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Bifunctional Zn(II) Complexes for Recognition of Non-Canonical Thymines in DNA Bulges and G-Quadruplexes

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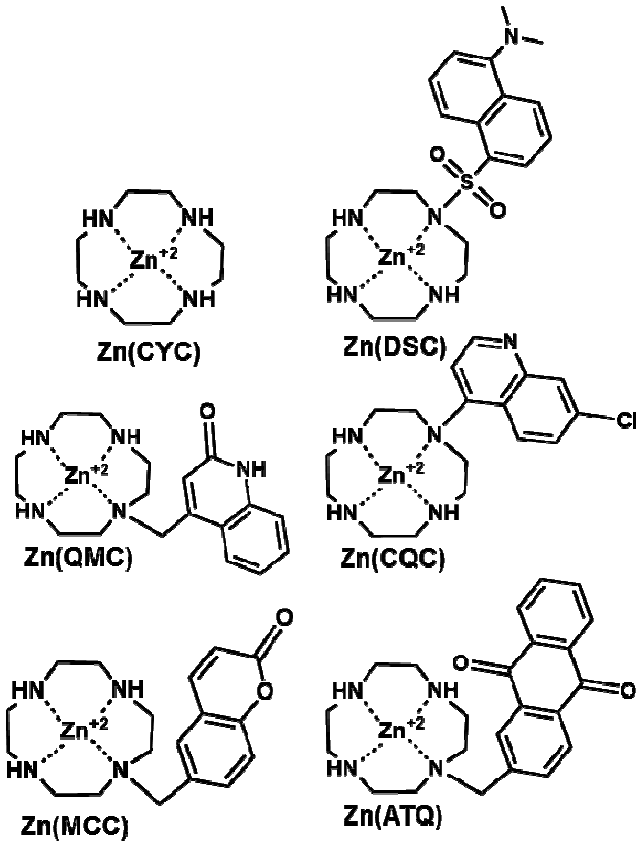
ABSTRACT

Six Zn(II) complexes of derivatives of 1,4,7,10-tetraazacyclododecane (cyclen) were studied for binding to DNA sequences containing non-canonical thymines, including a hairpin with a single thymine bulge (T-bulge) and a G-quadruplex (H-telo) containing thymine loops. The cyclen-based macrocycles contained pendents with either two fused rings to give planar groups including quinolinone (QMC), coumarin (MCC) and quinoline (CQC) derivatives or a non-planar dansyl group (DSC). Macrocyclic complexes with three fused rings including an anthraquinone pendent (ATQ) were also studied. All Zn(II) complexes were stable in solution for micromolar concentrations and neutral pH with the $Zn(L)(OH_2)$ species prevailing for L = QMC and CQC at pH 7.5 and 100 mM NaCl. Immobilized T-bulge or H-telo G-quadruplex was used to study binding of the complexes by surface plasmon resonance (SPR) for several of the complexes. For the most part, data matched well with that obtained by isothermal calorimetry (ITC) and, for fluorescent complexes, by fluorescence titrations. Data showed that Zn(II) complexes containing planar aromatic pendents with two fused rings bound to T-bulge more tightly than complexes with non-planar pendents such as dansyl. The H-telo DNA exhibited multiple binding sites for all complexes containing aromatic pendents. The complexes with two fused rings bound with low micromolar dissociation constants and two binding sites whereas a complex with three fused rings (ATQ) bound to three sites. This study shows that different pendent groups on Zn(II) cyclen complexes impart selectivity for recognition of non-canonical DNA structures.

INTRODUCTION

Nucleobases in non-canonical DNA structures often contain exposed Watson-Crick faces that may serve as important sites for small molecule binding and recognition.[1-5] For example, nucleobases that are part of DNA bulges, mismatches or abasic sites are common targets for the design of small molecule binders including both organic and inorganic compounds.[6-8] Organic heterocycles that act as recognition agents typically form hydrogen bonds to the exposed Watson-Crick face of the nucleobase and may also have groups that interact through aromatic stacking to increase the magnitude of binding.[9-11] Such heterocyclic compounds have been successfully used for the recognition of pyrimidines in non-canonical DNA or in analogous RNA structures. An alternative strategy that has been widely used for the recognition of bulges, abasic sites and mismatches is the use of coordination complexes.[6, 12-15] The metal ion centers in these complexes typically have a saturated coordination sphere and the complexes are kinetically inert towards ligand loss.[16-19] Aromatic heterocyclic ligands in these complexes facilitate interactions that involve stacking on nucleobases and shape recognition of the nucleic acid structure.[20, 21] A better understanding of these interactions as well as the development of alternative ways to recognize unusual nucleic acid structures may lead to improved design of small molecules that may one day be useful for therapeutic applications.

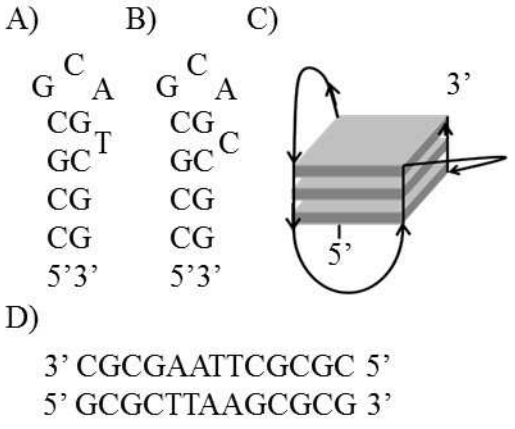


Scheme 1: Zn(II) macrocyclic complexes

An unusual DNA structure which is a potentially interesting therapeutic target is the G-quadruplex.[22-27] G-quadruplexes are four-stranded guanine-rich structures that are found in promoter regions of DNA or at the telomeric ends of DNA.[24, 28-30] Recent studies suggest that G-quadruplexes may be important in m-RNA as well.[31, 32] These structures contain four guanines held together by Hoogsteen hydrogen-bonds that form planar G-tetrads.[33] Many of the biologically important G-quadruplexes in DNA, such as human telomeric DNA (H-telo) contain thymine-rich sequences in their loop structures.[34, 35] Interestingly, these loops are generally not the targeted site for small molecule recognition

despite their variability in different G-quadruplex structures.[36] Molecules that are designed to bind to G-quadruplexes typically stack on the capping G-tetrad.[37-39] Many of these ligands consist of large organic heterocycles with attached cationic side chains.[40-42] Transition metals have been incorporated into G-quadruplex recognition agents, but only serve to orient the heterocycles in favorable geometries for stacking on the G-tetrad.[43-47]

