolyl)porphyrin tetrabromide salt), which possesses the same side chains as pPyNCP.

The binding ability was further elucidated using the Taq DNA polymerase stop assay to determine the NCP-induced stability of the quadruplex based on the concentration of the template strand at low K^+ concentration.[43] The primer extension assay using Taq polymerase stopped at the location of the DNA substrate that corresponded to the G-quadruplex structure. These results indicated that the pPyNCP led to the significant inhibition observed for Taq polymerase at the G-quadruplex site (Fig. S4, see Supplementary data). Also, with increasing concentrations of the ligand, there was enhanced pausing at the G-quadruplex site on the DNA template. This suggests that pPyNCP could bind to and stabilize the G-quadruplex.

2.2. Detection of the conformation of telomere sequence induced by ligands

Circular dichroism (CD) spectroscopy was used to confirm the above results and to investigate the conformation of the quadruplex induced by the ligand. Without the addition of any metal cations, the human telomeric sequence $(T_2AG_3)_4$ generated a positive peak near 256 nm. Interestingly, the human telomere sequence is highly polymorphic and can exist as parallel,[44] antiparallel,[45] and mixed-type parallel/antiparallel structures.[46] With the addition of increasing concentrations of pPyNCP, there was a sharp decrease in the 257 nm band accompanied by the detection of a small band with positive ellipticity near 270 nm and a dramatic increase in the band ellipticity observed around 295 nm (Fig. 1a). Thus the resultant CD spectroscopic profile indicated that pPyNCP could induce the formation of hybrid parallel/antiparallel G-quadruplex structures.[23,47] In a control experiment, pPyP was only prone to induce the formation of the G-quadruplex, but the conformational change



Scheme 2. Synthesis of pPyNCP. Reagents and conditions: (a) methanesulfonic acid (MSA), 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ), CH_2Cl_2, 40 °C, 30 min; (b) pyridine, 40 °C, 7 days; (c) $BF_3{\cdot}Et_2O$, chloranil, $CHCl_3$, 2 h; (d) pyridine, 110 °C, 1.5 h.

Table 1
CD melting data of 10 μM oligonucleotide (G4C) at different concentrations of pPyNCP and TMPyP4 in 10 mM Tris–HCl buffer (1 mM EDTA, pH 7.4), 100 mM KCl

Samples		Control (°C)	r = [pPyNCP]/[G4C]				
			1	2	3	4	5
pPyNCP	T_m	56.6 ± 0.8	60.4 ± 0.3	63.2 ± 0.6	64.9 ± 0.4	65.8 ± 1.0	65.9 ± 1.7
	ΔT_m		3.8 ± 0.4	6.6 ± 0.2	8.3 ± 0.3	9.2 ± 0.1	9.3 ± 0.6
TMPyP4	T_m		60.8 ± 1.9	64.1 ± 1.3	66.2 ± 0.9	67.8 ± 0.6	66.6 ± 1.1
	ΔT_m		4.2 ± 0.8	7.5 ± 0.3	9.6 ± 0.0	11.2 ± 0.2	10.0 ± 0.2
pPyP	T_m		58.8 ± 0.7	59.9 ± 1.1	61.6 ± 1.2	63.1 ± 1.3	64.2 ± 0.9
	ΔT_m		2.2 ± 0.1	3.3 ± 0.2	5.0 ± 0.2	6.5 ± 0.3	7.6 ± 0.0

