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Structure-specific recognition of quadruplex DNA by organic cations: Influence of shape, substituents and charge

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Abstract

Combining structure-specific recognition of nucleic acids with limited sequence reading is a promising method to reduce the size of the recognition unit required to achieve the necessary selectivity and binding affinity to control function. It has been demonstrated recently that G-quadruplex DNA structures can be targeted by organic cations in a structure-specific manner. Structural targets of quadruplexes include the planar end surfaces of the G-tetrad stacked columns and four grooves. These provide different geometries and functional groups relative to duplex DNA. We have used surface plasmon resonance and isothermal titration calorimetry to show that binding affinity and selectivity of a series of quadruplex end-stacking molecules to human telomeric DNA are sensitive to compound shape as well as substituent type and position. ITC results indicate that binding is largely enthalpy driven. Circular dichroism was also used to identify a group of structurally related compounds that selectively target quadruplex grooves.

Keywords: Synthetic cations; Telomere; DNA-quadruplex binding; G-tetrad; BIAcore; ITC

1. Introduction

Specific recognition of double helical sequences of base pairs in DNA, for example, by transcription control proteins [1] and designed synthetic molecules [2,3], has been thoroughly studied and many sequence-specific recognition complexes are now characterized in molecular detail. Therapeutic intervention or control of cellular function through specific recognition of a cellular duplex DNA sequence requires interaction with a large number of base pairs. This is routinely accomplished by cellular proteins but is more difficult to achieve with relatively small synthetic compounds that must have properties that allow them to pass through barriers, including cell membranes, in order to bind to DNA. Incorporating nucleic acid structure into the recognition motif, however, is a promising method to reduce the size of the recognition sequence required to gain the necessary specificity. Specific recognition of RNA structures by small molecules that interact with only a few bases or base pairs, for example, is well established for aminoglycoside antibiotics that target ribosomal RNA [4–6]. The recent discovery of small metabolites and analogs that bind specifically to RNA riboswitch structures is now a demonstrated method for specific control of translation [7]. Selection methods that yield nucleic acids that are capable of highly specific binding to small molecules provide an additional example of recognition by structure-specific motifs [8]. Examples of structure-specific targeting are also emerging for selective recognition of DNA. The mitochondrial kinetoplast DNA of kinetoplastid eukaryotic parasites, which cause serious diseases that affect millions of people, for example, has a complex structure that is composed of a few large circular DNAs in an interlocked, catenated array with thousands of minicircular DNAs [9–11]. The minicircular

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DNAs have phased AT sequence tracts that bend the DNA duplex [12] and provide an optimum sequence-structure target for drug design [13].

Eukaryotic cells of the parasites as well as of higher organisms have a 3′ terminal G-rich, single-stranded telomere DNA sequence that performs several essential functions in chromosome maintenance and protection. The G-rich strand can fold into a four-stranded quadruplex structure that is an attractive potential structure-specific target in rapidly dividing cells, such as eukaryotic parasites and cancer [14]. The key structural feature of the quadruplex is a series of stacked guanine tetrads held together in a coplanar cyclic array by Hoogsteen and Watson–Crick hydrogen bonds (Fig. 1). The quadruplex is also stabilized through π–π stacking interactions of the stacked tetrads as well as by coordination with cations located between or within the tetrads. Guanine-rich sequences, which are capable of forming quadruplex structures, are present in biologically significant regions of the genome including immunoglobulin switch regions [15], the transcriptional regulatory regions of a number of genes such as the insulin gene [16], and also the promoter regions of certain oncogenes, such as c-MYC [17,18].

Because of the critically essential roles of telomere DNAs in both cancer and parasitic cells, the telomere is a particularly attractive target for drug design [19–26]. The telomeric sequence and structure varies depending on the organism. In humans and other vertebrates and the eukaryotic parasites, telomeres consist of tandem T_2AG_3 repeats that can adopt a G-quadruplex conformation *in vitro* under physiological conditions [27,28]. The discovery of proteins such as transcription factors, nucleases and helicases that can bind to and even promote the formation of telomeric quadruplexes suggests that these structures may exist *in vivo* under certain conditions [29–31]. A very exciting recent finding is that a radiolabeled G-quadruplex binding ligand accumulated in nuclei of cultured cells and preferentially bound to the terminal regions of the chromosomes, indicating that G-quadruplexes do exist in vivo and are accessible to drugs [32]. Each time a cell replicates, DNA polymerase is unable to copy the extreme end of the 3′, lagging strand. This "end replication problem" results in shortening of the telomere by about 30–200 bases per cell doubling. After 60–70 rounds of cell replication, the telomeres reach a critical length and the cells enter a non-dividing state called senescence, which leads to apoptosis and eventually cell death [33]. However, in 85–90% of cancer cells and in parasites, telomerase is activated [34]. This enzyme is inactive in most normal somatic cells, providing a potentially specific therapeutic target. In order to extend telomere DNA the telomerase enzyme requires the telomere primer to be single stranded. The formation of higher ordered structures such as G-quadruplexes prevents hybridization of the telomerase RNA template onto the primer and thus inhibits telomerase activity through an indirect topological mechanism [35].