A research topic which is under investigation in our laboratory is the design of Zn(II) macrocyclic complexes for recognition of non-canonical thymine or uracil nucleobases.[6, 13, 14, 48] These complexes are bifunctional in that both the metal ion center and an aromatic pendent group are important in binding to the nucleobase. The Zn(II) center binds to the deprotonated thymine or uracil N3 site ($N3^-$), and the pendent aromatic group may stack on top of the pyrimidine.[49, 50] Alternatively, the pendent may interact with neighboring nucleotides of DNA or RNA within complex, folded structures.[14] Recent work in our laboratory showed that certain Zn(II) complexes bind tightly to single-thymine bulges with a subtle dependence on the bases flanking the bulge.[13] The strength of the Zn(II) complex interaction with the bulge depended on the linker to the aromatic group. Flexible methylene linkers facilitated binding to the bulge whereas less flexible linkers such as the sulfone group in dansyl-cyclen (DSC) did not. The flexibility of the methylene linker was suggested to be necessary for promoting stacking of the aromatic pendent on top of the bulged thymine. Whereas the thymine bulge was shown to be extrahelical in the unbound DNA, the bulged thymine interacted with groups in the major groove when bound to the Zn(II) complex. This docked thymine bulge appeared to form a binding pocket for the Zn(II) complex that may contribute to selective binding. We also recently reported that the Zn(II) complex of DSC (Zn(DSC)) binds tightly to the human telomeric G-quadruplex (H-telo).[48] H-telo has three loops containing the TTA sequence. The suggested binding mode involved binding of the Zn(II) center to the N3 of thymine with binding of at least two Zn(II) complexes to thymine in different loops. The dansyl pendent most likely interacts with a thymine in the loop of H-telo. Structural data for



Scheme 2: Oligonucleotides studied:
A) Thymine Bulge (5'CCGCGCAGTCGG 3')
B) Cytosine bulge (5'CCGCGCAGCCGG 3')
C) Telomeric G-Quadruplex (5'$A(G_3T_2A)_3G_3$ 3')
D) Dickerson Dodecamer (5' $CGCGA_2T_2CGCG$ 3')

the H-telo G-quadruplex suggests that the thymines in the loops are splayed outwards and are relatively accessible for binding to the Zn(DSC) complex.[35] The environment of the non-canonical thymines in the G- quadruplex thus appears to differ from that in thymine bulges.[13]

In this study, we compare a series of Zn(II) complexes (Scheme 1) with different aromatic pendent groups and associated linkers as recognition agents for two DNA sequences that have thymines in very different non-canonical structures (Scheme 2). One is a DNA sequence containing a thymine bulge and the second is a G-quadruplex with thymine containing loops. Our goal was to determine whether variation of the pendent group tunes the properties of the Zn(II) complexes for recognition of thymine in the different structural contexts. Biosensor-surface plasmon resonance was used to screen binding of several Zn(II) complexes to immobilized DNA. The most promising Zn(II) complexes were also studied for binding to DNA by using isothermal calorimetry. These complexes are of interest for their distinct binding mode that may be useful in the development of therapeutic agents that act by modulating the function of nucleic acids.

EXPERIMENTAL METHODS

The macrocycles DSC and CQC and complexes $[Zn(DSC)]Cl_2$ and $[Zn(CQC)]Cl_2$ were prepared as previously reported.[13, 51] DNA oligonucleotides, including 5-biotinylated DNA, were purchased from Integrated DNA Technologies (IDT) in desalted form. ^{1}H NMR spectra were taken on an Inova 400 and 500 MHz NMR spectrometers; ^{13}C NMR spectra were recorded on a Gemini 300 MHz NMR spectrometer. Surface plasmon resonance (SPR) measurements were done on a Reichert SR7500DC SPR instrument and data was analyzed using Scrubber 2.0a (Biologic Software Pty, Australia). Fluorescent studies were performed on a Cary Eclipse Varian Fluorometer with a Varian Temperature Regulator. Isothermal calorimetry data was collected on a MicroCal VP-ITC and data was analyzed using Origin software. Optical thermal melting data was collected using a Beckman Coulter DU 800 spectrometer with a Beckman Coulter High Performance Temperature Controller. Further details on experimental methods (surface plasmon resonance, isothermal calorimetry, pH potentiometric titrations, optical thermal melting) are given in the supplementary section.

Synthesis

6-(bromomethyl)-coumarin (6MC-Br). A modified literature procedure[52-54] was followed. of 6-methyl coumarin (53.3 mmol) was dissolved in 120 ml of CCl_4 and N-Bromosuccinimide (NBS, 59.2 mmol) and azobisisobutyronitrile (AIBN, 9.2 mmol) were added to the mixture. The mixture was allowed to reflux under Ar at 90 °C for 28 hours and was monitored by using TLC (eluent: 20:80 ethyl

acetate: hexane). Upon cooling of the reaction mixture, a precipitate formed. The precipitate was collected by using a glass frit filter and then redissolved in $CHCl_3$. The chloroform solution was washed with H_2O, and then dried. Evaporation of the chloroform yielded a light yellow powder. Yield: 28%. ^1H NMR (500 MHz, $CDCl_3$, 25 °C, δ): 7.7 (d, 1H J=9.5 Hz, Ar-H), 7.5 (d, 1H J=9.5, Ar-H), 7.5 (s, 1H, Ar-H), 7.4 (d, 1H J=8.0 Hz, Ar-H), 6.5 (d, 1H, Ar-H), 4.6 (s, 2H Ar-CH_2-Br). ^{13}C NMR (75 MHz, $CDCl_3$): 160.4, 153.7, 143.0, 134.3, 132.6, 128.2, 118.9, 117.3 (C-Ar), 32.0 (Ar-CH_2-Br).