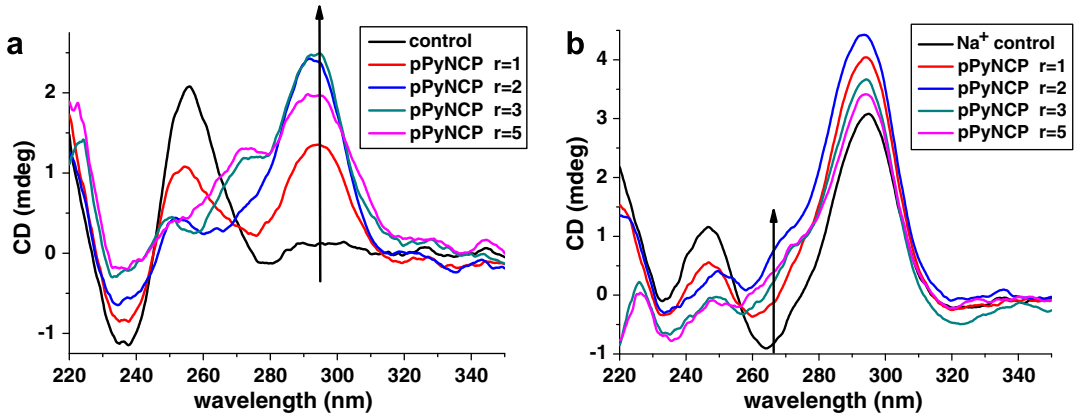


Figure 1. (a) CD titration of $(T_2AG_3)_4$ in 10 mM Tris–HCl 1 mM EDTA, pH 7.4; (b) CD titration of $(T_2AG_3)_4$ in 10 mM Tris–HCl, 1 mM EDTA, pH 7.4, 100 mM NaCl. Arrows from bottom to up indicated increments in r values from 0 to 5 (r = [pPyNCP]/[$(T_2AG_3)_4$]).

was not characteristic of an antiparallel or a hybrid of a parallel/antiparallel G-quadruplex structure (Fig. S5).

The results shown in Figure 1b indicated that pPyNCP was prone to inducing the structural transition from the antiparallel structure formed by Na^+ to the mixed-type hybrid G-quadruplex, as indicated by the increase in the CD signal at 265 nm from a negative peak to a small positive band. To our knowledge, only a few ligands are known to induce the structural transition of the human telomere sequence.[21,22,48,49] As for pPyP, the DNA sequence cannot change to the characteristic band known for a hybrid of a parallel/antiparallel G-quadruplex structure (Fig. S6). In the presence of a K^+ buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.4, and 100 mM KCl), the telomeric sequence $(T_2AG_3)_4$ formed a hybrid of parallel/antiparallel G-quadruplex structures in the absence of any compound.[47] It was easy to identify, when the addition of pPyNCP, the hybrid G-quadruplex structure still retained (Fig. S7, see Supplementary data). Therefore, it was reasonable to conclude that pPyNCP possessed the ability to induce and maintain the hybrid G-quadruplex structure formed from a telomeric sequence.

2.3. The binding selectivity between ligands and different kinds of G-quadruplex

We further investigated the binding abilities between G-quadruplex DNA and pPyNCP using surface plasmon resonance (SPR).[8,17,25,50,51] The human telomeric G-quadruplex sequence, the well-characterized c-myc and bcl2 promoter G-quadruplex sequences, and a duplex DNA hybridized with its complementary strand, which served as a control, had been chosen to evaluate whether pPyNCP could distinguish among various modes of quadruplex formation and between dsDNA. The SPR results showed that pPyNCP possessed high binding ability, significant selectivity for G-quadruplex DNA over dsDNA, and 108-fold selectivity for bcl2 over dsDNA (see Table 2 and Fig. S8 of Supplementary data). These results were also consistent with the preference of pPyNCP for the mixed antiparallel/parallel quadruplex mode as determined from the CD analysis, since the bcl2 promoter sequence had the same structural conformation. When compared with TMPyP4 and pPyP, pPyNCP showed a greater selectivity and preference for the hybrid of a parallel/antiparallel G-quadruplex structure (Figs. S9 and S10 of Supplementary data).

To further confirm the binding selectivity observed using the SPR analysis, the interaction of pPyNCP with G-quadruplex DNA in the presence of various concentrations of competitor dsDNA was studied using a competitive FRET melting assay.[52,53] The results clearly showed that a high level of stabilization of the G-quadruplex by pPyNCP was maintained even with a large excess of competitor ds22 (10 μM). This indicated that pPyNCP possessed high selectivity for G-quadruplex DNA versus dsDNA (Fig. 2).