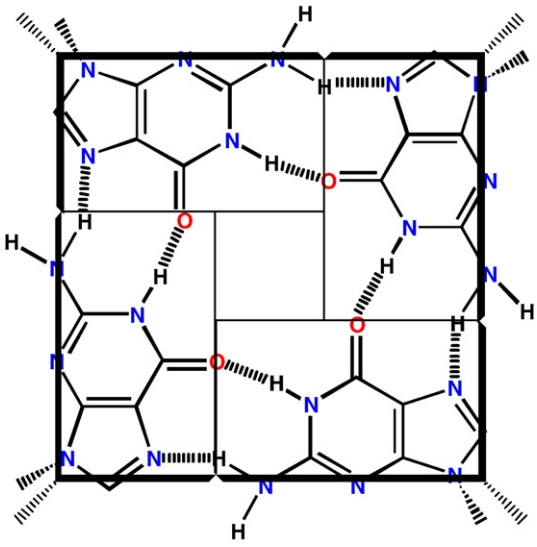

Fig. 1. A molecular representation of an H-bonded G-tetrad is shown overlaid with a schematic of the tetrad that we will use for a size reference for synthetic molecules in figures that follow. As can be seen, the G-tetrad has eight hydrogen bonds with a mix of Watson–Crick and Hoogsteen-type interactions. The four sugar-phosphate chains project away from the tetrad and create four grooves that can have different dimensions depending on the quadruplex geometry.

Stabilization of the quadruplex conformation of telomeres, such as by binding small molecules, has been shown to be an effective method to inhibit telomerase activity [19–26,36]. The development of small molecules that can selectively bind to and stabilize the G-quadruplex conformation of the telomere is therefore a current area of interest in anticancer as well as antiparasitic drug design. Compounds that have been shown to bind to quadruplex DNA have traditionally been planar, aromatic compounds that bind via external end-stacking to the G-quartet on either one end or both ends of the quadruplex [19–26]. These compounds, which include anthraquinones, cationic porphyrins, acridines, macrocyclic compounds and analogs, have planar aromatic surface areas that mimic the large planar surface of the G-tetrads in quadruplex DNA (Fig. 1) [37–40]. Since essentially all known quadruplex DNA binders are based on, or derived from duplex intercalators, many exhibit little selectivity for quadruplex over duplex structures and this can result in nonspecific cytotoxicity. Increasing the selectivity of telomerase inhibitors for their quadruplex targets is an important focus of research.

Groove binding has been a useful way to selectively recognize duplex DNA with relatively low nonspecific toxicity [2,3,13,41–43], but groove binding is an under-exploited design mode with DNA quadruplexes. The structural differences between duplex and quadruplex DNA grooves offer an attractive strategy for development of compounds to differentiate between these two structures. Since groove dimensions vary according to the type of quadruplex [44], groove binding also offers the opportunity for obtaining increased selectivity for a particular quadruplex structure. Unfortunately, no compounds to date have been found which bind to the grooves of the intramolecular human or parasite telomeres or to oncogene control quadruplexes. Shafer et al. obtained spectroscopic data suggesting that the dye 3,3′-diethyloxadicarbocyanine (DODC) binds in the quadruplex grooves of a dimeric hairpin G-quadruplex [45]. Satellite hole spectroscopy studies support the groove-binding model for this particular compound/DNA pair

[46,47]. Electrospray mass spectrometry fragmentation patterns upon ionization also indicate that the carbocyanine dye DTC binds in the quadruplex grooves [48].

Induced circular dichroism (CD) signals of molecules bound to duplex DNA have provided a very powerful method to distinguish intercalation-stacking type interactions from groove binding [49]. To initiate a search for compounds that can bind to quadruplex DNA grooves we have used CD spectroscopy and induced CD signals to distinguish end stacking from groove complexes. For quantitative comparison of binding affinities of quadruplex-binding agents under a defined set of conditions, we have used biosensor-surface plasmon resonance (SPR) methods. This method uses very little material and is applicable to a very large variety of small molecules with quite different properties, binding modes and affinities. We also report CD results for a number of compounds of quite different structure, and based on the CD results, a heterocyclic dication that appears to selectively target the grooves of telomere quadruplex DNA has been identified.

2. Materials and methods

2.1. Sample preparation

The oligonucleotides d[$AG_3(T_2AG_3)_3$], d[$AG_3TG_4AG_3TG_4A$], d[$(GC)_7$], d[GCGAATTCGC], 5′–Biotin–d[$AG_3(T_2AG_3)_3$], 5′–Biotin–d[$CGCGCGCGT_4CGCGCGCG$] and 5′–Biotin–d[CGAATTCGTCTCCGAATTCG] were purchased from Midland Certified Reagent Company with HPLC purification and mass spectrometry characterization. NMR analysis verified the structure and purity of the DNAs. The absorbance of each sample was measured at 260 nm at 80 °C, and extrapolated to 20 °C. The concentration of each DNA sample was then calculated using the nearest neighbor extinction coefficient, and the results are in good agreement with concentrations determined using the extinction coefficient from a colorimetric phosphate assay by Li et al. [50]. DODC, distamycin and ethidium bromide were purchased from Sigma-Aldrich. TMPyP4 was purchased from Midcentury. The synthesis of DB832, DB914, and DB1093 will be described elsewhere. The disubstituted acridines and anthraquinones were synthesized as previously described [51–55] and BRACO-19 was synthesized as described [56]. Se2SAP was synthesized as previously described [57] and provided by Professor Laurence Hurley. RHPS4 was synthesized as previously described [58] and provided by Professors Malcolm Stevens and Charles Laughton. A 1 mM stock solution of each compound was prepared in double deionized water and diluted to working concentrations with buffer immediately before use.

2.2. CD measurements

All measurements were performed at 20 °C in a 10 mM HEPES buffer (pH 7.4) containing 3 mM EDTA and 50 mM KCl. CD spectra were recorded using a Jasco J-810 instrument with a 1-cm cell and a scan speed of 50 nm/min with a response time of 1 s. The spectra were averaged over four scans. A buffer baseline scan was collected in the same cuvette and subtracted from the average scan for each sample. Appropriate amounts of stock solution of ligand were added sequentially to the desired molar ratios. Data manipulation and plotting were performed with Kaleidagraph.