6-((1,4,7,10-tetraazacyclododecan-1-yl)methyl)-Coumarin (MCC). 6-(bromomethyl)-coumarin (0.658 mmol) was dissolved in chloroform and added slowly to 1,4,7,10-tetraazacyclododecane (cyclen) (4.12 mmol) in chloroform under N_2 at 0 ^OC. The reaction mixture was then allowed to stir at 0 ^OC for 30 minutes. The crude reaction mixture was washed first with H_2O, then with 1N NaOH, followed by H_2O wash. The organic layer was dried over anhydrous Na_2SO_4 and solvent was removed under vacuum to give a white powder. Yield: 95% ESI-MS (MH^+): 329.2, 331.2.^1H NMR (500 MHz $CDCl_3$, 25 °C, δ) 7.7 (d, 1H J=9.0 Hz, Ar-H), 7.6 (d, 1H J=8.5 Hz, Ar-H), 7.5 (s, 1H, Ar-H), 7.3 (d, 1H J=8.0 Hz, Ar-H), 6.5 (d, 1H J=9.0 Hz, Ar-H), 3.7 (s, 2H, Ar-CH_2-N), 2.8 (t, 4 H J=5.0 Hz, NCH_2), 2.7 (t, 4 H J=5.0 Hz, NCH_2), 2.6 (m, 8 H J=6.0 Hz, NCH_2) ^{13}C NMR (75 MHz, $CDCl_3$): 160.8, 153.3, 143.5, 135.5, 132.5, 130.1, 128.0, 118.7, 117.0, 116.7 (C-Ar), 59.1 (Ar-CH_2-N), 51.4, 47.8, 46.0, 45.9 (CH_2N).

[Zn(MCC)]Cl_2. The free-base macrocycle was dissolved in a minimal amount of ethanol at room temperature, then 1.05 molar equivalents of ethanolic zinc(II) chloride was added. The reaction was allowed to stir overnight at room temperature. The resulting precipitate was collected by vacuum filtration and washed with several portions of cold ethanol. The resulting fine dark yellow powder was vacuum dried. Yield: 49% ESI-MS ($[M-Cl]^+$): 429.2, 431.2, 433.2; (MNa^+): 487.2. ^1H NMR (500 MHz, D_2O, 25 °C, δ) δ 7.9 (d, 1H J=9.0 Hz, Ar-H), 7.6 (d, 1H J=8.5 Hz, Ar-H), 7.5 (s, 1H, Ar-H), 7.4 (d, 1H J=8.0 Hz, Ar-H), 6.4 (d, 1H J=9.0 Hz, Ar-H), 4.0 (s, 2H, Ar-CH_2-N), 3.1 (m, 2 H, NCH_2), 2.8 (m, 4 H, NCH_2), 2.7 (m, 8 H, NCH_2), 2.6 (m, 2H, NCH_2). ^{13}C NMR (75 MHz, D_2O): 163.8, 153.0, 145.6, 134.8, 130.8, 128.6, 118.9, 116.8, 115.7 (C-Ar), 55.3 (Ar-CH_2-N), 48.8, 44.4, 43.5, 42.0 (CH_2N).

4-((1,4,7,10-tetraazacyclododecan-1-yl)methyl)quinoline-2(1*H*)-one (QMC). A solution containing 4-bromomethyl-2(1H)-quinolinone (0.15 g, 0.63 mmol) in 7 mL of chloroform was added dropwise to a stirred solution of cyclen (0.74 g, 4.3 mmol) in chloroform (20 mL) with the temperature maintained between 0-10 °C. The solution was removed from the ice bath and hexanes were added dropwise until QMC began to precipitate. The solution was left to stir overnight at room temperature and filtered the next day to give a white solid. Yield: 60 %. ESI-MS (MH^+): 330.4. ^1H NMR (400 MHz, DMSO, 25 °C) δ 11.7 (s, 1H, Ar-NH), 7.9 (d, J = 7.6, 1H, Ar-H), 7.4 (t, J = 7.6 Hz, 1H, Ar-

H), 7.3 (d, *J* = 7.6 Hz, 1H, Ar-H), 7.2 (t, *J* = 7.6 Hz, 1H, Ar-H), 6.5 (s, 1H, Ar-H), 3.9 (s, 2H, Ar-C$\underline{H}_2$-N), 2.7 (m, 4H, N-C$\underline{H}_2$), 2.6 (m, 4H, N-C$\underline{H}_2$), 2.5 (m, 8H, N-C$\underline{H}_2$). ^{13}C NMR (300 MHz, D_2O, δ): 163.9, 150.6, 137.7, 131.4, 124.4, 123.4, 120.4, 119.1, 116.8 (C-Ar); 56.4, 50.8, 43.1 (C$\underline{H}_2$-N).

[Zn(QMC)]Cl_2. A stock solution (mM) of the Zn(II) complex of QMC was prepared in water by dissolving the free-base macrocycle and adding $ZnCl_2$ in small aliquots until reaching a 1:0.95 molar ratio. The pH was then adjusted to 7. ESI-MS [$(Zn(M))^{2+}$]: 392.5 [$(Zn(M)-Cl)^{+}$]: 428.4, 430.4, 432.4 ^{1}H NMR (500 MHz, D_2O, 25 °C) δ 11.7 (s, 1H, Ar-NH), 7.9 (d, *J* = 8.4, 1H, Ar-H), 7.8 (d, *J* = 8.0 Hz, 1H, Ar-H), 7.6 (t, *J* = 8.2 Hz, 1H, Ar-H), 7.4 (m, 1H, Ar-H), 6.7 (s, 1H, Ar-H), 4.1 (s, 2H, Ar-C$\underline{H}_2$-N), 3.0 (m, 4H, N-C$\underline{H}_2$), 2.9 (m, 4H, N-C$\underline{H}_2$), 2.7 (m, 8H, N-C$\underline{H}_2$).