To understand the strong interaction between NCP and G-quadruplex, a close examination of the interaction between pPyNCP and G-quadruplex DNA was studied by qualitative molecular modeling using molecular dynamics and energy minimization methods. The results clearly illuminated sufficient overlap between the aromatic macrocycle scaffold and the guanine quartets. This binding mode is characterized by favorable π–π stacking interactions between pPyNCP and the G-quadruplex structure (Fig. 3). The four cationic side chains were properly oriented and directed into the DNA groove toward the negatively charged sugar–phosphate backbone.

3. Conclusion

To our knowledge, these data demonstrate for the first time that NCP can serve as G-quadruplex structure stabilizer. A variety of experimental results demonstrated that pPyNCP could induce and stabilize the formation of G-quadruplex DNA. For example, SPR sensorgrams showed the high affinity and significant selectivity of pPyNCP for G-quadruplex DNA over dsDNA, and CD titration experiment indicated that it possess the capacity to induce a

Table 2
Kinetic rate constants and the chi2 (χ^2) for DNA–drug interactions using kinetics simultaneous algorithm by the model of binding with mass transfer

Compd	DNA	k_a[a] (M^{-1} s^{-1})	k_d[a] (s^{-1})	K_A[b] (M^{-1})	K_A(G4)/K_A(ds)	χ^2[c]
pPyNCP	bcl2	1.43×10^5	1.01×10^{-2}	1.42×10^7	108	1.6
	c-myc	8.79×10^4	1.17×10^{-2}	7.54×10^6	57	2.1
	htelo	2.45×10^4	4.6×10^{-3}	5.32×10^6	40	1.1
	dsDNA	4.21×10^2	3.2×10^{-3}	1.32×10^5	1	1.3
TMPyP4	bcl2	1.42×10^6	4.6×10^{-2}	3.09×10^7	9	1.4
	c-myc	7.86×10^5	7.78×10^{-3}	1.01×10^8	28	1.5
	htelo	1.33×10^5	2.87×10^{-3}	4.65×10^7	13	0.8
	dsDNA	3.64×10^3	1.02×10^{-3}	3.62×10^6	1	1.8
pPyP	bcl2	1.86×10^4	1.07×10^{-3}	1.74×10^7	25	0.9
	c-myc	2.64×10^5	4.54×10^{-3}	5.82×10^7	85	1.4
	htelo	3.53×10^4	1.49×10^{-3}	2.37×10^7	34	1.7
	dsDNA	1.7×10^3	2.48×10^{-3}	6.85×10^5	1	1.2

[a] Kinetic constants (k_a and k_d are the corresponding association and dissociation rate constants) determined from BIAcore analysis by using 1:1 binding with mass-transfer fitting of 240 s association and 240 s disassociation.
[b] K_A determined from k_a/k_d, K_D determined from k_d/k_a.
[c] The chi2 (χ^2) value is a standard statistical measure of the closeness of fit. χ^2 is of the same order of magnitude as the noise in RU. And the fitting process is terminated automatically when a minimum value is found for χ^2.

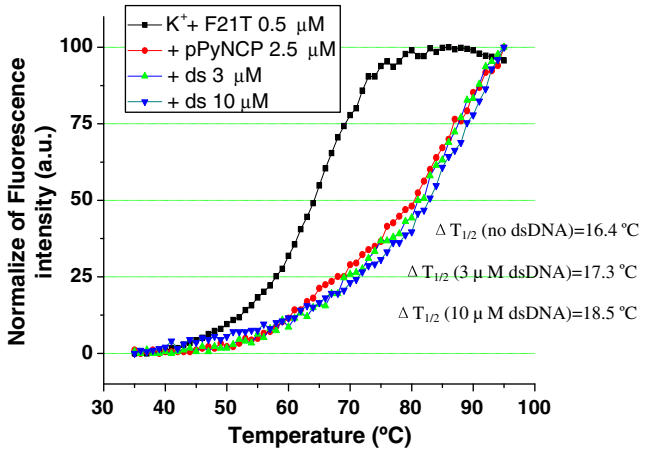


Figure 2. FRET melting curves of 0.5 μM labeled oligonucleotide F21T with 2.5 μM pPyNCP in the absence or presence of competitor ds26 in 10 mM lithium cacodylate buffer, 100 mM KCl.

structural transition from the antiparallel to the mixed-type hybrid G-quadruplex structure, which could implicate its recognition to different G-quadruplex. In addition, when compared with pPyP, which possessed the same substitution patterns, the N-confused porphyrin derivative could enhance the binding potential and selectivity relative to the normal porphyrin derivative. These studies suggested that the NCP derivative was a better class of quadruplex binding ligands. Further investigation of its mechanism, biological activities and structural relationship is under going.