2.3. Surface plasmon resonance studies

Surface plasmon resonance measurements were performed with a four-channel BIAcore 3000 optical biosensor system (BIAcore Inc.). 5′-biotin labeled DNA was immobilized onto streptavidin-coated sensor chips (BIAcore SA) as previously described [42,43,59–61]. Three flow cells were used to immobilize the DNA oligomer samples, while a fourth cell was left blank as a control. The SPR experiments were performed at 25 °C in filtered, degassed, 10 mM HEPES buffer (pH 7.4) containing 100 mM or 200 mM KCl, 3 mM EDTA and 0.005% surfactant P20. Compound solutions were prepared by serial dilutions from stock solution and injected from 7 mm plastic vials with pierceable plastic crimp caps. Solutions of known ligand concentration were injected through the flow cells until a constant steady-state response was obtained. Compound solution flow was then replaced by buffer flow resulting in dissociation of the complex. The reference response from the blank cell was subtracted from the response in each cell containing DNA to give a signal (RU, response units) that is directly proportional to the amount of bound compound. A set of sensorgrams at different concentrations for binding of each compound to human telomeric DNA was obtained. The instrument response (RU) in the steady-state region was determined by linear averaging over a selected time span. The predicted maximum response per bound compound in the steady-state region (RU_{max}) was determined from the DNA molecular weight, the amount of DNA on the flow cell, the compound molecular weight, and the refractive index gradient ratio of the compound and DNA, as previously described [42,43]. The number of binding sites and the equilibrium constant were obtained from fitting plots of RU versus C_{free}. The data were fitted to a two-site equilibrium model using Kaleidagraph for nonlinear least squares optimization of the binding parameters:

$$RU = RU_{max}*(K_1*C_{free} + 2*K_1*K_2*C_{free}^2)/(1 + K_1*C_{free} + K_1*K_2*K_{C_{free}}^2)$$

where RU_{max} is the maximum response per bound compound and K_1 and K_2 are the macroscopic binding constants for a two-site binding model. For a single binding site model, K_2 is equal to zero.

2.4. Isothermal titration calorimetry (ITC)

ITC experiments were performed with a MicroCal VP-ITC (MicroCal Inc., Northampton, MA, USA). Data were collected and processed with Origin. CAC15 buffer containing 10 mM cacodylic acid, 1 mM EDTA, and 150 mM KCl and pH adjusted to 6.25 was used for the ITC experiments. The G-quadruplex telomere sequence [$AG_3(T_2AG_3)_3$] sample was dissolved in

buffer to the desired concentration, heated to 85 °C and cooled slowly to insure the folding of the G4. The compound was injected into the DNA in the sample cell in 5 μL increments. The observed heat for each injection was determined by integration of the injection peak areas with respect to time. Blank titrations were conducted by injecting the compound into the sample cell containing only buffer under the same conditions. The corrected interaction heat was determined by subtracting the blank heat from that for the compound/DNA titration. Reaction heats, equilibrium constants and number of binding sites were determined by fitting with Origin.

3. Results and discussion

3.1. Compounds that stack on the ends of DNA quadruplex structures

Sun et al. provided an exciting first example of a compound that caused significant inhibition of telomerase through stabilization of a DNA quadruplex structure in telomeres [36]. The 2,6-anthraquinone dication from that discovery is shown in Fig. 2 (top left) as an overlay on the G4 reference size schematic from Fig. 1. As can be seen, the anthraquinone ring system can stack on a G-tetrad at the end of quadruplex structures with the cationic substituents positioned to interact with the grooves of the G4 structure. Evidence for end-stacking of the compounds described in this section is included in the original references for the compound-quadruplex complexes and will not be presented here. In an effort to optimize quadruplex affinity and selectivity, Neidle et al. synthesized additional anthraquinones with substituents at different positions [53,54] (Fig. 2). Quantitative analysis of the anthraquinone-quadruplex interactions has been evaluated by biosensor-SPR methods with an immobilized DNA telomere model system, d[$AG_3(T_2AG_3)_3$] (Materials and methods). The anthraquinones have similar structures, sizes and charges, and the telomere binding of the compounds falls within a fairly narrow range ($K_a \sim 1 \times 10^6\ M^{-1}$). Sensorgrams for the 1,5-compound, 9019, which has a slightly higher binding constant ($K_a \sim 2 \times 10^6\ M^{-1}$) than the average for the compounds in Fig. 2, are also shown in the figure as examples. The results