2-((1,4,7,10-tetraazacyclododecan-1-yl)methyl)anthracene-9,10-dione (ATQ). Cyclen (0.4784 g, 0.0028 mol) was dissolved in chloroform and brought to reflux under N_2. 2-(chloromethyl)anthracene-9,10-dione (0.0975g, 0.00038 mol) was added dropwise over 5 hours. After the addition was complete, the reaction was allowed to cool to room temperature. The crude reaction mixture was washed with deionized H_2O twice, 1N NaOH twice and deionized H_2O twice more. The organic layer was dried over anhydrous Na_2SO_4 and vacuum dried to give a white powder. Yield: 96%. ESI-MS (MH^+) : 393.3; (MNa^+): 415.3. ^{1}H NMR (500 MHz, $CDCl_3$, 25 °C, δ): 8.3 (m, 4H, Ar-$\underline{H}$), 7.8 (m, 3H, Ar-$\underline{H}$), 3.7 (s, 2H, Ar-C$\underline{H}_2$-N), 2.8 (t, *J* = 5.8 Hz, 4H, N-C$\underline{H}_2$), 2.7 (t, *J* = 6.3 Hz, 4H, N-C$\underline{H}_2$), 2.6 (m, 4H, N-C$\underline{H}_2$), 2.5 (t, *J* = 6.5 Hz, 4H, N-C$\underline{H}_2$). ^{13}C (300 MHz, $CDCl_3$, 25 °C, δ): 183.2, 182.7, 146.6, 134.5, 134.0, 133.9, 133.5, 133.5, 133.5, 132.5, 127.6, 127.4, 127.1, 127.0 (C-Ar); 59.1 (Ar-C$\underline{H}_2$-N), 51.6, 47.2, 46.4, 45.2 (C$\underline{H}_2$-N).

[Zn(ATQ)]Cl_2. [Zn(ATQ)]Cl_2 was prepared in a similar manner to [Zn(MCC)]Cl_2. The resulting beige powder was vacuum dried. Yield: 52%. ESI-MS ($[M-Cl]^+$): 491.4, 493.3, 495.3. ^{1}H NMR (500 MHz, H_2O, 25 °C, δ): 8.3 (m, 3H, Ar-$\underline{H}$), 8.0 (m, 1H, Ar-$\underline{H}$), 7.8 (m, 3H, Ar-$\underline{H}$), 3.9 (s, 2H, Ar-C$\underline{H}_2$-N), 3.1 (m, *2*H, N-C$\underline{H}_2$), 2.9 (m, 4H, N-C$\underline{H}_2$), 2.7 (m, 8H, N-C$\underline{H}_2$), 2.6 (m, 2H, N-C$\underline{H}_2$).

RESULTS

Synthesis and characterization of complexes. Six different Zn(II) complexes were studied (Scheme 1) as DNA recognition agents. All contained a derivative of the 1,4,7,10-tetraazacyclododecane (cyclen) macrocycle and five of them also contained a single aromatic pendent group. Most pendent groups contained two fused rings including a quinoline (CQC), quinolinone (QMC), coumarin (MCC) and dansyl (DSC). One pendent, with three fused rings, anthraquinone (ATQ), was studied. The macrocycles were synthesized by alkylation of cyclen with the cyclen present in excess in order to prevent the multiple alkylations of the macrocycle. The Zn(II) complexes were prepared as the chloride

salts either in ethanolic solution or in water upon combination of the equimolar concentrations of Zn(II) and macrocycle at neutral pH.

To study binding of Zn(II) to the macrocycles as well as solution speciation, pH-potentriometric titrations were carried out for two of the macrocyclic complexes, Zn(CQC) and Zn(QMC). Data from pH potentiometric titrations is fit to give a pK_a value of 8.44 for Zn(CQC)(OH_2) with a binding constant for the neutral macrocycle to Zn(II) of log K = 10.59. Zn(QMC)(OH_2) had a water pK_a of 8.30 and a formation constant of log K = 12.03, similar to other complexes with methylene linkers.[13] Additional data for pH-potentiometric titrations (Fig. S1a ESI†) and all equations (Eq. S1-S16) used for fitting the data are given in supplementary sections. The data is consistent with previous pH-potentiometric studies of Zn(DSC) that gave a formation constant of log K = 10.9 whereas macrocycles with methylene group linkers typically have even higher formation constants of log K = 11-14.[13] The pK_a values of the Zn(II) complexes are in the range of values previously reported in 100 mM NaCl for Zn(II) complexes that have simple aromatic pendent groups (7.9 to 8.4).[13]

Typical speciation diagrams are shown in Figure S2b for Zn(QMC) and Zn(CQC) as a function of pH (Fig. S1b ESI†). Diagrams are given for 1.00 mM, and for 50 μM as typical of the higher concentrations used in ITC experiments and another is given for 2 μM as the lowest concentration used for the SPR experiments. These data show that the major species in solution at neutral pH for both complexes and at all concentrations is the Zn(L)(OH_2) complex for L = QMC and CQC. Analysis of the speciation diagrams shows that the Zn(L)(OH_2) species is dominant at pH 7.5 and little dissociation of Zn(II) occurs at this pH. Zn(II) complexes of cyclen-based macrocycles are stable in solution at near neutral pH even at micromolar concentrations.[55] Notably, at solution concentrations of 10 μM or higher, only ≤ 2% of the Zn(II) complex in solution are typically in dissociated form.

For complexes that did not have sufficient solubility for pH-potentiometric titrations, including Zn(MCC) and Zn(ATQ), UV-vis spectroscopy was used to monitor complex stability in the micromolar concentration range. Similar experiments were carried out on Zn(QMC), Zn(CQC), and Zn(DSC) for comparison. The absorbance of the complexes was monitored as a function of concentration from low micromolar to 100 μM. Notably, the absorbance of the macrocyclic ligand alone under similar conditions differs from that of the complex (data not shown). The UV-vis absorbance characteristic of each of the five complexes, Zn(MCC), Zn(QMC), Zn(CQC), Zn(DSC) and Zn(ATQ) all followed Beer's law. This is consistent with a single Zn(II) complex being the predominant species under these conditions for each case (Fig. S2 ESI†).