4. Experimental section

4.1. Synthesis of compound 1, 2, pPyNCP and pPyp

4.1.1. Synthesis of compound 1 (4-(bromomethyl)benzaldehyde)[54]

A solution of *p*-xylylene dibromide (4 g, 15 mmol) and activated manganese dioxide (6 g, 69 mmol) in chloroform (150 mL) was refluxed for 3 days. The reaction mixture was filtered and washed with chloroform. The combined filtrates were evaporated and isolated by silica gel with petroleum ether–dichloromethane (3:1) as eluent. Compound was obtained as white solid in 21% yield (627 mg). ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.46 (s, 2H), 7.50 (d, 2H, J = 7.8 Hz), 7.81 (d, 2H, J = 7.8 Hz), 9.95 (s, 1H).

4.1.2. Synthesis of compound 2 (5,10,15,20-tetrakis(4′-bromomethylphenyl)-2-aza-21-carbaporphyrin)[39]

Pyrrole (138 μL, 2 mmol) and **1** (398 mg, 2 mmol) were dissolved in 200 mL of CH_2Cl_2 and the reaction was initiated by adding methanesulfonic acid (MSA) (90 μL, 1.4 mmol). The reaction mixture was stirred at room temperature for 30 min. 2,3-Dichloro-5,6-dicyano-*p*-benzoquinone (DDQ) (400 mg, 1.76 mmol) was added and the mixture was allowed to stir for 1 min and then the reaction was quenched by adding 360 μL triethylamine. The solvent was evaporated and purified by silica gel column chromatography with CH_2Cl_2–CH_3OH (v/v = 100: 3) as eluents and precipitated from CH_2Cl_2–CH_3OH. Compound **2** was obtained as dark green solid, which was subjected for subsequent reaction as soon as possible after precipitation without further drying because **2** was unstable under condensed conditions. ^{1}H NMR (300 MHz,

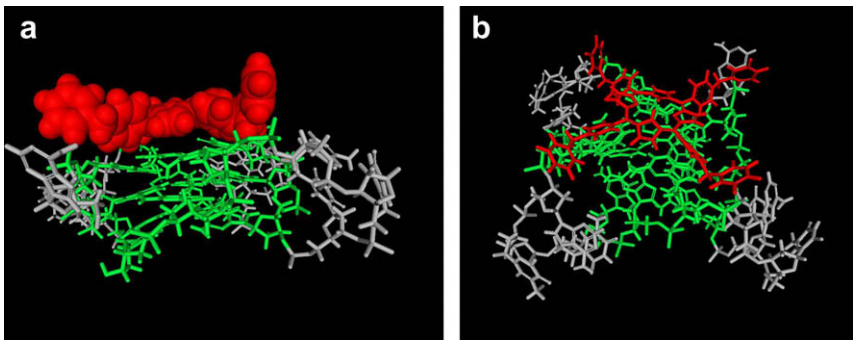


Figure 3. Minimized model of a complex between pPyNCP and the human parallel d($AG_3[T_2AG_3]_3$) quadruplex. pPyNCP is colored in red, the bases G are colored in green, and the other bases are colored in gray. (a) The side view of complex; (b) the top view of complex.

$CDCl_3$): δ −5.04 (s, 1H), −2.56 (br, 2H), 4.74 (s, 2H), 4.77 (s, 6H), 7.72 (d, 4H, J = 6.9 Hz), 7.82 (m, 4H), 8.07 (m, 4H), 8.23–8.31 (m, 4H), 8.48–8.54 (m, 4H), 8.65 (s, 1H), 8.88 (d, 1H, J = 3.6 Hz), 8.93 (d, 1H, J = 5.7 Hz). ESI-MS: m/z: calculated for: M+H = 987.4, found: 986.9.