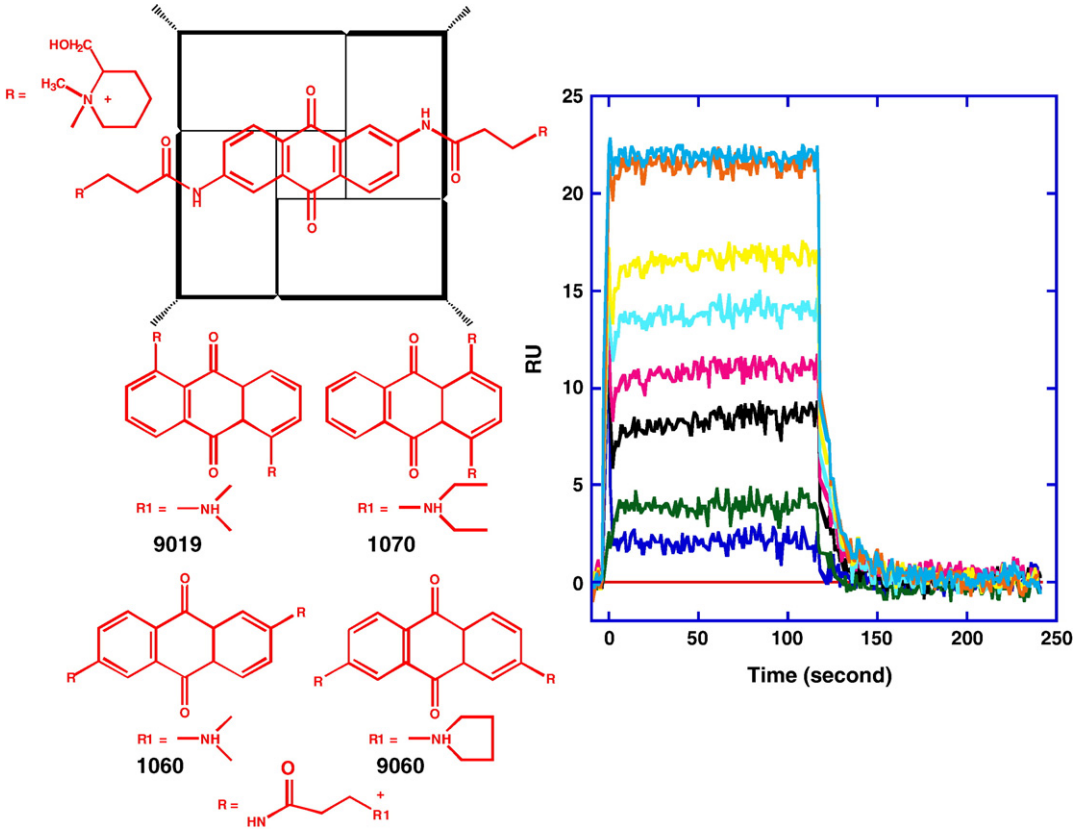

Fig. 2. A 2,6-substituted anthraquinone dication, the first G-quadruplex-specific compound shown to significantly inhibit telomerase, is shown at the top left as an overlay on the G4 reference diagram for size comparison [36]. As part of a search for optimum telomere-binding compounds, additional anthraquinones, with the charged groups moved to different positions on the anthraquinone ring, were prepared by Neidle et al. (unpublished) to elaborate on the anthraquinone–dication system (compound numbers are shown with each structure). All of the compounds have similar charged side chains that are shown in the figure. Sensorgrams for the interaction of 9019 (left) with the telomere quadruplex model are shown on the right. The concentrations of 9019 range from 0.05 μM in the lower sensorgram to 3.0 μM at the top. Before the zero time point, the buffer was flowing over the DNA derivatized sensor chip surface as well as through a blank flow cell. At zero time, 9019 was injected at a desired concentration and flow was continued for 120 s. A stable, steady-state plateau was reached even at the lowest concentrations in under 20 s. Buffer injection was restarted at 120 s and the complex rapidly dissociated. The average response in the steady-state region was obtained over a 50 s time period (50–100 s) and, after subtraction of the blank response, was plotted against the flow concentration of 9019 (the unbound concentration) as described in Materials and methods to determine the *K* values for 9019. The strongest binding constant can be determined with confidence and is $K = 1.6 \times 10^6\ M^{-1}$ in HBS buffer: pH 7.4, 0.01 M HEPES, 0.2 M KCl, 3 mM EDTA.

were fitted by using the response averages from the steady-state regions of the sensorgrams, the plateau in Fig. 2, where the rates of association and dissociation are equal. The best fit is for two binding sites for the anthraquinone with the DNA quadruplex. The binding constants given above are for the strongest binding site which is the most important for telomerase inhibition. Binding to the second site is at least 10 times weaker and cannot be determined accurately for this system with the experimental concentration range used. Binding to DNA duplex sequences was also evaluated by SPR methods and the binding constants for all sequences with anthraquinones are in the range of $K_a \sim 5 \times 10^5\ M^{-1}$, around 2–5 times weaker than for quadruplex primary binding.

As noted above, an advantage of the SPR method of evaluating biomolecular interactions is that it also can provide kinetics information on the binding process. The anthraquinone-quadruplex binding was also evaluated by kinetics methods at lower concentrations where the secondary binding is not significant. Binding kinetics could also be analyzed at higher concentrations by fitting to a two-site model, but this introduces significant additional uncertainty in the results. Global fitting was used to simultaneously fit all sensorgrams for both association (k_a) and dissociation (k_d) rate constants. A plot with the results and best fit lines is shown in Supplementary Materials (Fig. S1). The K_a for the strong binding site, obtained from the ratio of k_a/k_d, was very similar to that from the steady-state analysis. The structure and properties of the anthraquinone aromatic system limit more extensive synthetic modifications that are necessary to improve quadruplex binding affinity and specificity. The anthraquinone results are exciting, however, because they clearly show the potential for developing compounds that bind selectively to G4 DNA structures.