SPR studies. Biosensor surface plasmon resonance (SPR) studies were carried out to screen Zn(II) complexes for interaction with a DNA hairpin containing a thymine bulge. A related DNA hairpin containing a single cytosine bulge was studied as a control sequence. All DNA was immobilized on a streptavidin chip by using a 5'-biotinylated DNA as detailed in the experimental section. The DNA sequences are shown in Scheme S1 ESI†.

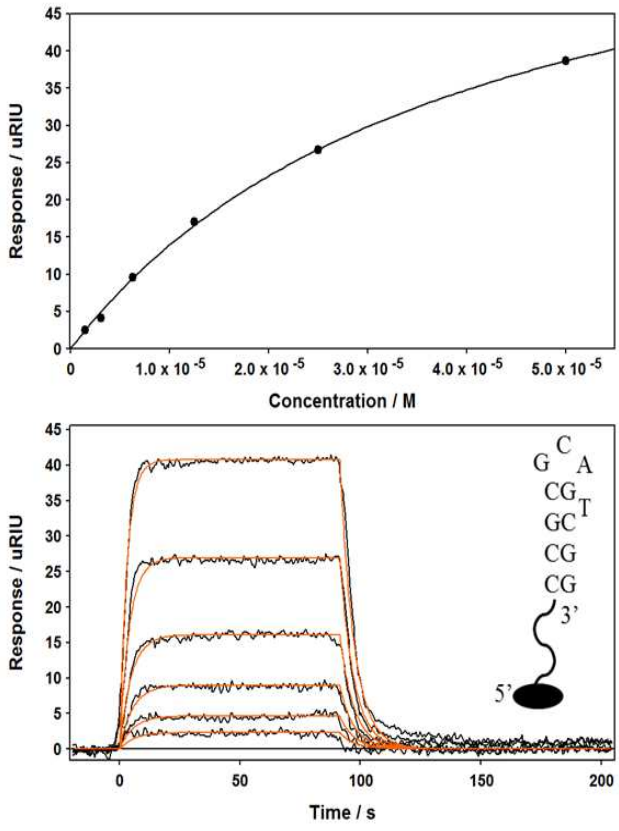


Fig. 1 T-bulge-Zn(QMC) SPR studies (Top) Change in response (μRIU-refractive index units) vs Zn(II) complex concentration with data fit to binding isotherm. (Bottom) Change in response (μRIU) vs time (s) determining on/off rate constants. (Inset) 5-Biontinlyated Thymine Bulge used for SPR Studies. Concentration of Zn(II) complex ranged from 1.5 μM – 50 μM. All samples were prepared in 0.10 M NaCl, 0.02 M HEPES (pH 7.5) and 0.05% Tween 20.

A typical sensorgram for binding of Zn(QMC) to the T-bulge is shown in Fig.1 and binding constants are given in Table 1. Binding constants were obtained by fitting the average response from the steady state (plateau) region of the sensorgram where the rates of dissociation and association are equal. In general, there is good agreement between dissociation constants obtained from binding isotherms and from rate constant ratios (Table S2 and S3 ESI†). Data was fit to a single site binding equation to give dissociation constants given in Table 1. SPR studies with the Zn(ATQ) complex were not carried out in order to avoid poisoning the chip from potentially strong interaction of the aromatic component with streptavidin. Studies with Zn(CYC) showed limited interaction with the T-bulge and no measurable binding. Biosensor SPR studies of immobilized C-bulge showed limited interaction with any of the Zn(II) complexes. Sensorgrams of the immobilized C-bulge plotted as a function of complex concentration showed scattered plots consistent with weak or nonspecific binding (Fig. S3 ESI†).

Table 1: Apparent Dissociation constants ($K_{d\,(app)}$ x 10^{-6}M) of Zn^{2+} complexes to T-Bulge

Complex	$K_{d\,(app)}$ by SPR[a]	$K_{d\,(app)}$ by ITC[b]	$K_{d\,(app)}$ by Fluorescence[c]
Zn(CQC)	30.4 µM ± 1.8	14.4 ± 8.6	nf[c]
Zn(QMC)	38.6 µM ± 1.2	44.9 ± 0.6	nf[c]
Zn(MCC)	31.5 µM ± 1.0	31.2 ± 13.7	nf[c]
Zn(DSC)	1.1 mM ± 0.4	nd[e]	152 ± 32[d]
Zn(ATQ)	nd[f]	21.7 ± 4.0	nf[c]
Zn(Cy)	Weak	nd[e]	nf[c]
Zn(ACR)	nd[g]	nd[g]	≤ 1

[a] Apparent dissociation constants of Zn^{2+}complexes binding to immobilized T-Bulge in 0.10 M NaCl, 0.02 M HEPES (pH 7.5) and 0.05% Tween 20 at 25 °C. [b] Apparent dissociation constants of Zn^{2+}complexes binding to a solution of T-Bulge in 0.10 M NaCl and 0.02 M HEPES (pH 7.5) at 25 °C, using an n=1 stoichiometry. [c] Complex not fluorescent. [d] From previous work (Ref.13) [e] Not determined using ITC because of weak binding. [f] Not determined using SPR. [g] Not determined using SPR or ITC because of overly strong binding to matrix or to DNA.

Binding of Zn(II) complexes to the immobilized H-telo G-quadruplex was also studied by SPR. Data from these studies was fit to binding curves with either a 1:1 or a 2:1 stoichiometry (Table 2, Table S4 and Fig. S4 ESI†). The three complexes Zn(CQC), Zn(QMC) and Zn(MCC) all showed similar binding constants, when fit to an equivalent site binding model (Eq. S17). The fits of these data using a sequential site binding model (Eq. S18) were consistent with one strong (2-3 µM) and one weaker (20-120 µM binding) site. In contrast, the Zn(DSC) complex did not exhibit such strong binding by SPR studies. This complex, however, typically gives lower dissociation constants by SPR studies than those obtained by fluorescence or ITC experiments, which is attributed to aggregation of the Zn(DSC) complex in the solution flowed over the streptavidin chip to which the DNA is bound. Solution studies of binding by using ITC or fluorescence typically involve continuous mixing that minimizes aggregation. Zn(CYC) showed limited interaction with H-telo G-quadruplex and no measurable binding.