4.1.3. Synthesis of compound pPyNCP (5,10,15,20-tetrakis(α-pyridinio-*p*-tolyl)-2-aza-21-carbaporphyrin tetrabromide salt)[39]

Compound **2** was prepared from 2 mmol pyrrole and 2 mmol aldehyde and was used directly after precipitation. Compound **2** was dissolved in 150 mL of pyridine. The mixture was vigorously stirred for 7 days at 40 °C. The solution was filtrated. A dark green solid of pPyNCP was obtained as a hygroscopic solid in 5.1% yield from 1 (36 mg, 25 mmol as $7H_2O$ adduct). ^{1}H NMR(300 MHz, DMSO-d_6): δ −3.01 (s, 2H), 2.06 (s, 1H), 6.18 (s, 8H), 7.50 (s, 2H), 7.81 (m, 6H), 7.91 (m, 9H), 7.99 (m, 5H), 8.30 (s, 9H), 8.71 (s, 4H), 9.41 (s, 8H), 12.94 (s, 1H); UV–vis (CH_3OH) λ_{max}/nm (log ε) 411 (4.53), 542 (3.39), 591 (3.42), 725 (3.35); HRMS, m/z: calculated for: (M−4Br−)/4 = 245.6112, found: 245.6106. Elemental analysis calcd for $C_{68}H_{54}N_8Br_4{\cdot}7H_2O$: C, 57.16; H, 4.80; N, 7.84. Found: C, 57.10; H, 4.73; N, 8.06%.

Compound of 5,10,15,20-tetrakis(4′-bromomethylphenyl)porphyrin was synthesized according to the Ref. [55]

4.1.4. Synthesis of compound pPyP (5,10,15,20-tetrakis(α-pyridinio-*p*-tolyl)porphyrin tetrabromide salt)

Compound 5,10,15,20-tetrakis(4′-bromomethylphenyl)porphyrin (98 mg, 0.1 mmol) was dissolved in 20 mL of pyridine. The mixture was refluxed with vigorous stirring for 1.5 h. After cooling to room temperature, the solution was filtrated and precipitated from CH_3OH–Et_2O. Compound pPyP was obtained as purple solid in 91% yield (118 mg). ^{1}H NMR(600 MHz, DMSO-d_6): δ 0.42 (s, 1H), 6.27 (s, 8H), 7.97 (d, 8H, J = 7.2 Hz), 8.28 (d, 8H, J = 7.8 Hz), 8.36 (d, 8H, J = 6.6 Hz), 8.78 (m, 12H), 9.53 (d, 8H, J = 6 Hz); ESI-MS, m/z: calculated for: (M−4Br−)/4 = 245.8, found: 245.9.

4.2. Biology

4.2.1. Materials

All oligonucleotides/primers except fluorescent oligonucleotides P18 and F21T were synthesized and purified by Invitrogen Technology (Shanghai, China). P18 and F21T were purchased from TaKaRa, Dalian, China. Taq DNA polymerase was purchased from Toyobo (China). The oligonucleotides Telo24 (5′-TTAGGGTTAGGGTTAGGGTTAGGG-3′) was used in CD spectroscopy, the sequence HT4 (5′-TCCAA CTATG TATAC TTAGGG TTAGGG TTAGGG TTAGGG ACATA TCGAT GAAAT TGCTA TAGTG AGTCG TATTA-3′) and 5′Tamra labeled P18 (5′-TAATA CGACT CACTATAG-3′) were used for DNA polymerase stop assay, the sequence G4C (5′-CATGG TGGTTTGGGTTAGGGTTAGGGTTAGGGTTACCAC-3′) was used in the CD melting experiments, F21T (FAM-G3[TTAG3]3-Tamra, FAM: 6-carboxyfluorescein, Tamra: 6-carboxytetramehtylrhodamine) and ds22 (the self-complementary 5′-GGCATAGTGCGTGGGCGTTAGC-3′) was used in FRET melting assay. The human telomeric DNA (5′-biotin-AGGGTTAGGG TTAGGGTTAGGG-3′), the two promoter DNA c-myc (5′-biotin-GAGGGTGGGGAGGGTGGGG-3′) and bcl2 d(5′-biotin-GGGCGCGGGAGGAATTGGGCGGG-3′), and the duplex DNA d(5′-biotin-GGCATAGTGCGTGGGCGTTAGC-3′) hybridized with its complementary were used in SPR.