The acridine nucleus is known to interact favorably with DNA bases, and it provides an attractive system for development of quadruplex targeted compounds. Depending on the substituents, the acridine nitrogen can be charged when bound to DNA, and with the ring stacked on a G-tetrad, the charge would occupy a position similar to that of the coordinated metal cation that stabilizes the G-tetrad interaction [39,51]. Unlike the anthraquinone system, the acridine nucleus can be substituted in all three rings with groups that can interact with the grooves of DNA quadruplexes. In an effort to develop compounds with improved selectivity and affinity for the human telomere, Neidle et al. replaced the anthraquinone system with a 3,6-substituted acridine [39,51]. Results for disubstituted acridines, such as BSU6048 (Fig. S2), indicate that the acridines have affinities that are similar to those of the anthraquinones in Fig. 2 for the human telomere quadruplex ($K_a \sim 3 \times 10^6\ M^{-1}$) with approximately 10-fold weaker binding to DNA duplexes. A breakthrough in the design of compounds of this type occurred with the synthesis of 3,6,9-trisubstituted acridines such as BRACO-19 (Fig. 3) [39,56,62–64]. Sensorgrams for binding of BRACO-19 to the telomere quadruplex are shown in Fig. 3 along with steady-state binding curves for both the telomere and DNA duplex model systems. Comparison with the results for the disubstituted compounds (Fig. 2 and Fig. S2) clearly shows

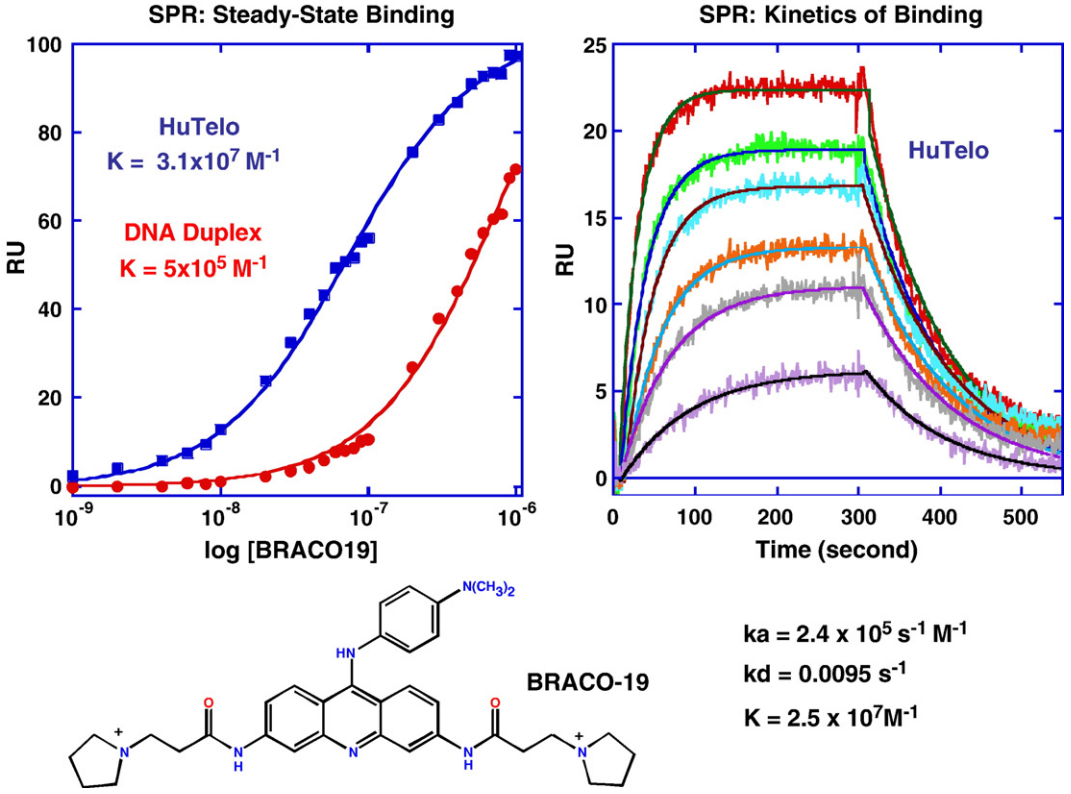

Fig. 3. Sensorgrams for binding of the strong telomerase inhibitor, BRACO-19 (structure at the bottom of the figure) to the human telomere sequence are on the right and binding plots for the compound interactions with the telomere and with a DNA duplex are on the left. Kinetics constants for BRACO-19 binding to the human telomere are shown along with equilibrium constants for binding to both the telomere and duplex.

the slower kinetics for dissociation of the trisubstituted acridine. The dissociation constant decreases from approximately 0.1 s^{-1} for the disubstituted acridine to 0.01 s^{-1} for BRACO-19. The equilibrium constant from kinetic fits to the data (Fig. 3, right) is in excellent agreement with the equilibrium constant from the steady-state fit. In agreement with the much slower dissociation of BRACO-19 from the telomere, it has an equilibrium binding constant ($K_a \sim 3\times10^7$ M^{-1}) that is at least 10 times larger than for the disubstituted compounds. Much of the improvement in affinity comes from the decrease in dissociation rate constant. As with the disubstituted compounds, BRACO-19 also has a second binding site on the telomere that binds the compound approximately 10 times more weakly than the strong site. Sensorgrams for BRACO-19 binding to the telomere and duplex DNA model systems are shown in Fig. S3 for comparison. The much stronger binding of the compound to the telomere can be seen by comparison of the SPR response at the same compound concentrations. As a reference, the binding of the antimalarial, dicationic-acridine quinacrine was evaluated by SPR methods under the same conditions (not shown). The quinacrine binding to the telomere quadruplex was at least 1000 times lower (K_a near 1×10^4 M^{-1}) than for BRACO-19, and duplex binding was stronger than for the quadruplex interactions.