Isothermal calorimetry studies. Binding of four of the five new Zn(II) complexes with pendent aromatic groups (Scheme 1) to the T-bulge DNA was studied by ITC. Neither Zn(CYC) nor Zn(DSC) were studied because both complexes bind very weakly to thymine bulges as shown by SPR and, for Zn(DSC), by fluorescence spectroscopy.[48] A typical binding curve obtained from fitting a plot of heat versus molar ratio of Zn(II) complex to DNA is shown in Fig.2 and Fig. S5 ESI†. Data was fit to a single site binding equation (Eq. S17 ESI†) to give dissociation constants listed in Table 1. Binding constants obtained by ITC for Zn(CQC), Zn(MCC) and Zn(QMC) were in good agreement with those obtained by SPR methods. ITC data for Zn(ATQ) binding to T-bulge were fit to give a binding constant of 22 μM, similar to that of the other three complexes. ITC experiments were also conducted with Zn(ATQ) and duplex DNA (Dickerson dodecamer, (Fig. S6 ESI†) for comparison, given that binding of this complex to DNA could not be studied by using SPR. A binding constant of 20 μM for single site binding to the dodecamer duplex was obtained, showing that there is little selectivity of the Zn(ATQ) complex for the T-bulge.

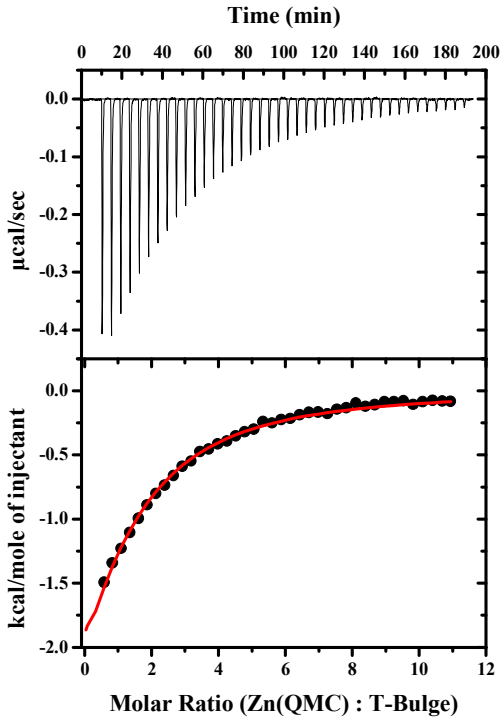


Fig. 2 (Top) Isothermal Calorimetric Plots for titration of Zn(QMC) into 20 μM T-Bulge, in 100 mM NaCl and 0.02 M HEPES (pH: 7.5). (Bottom) Plots of heat evolved (kcal/mol) versus molar ratio (Complex: T-Bulge) fit to Eq. S17.

Table 2: Apparent Dissociation constants ($K_{d\,(app)}$ X 10^{-6}M) of Zn^{2+} complexes to H-Telo

Complex	$K_{d\,(app)}$ by SPR[a]	$K_{d\,(app)}$ by ITC (n)[b]	$K_{d\,(app)}$ by Fluorescence (n)[b]
Zn(CQC)	13.0 ± 0.25	6.0 ± 0.7 (1.6)	nf[c]
Zn(QMC)	15.1 ± 4.6	5.6 ± 1.8 (1.6)	nf[c]
Zn(MCC)	32.5 ± 2.5	2.9 ± 0.0 (2.0)	nf[c]
Zn(DSC)	94.0 ± 4.0	6.0 ± 0.8 (1.7)[d]	2.5 ± 0.1 (2)[d]
Zn(ATQ)	nd[e]	2.8 ± 1.3 (3.1)	nf[c]
Zn(CYC)	weak	nd[f]	nf[c]
Zn(ACR)	nd[g]	nd[g]	1.0 ± 0.04 (5)[d]

[a] Apparent dissociation constants of Zn^{2+}complexes binding to immobilized H-Telo in 0.10 M KCl, 0.02 M HEPES (pH 7.5) and 0.05% Tween 20 at 25 °C. [b] Apparent dissociation constants of Zn^{2+}complexes binding to H-Telo in 0.10 M KCl and 0.02 M HEPES (pH 7.5) at 25 °C; (n) denotes stoichiometric constant determined using a multiple site binding equation with one type of site.[c] Complex not fluorescent. [d] From previous work (Ref. 48). [e] Not determined using SPR. [f] Not determined using ITC because of weak binding. [g] Not determined using SPR or ITC because of overly strong association with matrix or with DNA.

Binding of five Zn(II) complexes to H-telo G-quadruplex was reported by using ITC (Fig. S7 ESI†). Data was fit to binding curves with equations that allow variation in the number of binding sites (Table 2). All complexes with two fused rings in the pendent gave comparatively tighter binding values for G-quadruplex binding in comparison to T-bulge. Data was consistent with approximately two binding sites on H-telo. Alternatively, data could be fit to a model that includes two distinct binding sites with two different binding constants (Table S4, Eq. S18 ESI†). Data is in reasonable agreement with SPR studies for Zn(CQC) and Zn(QMC), given the rather large uncertainties in fitting the data to multiple binding sites. Binding constants for Zn(MCC) are in agreement with those derived from ITC for fitting of the data to two distinct binding constants. ITC-derived binding constants for Zn(DSC) to H-telo G-quadruplex does not match as closely to that of SPR. Once again, this is attributed to aggregation of the Zn(DSC) complex in solution under conditions of the SPR studies. ITC data for the Zn(ATQ) complex is consistent with somewhat tighter binding of the complex to H-telo G-quadruplex in comparison to the other complexes, but with three binding sites.