4.3. CD experiments

Experimental conditions were set up according to our previous reported procedures.[23,51] In the absence or presence of 100 mM KCl or NaCl, we observed changes of the oligomer Telo24 $d(T_2AG_3)_4$. Various amounts of a stock solution of the pPyNCP were subsequently added to increase the concentration ratio of pPyNCP/DNA strand. All the CD spectra were baseline corrected for signal contributions from the buffer. In CD melting experiment, the samples contain 10 mM Tris–HCl buffer (1 mM EDTA, pH 7.4), 100 mM KCl, 20 μM G4C and different concentrations of pPyNCP, pPyP or TMPyP4. Before the melting experiment, the samples were boiled at 95 °C for 5 min, and then cooled down to room temperature. The CD melting curves were obtained by monitoring absorbance at 290 nm, while the temperature was ramped from 25 °C to 95 °C at about 1.5 °C/min.

4.4. Taq DNA polymerase stop assay

General procedures were described by our and other group.[43,56] Then various concentrations of compounds pPyNCP, dNTPs (final concentration 200 μM) and Taq DNA polymerase (1.5 U/reaction, Bioready) were added, the mixture was incubated at 55 °C for 30 min. Finally, the products were separated on a 20% denaturing polyacrylamide gel for electrophoresis and autoradiographed on a Typhoon phosphor imager (Amersham Biosciences, AB, Uppsala, Sweden).

4.5. Surface plasmon resonance

The measurements were finished by using BIAcore 3000 optical biosensor system (BIAcore AB, Uppsala, Sweden) using a CM5 sensor chip covered streptavidin by an Amine Coupling Kit. Experimental procedures were described in the reported papers.[25,57]

4.6. FRET melting assay[53]

Fluorescence melting curves were determined with a real-time PCR machine (Rotor-Gene 2000, Corbett, Australia) using F21T, which was performed as a high-throughput screen in a 36-well format. The total reaction volume of samples was 20 μL, and it contained 0.5 μM of labeled oligonucleotide F21T in a buffer of 10 mM Cacodylate Lithium, pH 7.4 and 100 mM KCl. The fluorescence DNA solution without compounds were boiled at 95 °C for 5 min first and then cooling down to room temperature to form G-quadruplex structures completely. Then adding compounds and variable concentrations of double-stranded competitor ds22 into different samples and lay them at 4 °C for more than 6 h. The temperature procedure in real-time PCR was as following: 35 °C for 5 min, and then a stepwise increase of 1 °C every minute from 35 °C to reach 95 °C was performed and measurements of FAM were made after each stepwise.

4.7. Molecular modeling

The structure of porphyrin was built manually and energy minimized by GAUSSION03[58] using DFT method at 6-31G level. The coordinates of the parallel G-quadruplex were obtained from the Brookhaven Protein Data Bank (PDB ID: 1KF1). Discovery STUDIO 2.1 package was used for molecular dynamics calculation. Porphyrin was dock manually to the G-quadruplex and the complex was allowed to minimized using CHARMm force field. First 1000 steps steepest descent followed by 1000 steps conjugate gradient, then the system was healing from 50 k to 300 k in 2000 steps, equilibrate 2000 steps, and product 5000 steps. Then the final conformation was minimized by 500 steps steepest descent and 500 steps conjugate gradient.

Acknowledgments

This work was partially supported by the National Science of Foundation of China (Nos. 20672084, 90813031, 20621502,

30973605), National Key Foundation for Infectious Diseases (Protection and treatment of AIDs, virus hepatitis, 2008ZX10003-005). We thank Professor Guangfu Yang, Key Laboratory of Pesticide & Chemical Biology of Ministry of Education, College of Chemistry, Central China Normal University, Hubei, Wuhan 430079, PR China and he allows us use his INSIGHT II 2005 software package for modelling calculation.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.12.049.

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