The results above clearly illustrate that it is the type and position of the substituents on the acridine ring that provide the exceptional quadruplex binding affinity of BRACO-19. To understand the thermodynamic basis for the exceptionally strong binding of BRACO-19, ITC experiments with the G-quadruplex telomere DNA sequence used in SPR studies were conducted (Fig. 4). Plots for the titration of BRACO-19 into buffer and into a DNA solution are shown in Fig. 4. Plots of the observed net binding heat/mole versus molar ratio were obtained by subtracting the integrated peak areas for the blank titration from the areas in the DNA interaction titration. Even though the K_a value for BRACO-19 binding to G4 is large, the value obtained from fitting the ITC titration (Fig. 4) is in agreement with that obtained by SPR at much lower concentrations. For molar ratios of less than approximately 1.0, the observed heat/mole is constant, since most of the added compound binds to the strong telomere site under these conditions and is essentially the enthalpy for binding,

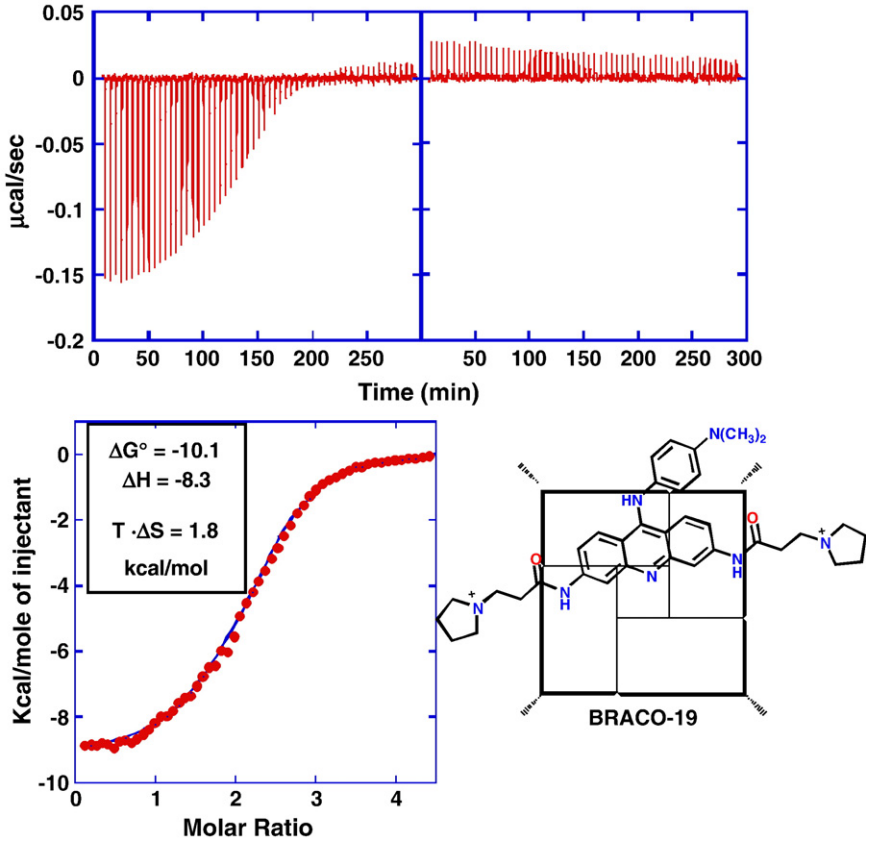

Fig. 4. ITC experiments with the G-quadruplex telomere sequence [$AG_3(T_2AG_3)_3$] DNA oligomer used in SPR studies were conducted to get a full thermodynamic understanding of the BRACO-19/G4 interactions. Plots for titration of BRACO-19 into buffer (top right) and into a DNA solution (top left) are shown. Plots of heat versus molar ratio (bottom left) were obtained by subtracting the integrated peak areas for the blank, buffer titration from the DNA interaction titration and a two-site fit to the results is shown in the same panel. A structure of BRACO-19 with the G4 size reference from Fig. 1 is shown at the bottom right. Due to the large K value for BRACO-19 binding to G4, only the enthalpy, ΔH, could be obtained accurately in the ITC experiments. For molar ratios of less than approximately 1.0, the observed ΔH is constant since essentially all of the added compound binds to DNA in the calorimeter. The observed binding enthalpy in CAC15 buffer at 25 °C is −8.3 kcal/mol. The calculated $-T\cdot\Delta S$, based on the Gibbs energy ($\Delta G^\circ = -RT\cdot\ln K_a = -10.1$ kcal/mol) from SPR equilibrium constants and ΔH from ITC, is −1.8 kcal/mol. These are the constants for the strong binding site, which is of interest for biological activity. The BRACO-19 interaction with G-quadruplex telomere sequence [$AG_3(T_2AG_3)_3$] is principally driven by the interaction enthalpy and not by the entropy term.

−8.3 kcal/mol. At higher concentrations secondary binding is observed in the ITC experiments as with SPR. The calculated $-T\cdot\Delta S$, based on the Gibbs energy ($\Delta G^\circ = -RT\cdot\ln K_a$ = −10.1 kcal/mol) for the strong binding site from SPR equilibrium constants and ΔH from ITC, is −1.8 kcal/mol. The BRACO-19 interaction with the G-quadruplex telomere sequence is, thus, principally driven by the interaction enthalpy with a smaller favorable contribution from the entropy term.

Another group of compounds that has been investigated extensively contains porphyrins and related analogs. The tetracationic porphyrin, TMPyP4, was observed in early studies to stabilize DNA quadruplexes and have antitelomerase activity [38,65–68]. Biological studies have clearly shown the potential of the porphyrin to target cellular quadruplexes as an approach for development of anticancer agents [68,69]. Sensorgrams for TMPyP4 binding to the telomere DNA quadruplex model described above as well as to a duplex DNA are shown in Supplementary Materials (Fig. S4) with the porphyrin ring shown as an overlay on the G4 size reference (Fig. 1). The porphyrin binds strongly to the quadruplex ($K_a \sim 3\times10^7$ M^{-1}) but with little specificity over DNA duplexes. In addition, the dissociation rate constant is much larger than with the trisubstituted acridine, BRACO-19. The porphyrin results clearly show, however, that porphyrin-type systems can be designed with strong quadruplex interactions.