Optical thermal melting. Optical thermal melting experiments on H-telo G-quadruplex DNA in the presence of four different Zn(II) complexes were determined. The melting temperatures (T_m) for a 1:1 or a 1:2 DNA to Zn(II) complex ratio are shown in Fig. S8 ESI†. These data show that the Zn(MCC), Zn(CQC) and Zn(ATQ) complexes stabilize the G-quadruplex by 6, 7 and 8 ^{o}C, respectively. In contrast, Zn(QMC) slightly destabilizes the G-quadruplex by -3 ^{o}C at a 1:2 ratio. Previous studies of optical thermal melting as a function of concentration showed that the T-bulge DNA was in hairpin form under conditions similar to those studied here.[13]

Fluorescence spectroscopy studies. For comparison, dissociation constants obtained by fluorescence spectroscopy are included in Tables 1 and 2 for the two complexes that are fluorescent including Zn(DSC) and Zn(ACR). These studies show that the fluorescence data for Zn(DSC) is in reasonable agreement with the ITC data for binding of the complex to the H-telo G-quadruplex. Binding data from fluorescence titrations of Zn(DSC) does not correspond as well to SPR studies of H-telo or T-bulge as discussed above. Florescence titrations of Zn(ACR) with DNA show that the complex binds very tightly to both the thymine bulge (Fig. S9 ESI†) and to the H telo G-quadruplex.[48]

DISCUSSION

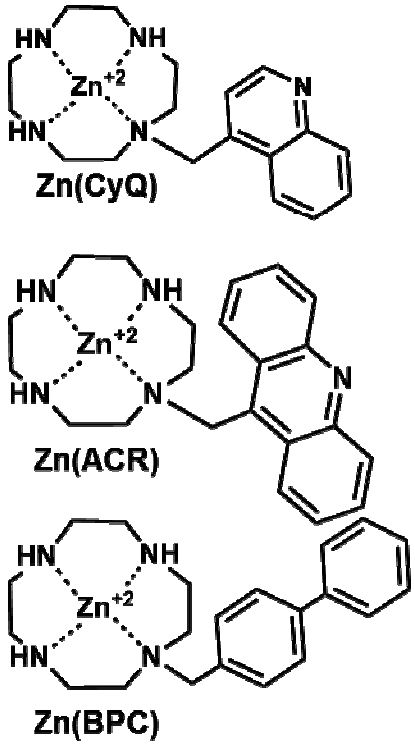
Zn(II) macrocyclic complexes with aromatic pendents are unusual examples of coordination complexes that are bifunctional recognition agents for non-canonical thymines or uracil nucleobases. Recognition is mediated by both the Zn(II) center and by the aromatic pendent. The Zn(II) center in the azamacrocyclic complex is uniquely tuned to be sufficiently Lewis acidic to bind to the $N3^-$ of thymine or uracil, but not so Lewis acidic that hydroxide complexes form to inhibit binding to the nucleobases.[13] The importance of the Zn^{2+} center is demonstrated by studies showing only weak binding of the free macrocycle to structured DNA sequences.[13, 48] Furthermore, analogous cyclen complexes containing Cu(II), Co(II) or Fe(II) do not bind to non-canonical thymines, demonstrating the importance of the Zn^{2+} ion.[48] Bifunctional recognition of thymine/uracil may be tuned by modification of the Lewis acidity of the Zn(II) center through variation in macrocycle donor groups.[50] However, the cyclen macrocycle was the focus of our studies here given that it binds sufficiently strongly to remain complexed with Zn(II) even at the low (micromolar) concentrations used for these studies. The Lewis acidity of the Zn(II) complexes in our study (Scheme 1) does not vary substantially as evidenced by similarity in bound water pK_a values.

Differences in the recognition properties of the Zn(II) complexes studied here are attributed to their different aromatic pendent groups. Notably, one pendent lacks a methylene group (CQC) to produce a more rigid recognition system, two pendents have methylene linkers at different points of attachment to aromatics which contain two fused rings and different hereroatoms (MCC and QMC), and the dansyl pendent (DSC) contains a sulfone linker and a non-planar dimethylamino group. The anthraquinoline pendent (ATQ) has three fused aromatic rings for comparison to the previously studied Zn(ACR). This variation in pendent group size and linker type was studied in order to determine the limitations of recognition of non-canonical thymines. Structured DNA or RNA is very challenging to recognize and provides binding opportunities to non-canonical bases in unique and different environments. By comparison, early work by Kimura and co-workers reported binding of Zn(II) macrocyclic complexes to thymine in single-stranded DNA or to double-stranded DNA but did not study the discrimination of non-canonical thymines in more complicated structures.[56-58]

The two DNA structures studied here have non-canonical thymines in different structural contexts. The H-telo G-quadruplex has thymines that are relatively accessible and unencumbered for Zn(II) complex binding. NMR studies show that there are thymines in two different loops of H-telo which are splayed out and away from the G-tetrad. Zn(DSC) can be readily docked into these thymine binding sites.[34, 35] In contrast, thymine bulges in sequences similar to those studied here have the thymine in an extrahelical position. Notably, the bulged thymine is abutted by groups in the major groove.[14] Thus, the single thymine bulge is likely less accessible for Zn(II) binding than are the thymines in the loops of H-telo G-quadruplex.

The different pendent groups do modulate the magnitude of the binding strength of Zn(II) complexes to non-canonical thymines in single-base bulges. Previous work on thymine bulges showed that Zn(CyQ), which contains the planar quinoline pendent (Scheme 3), bound to the thymine bulge with an apparent K_d of 1-3 μM as shown in studies that used a fluorescence displacement assay. A 100-fold selectivity for binding T-bulge over duplex DNA or a 50-fold selectivity over C-bulge DNA was reported for the Zn(CyQ) complex. NMR studies of this complex suggested that the pendent quinolone stacked on top of the bulged thymine.[14] The Zn(BPC) complex which contains a twisted, non-planar pendent, showed weaker binding to thymine bulges and low selectivity over DNA lacking thymine bulges.[13] This initial work led us to propose that only planar aromatic pendents would bind effectively to the restricted bulge structure.

Most of the Zn(II) complexes studied here that contain two fused aromatic rings bind to the thymine bulge with a 1:1 binding stoichiometry with dissociation constants in the range of 10-40 μM.