Based on an analysis of the macrocyclic natural product, telomestatin [25], which strongly stabilizes quadruplex conformations as well as the porphyrin results described above, Hurley et al. designed a series of seleno-sapphyrin derivatives to selectively target quadruplexes [57]. The compounds have features that are similar to the porphyrins but they are closer in size to telomestatin. A set of sensorgrams for one of the sapphyrin derivatives, Se2SAP, binding to the telomere model system is shown in Fig. 5 along with binding plots for the telomere model system. Results for a duplex DNA are shown for comparison. As can be seen, the Se2SAP compound binds strongly ($K_a \sim 6\times10^7$ M^{-1}) and has significant selectivity for the quadruplex [70]. The compound binds even more strongly to a quadruplex from the promoter region of the c-MYC oncogene (Fig. 6) and about 40 times more weakly to duplex

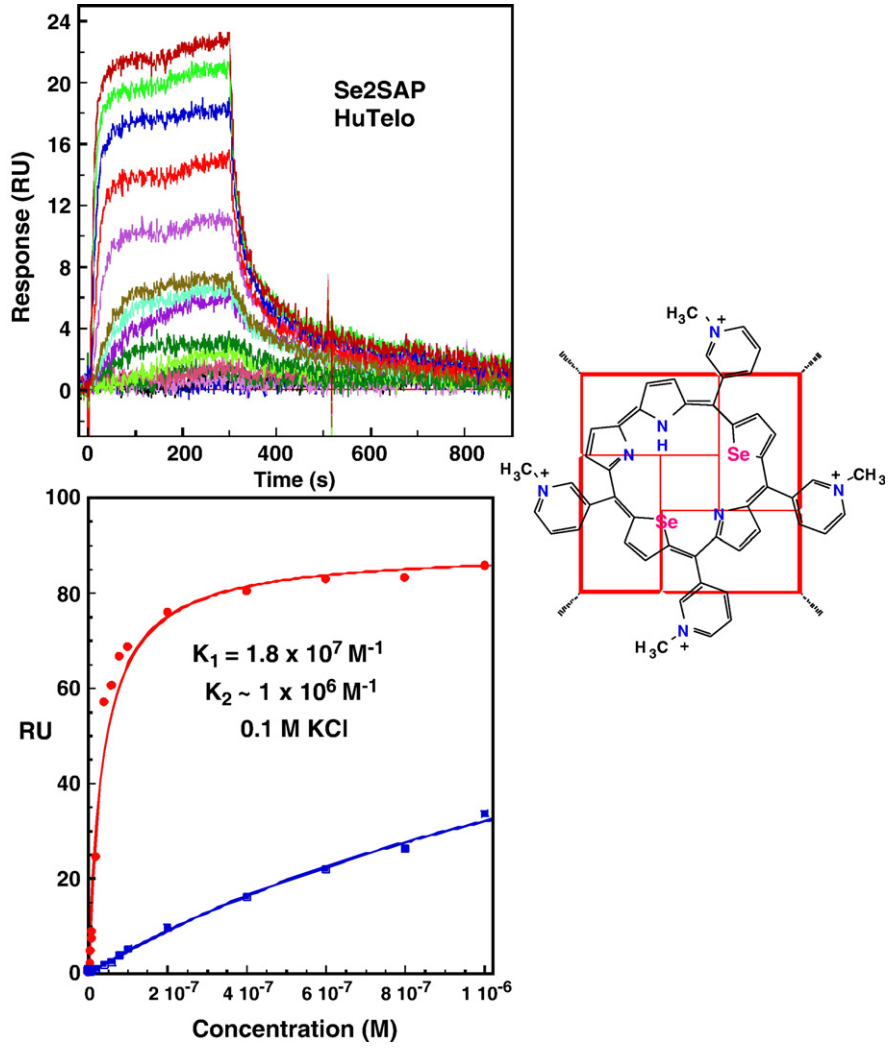

Fig. 5. Sensorgrams for binding of the expanded porphyrin, Se2SAP (structure at the right with the size reference) to the human telomere sequence are on the top left and a binding plot for the compound interactions with the telomere (circles) and with a DNA duplex (squares) are on the bottom left. Equilibrium constants for binding of Se2SAP to the two sites on the telomere quadruplex are shown in the bottom panel in buffer with 0.1 M KCl.