Scheme 3: Previously studied Zn(II) macrocyclic complexes

These complexes, including Zn(MCC), Zn(CQC) and Zn(QMC), are selective for the thymine bulge over analogous DNA with a cytosine bulge. The exceptions are Zn(DSC), which lacks the methylene linker and also lacks a planar aromatic group, as well as the parent Zn(CYC) complex. These two complexes bind only weakly to the thymine bulge, consistent with the requirement of a planar aromatic group that stacks on top of the thymine in a relatively restricted space. In contrast, complexes with three fused rings show little specificity for thymine bulge. This includes the Zn(ACR) complex which binds tightly to T-bulge but also to duplex DNA, presumably due to acridine intercalation into the duplex.[48] Zn(ATQ) binds more weakly to both the T-bulge and duplex DNA than does the Zn(ACR) by at least 20-fold. This complex also shows a lack of specific binding to the thymine bulge over duplex DNA.

The binding mode of the Zn(II) complex to the H-telo is more difficult to elucidate, given the complexity of the G-quadruplex structure. There are three loops containing a total of six thymines. In addition, there is the G-tetrad that forms the platform on which many planar aromatic ligands bind through stacking. Previous studies were consistent with binding of a Zn(II) complex to the thymines in H-telo.[48] Namely, the binding strength of Zn(DSC) increased with pH over the range of 6 to 7.5, consistent with the Zn(II) center binding to the deprotonated thymine ($N3^-$). In addition, Zn(DSC) did not bind strongly to consecutive thymines leading us to propose that the two site binding observed for this system was attributed to Zn(DSC) binding to two different loops of H-telo G-quadruplex. Consistent with this postulate, structural studies of H-telo show that only two of the three loops have exposed thymines.

Fitting of the binding data for the Zn(II) complexes studied here to the H-telo G-quadruplex is also complicated by the potential of multiple thymine binding sites. All of the other Zn(II) complexes that contain two fused rings (Zn(MCC), Zn(CQC), Zn(QMC)) bound to H-telo, as shown by ITC, with approximately two binding sites and micromolar binding constants, similar to Zn(DSC). This suggests that binding of the Zn(II) complexes to loop thymines is not particularly selective for the different heteroatoms or for different points of attachment in the pendent group. This corresponds to the binding model previously proposed which has the Zn(II) complex bound to two thymines in two distinct loops with space for an extended pendent group to interact with the thymine with different stacking orientations.

By contrast, Zn(II) complexes that contain three aromatic rings such as Zn(ATQ) and Zn(ACR) bind to further additional sites on the H-telo. Data is consistent with Zn(ATQ) binding to three sites and Zn(ACR) to five sites in the H-telo G-quadruplex. The locations of these extra binding sites likely involve binding by capping the G-tetrad. The stabilization of the H-telo quadruplex by Zn(ATQ) is larger than that of any of the other Zn(II) complexes (9 ^{o}C), consistent with interaction of the complex with the G-tetrad. Notably, none of the pendents with two fused rings stabilize the H-telo to this extent.

CONCLUSIONS

Modification of the pendent groups in Zn(II) cyclen produce complexes that bind to non-canonical DNA with moderately different binding strengths. For thymine bulges, the best choices are planar pendent groups with two fused rings with either a methylene linker or direct linkage to the macrocyclic amine. This is presumably an important characteristic of the complex so that the pendent can interact with the thymine nucleobase by stacking as shown for Zn(CyQ) in structural studies.[13] Somewhat surprisingly, aromatic groups with differing points of attachment to the methylene linker do not markedly change the binding constant. This suggests that the binding interaction is not unduly restrictive for Zn(II) complexes that bind to T-bulge DNA as long as the pendent has a planar aromatic group.

Zn(II) complexes with pendents containing two fused rings bind to the thymines of the H-telo G-quadruplex loops more tightly than to the thymine bulge by 5-10 fold. Structural studies are consistent with the thymines in the G-quadruplex loops being more accessible than in the T-bulge. These studies support the observation that the different pendents, including the non-planar dansyl pendent, produce tightly binding Zn(II) complexes. Stacking interactions, however, do depend on the heteroatoms in the pendents and thus binding interactions might be expected to differ.[59] The use of three fused rings in the pendent indeed increases binding to the G-quadruplex, as shown for both the Zn(ACR) and Zn(ATQ) complexes. However, the selectivity of the Zn(ACR) complex is very low as it binds tightly to all other DNA sequences studied. The Zn(ATQ) complex has higher selectivity for the G-quadruplex and is intriguing in that binding of the complex stabilizes the quadruplex. Further studies will focus on better defining the mode of H-telo binding to address the structural requirements for interaction of the Zn(II) complexes. Such structural studies may aid in the further functionalization of Zn(II) complexes for the development of highly selective recognition agents for DNA structures containing non-canonical thymines.

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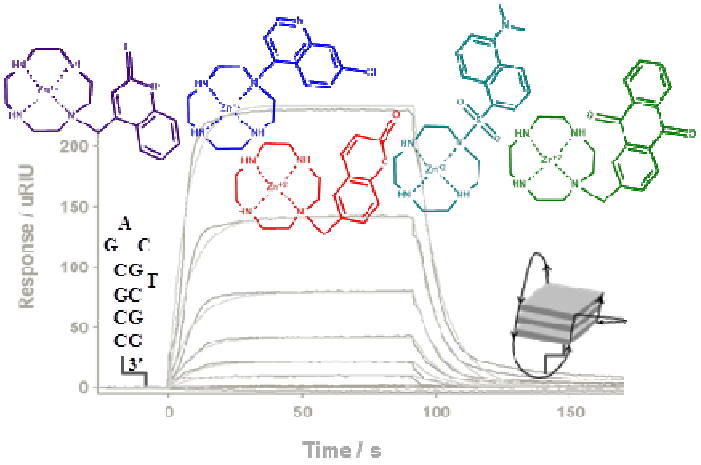

The aromatic pendent groups of Zn(II) tetraazamacrocyclic complexes were varied to study their role in the recognition of non-canonical thymines in a DNA bulge and in the human telomeric G-quadruplex.