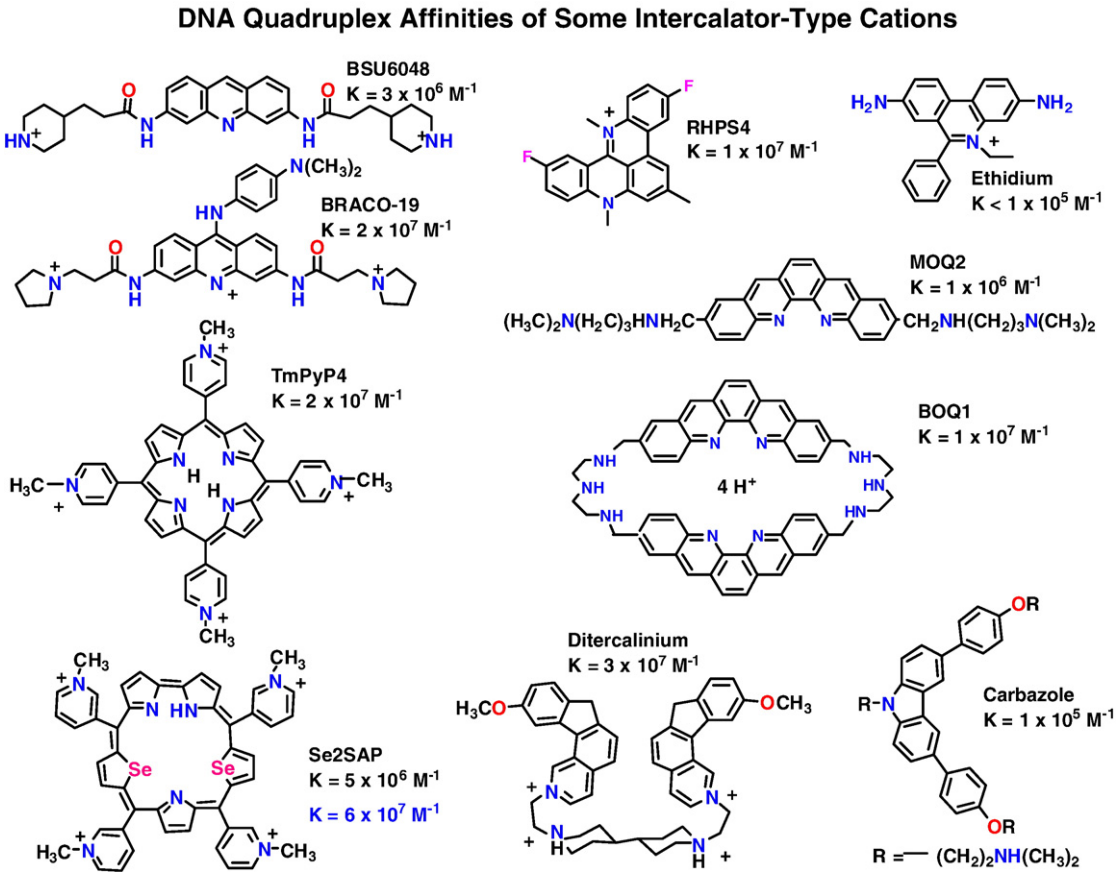

Fig. 6. Some quadruplex compound structures are shown with their SPR determined equilibrium constants with the human telomere (HEPES buffer with 0.2 M KCl). The lower *K* for Se2SAP is for binding to the c-MYC quadruplex. The error in the *K* values is generally ±25%. SPR methods can frequently provide equilibrium information with a lower error but the two sets of equilibrium constants for these compounds (strong and weaker sites) as well as the difficulty of working with many of the compounds in the figure yields the ±25% error.

DNAs [57]. As with other compounds that have strong affinity and specificity for quadruplex conformations, Se2SAP dissociates much more slowly from the c-MYC quadruplex complex (k_d=0.003 s^{-1}) than from duplex DNA (k_d>0.2 s^{-1}) and much of the improved affinity arises from the slower dissociation rate constant [57]. The sapphyrin compound thus has strong quadruplex affinity with a significant improvement over TMPyP4 in binding selectivity. It illustrates the potential for design of compounds that can selectively recognize different quadruplexes as well as differentiate quadruplexes from duplexes.

The binding constants with the DNA model systems described above were also determined by the SPR method for a number of additional compounds that have been shown to have significant interactions with DNA quadruplexes. Structures and quadruplex binding constants are collected in Fig. 6 for the compounds described above as well as the other systems. It should be noted that there are important series of compounds, such as the perylene diimides [71], benzoindoloquinolines [72] and pyridine carboxamides [32], which have very favorable telomere binding properties but are not listed in Fig. 6. These compounds have similar sizes and physical characteristics to the compounds in the figure and should have similar quadruplex binding constants. The disubstituted anthraquinones and acridines (top left in Fig. 6) have significant quadruplex interactions but limited selectivity over duplex interactions. The porphyrin derivative, TMPyP4, binds even more strongly to the telomere quadruplex but again with little selectivity over duplex interactions. Significant improvements in quadruplex targeting were made with the trisubstituted acridines [39,56,63] and seleno-sapphyrins [57,70] as illustrated in Fig. 6 with BRACO-19 and Se2SAP. A set of expanded acridine derivatives, such as RHPS4, shown at the top middle of Fig. 6, was designed by Stevens et al. [58,73–78] with a large planar pentacyclic ring system. NMR studies indicate that as with the other compounds of Fig. 6, RHPS4 selectively recognizes G-quadruplex DNA by stacking on the ends of the structure [74]. The compound is a strong inhibitor of telomerase and appears to target telomeres as the key component of its anticancer mechanism of action. It is the optimum therapeutic compound of a series synthesized by Stevens et al. [58]. SPR binding studies indicate that the compound has a telomere binding constant ($K_a \sim 1 \times 10^7$ M^{-1}) that is in the same range as the compounds on the left of the figure and is approximately 10 times higher than for duplex binding (unpublished results).

Macrocycles such as BOQ1 (right side of Fig. 6) [40] would appear to have an ideal structure to stack on G-tetrads with strong affinity and selectivity for quadruplex binding. BOQ1 does have a quadruplex binding constant in the same range as the other compounds in Fig. 6 with a selectivity over duplexes

of approximately 10. Given the size and synthetic complexity of this system, it is somewhat disappointing not to find a higher level of quadruplex affinity and selectivity. The macrocycle is, however, a much better quadruplex targeting agent than similar non-macrocycles, such as MOQ2 (Fig. 6), which have quadruplex binding constants that are approximately 10 times less than for BOQ1 with little selectivity over duplex binding. The bisintercalator, ditercalinium (bottom of Fig. 6), binds strongly to the telomere quadruplex but, as with the porphyrins, the bisintercalator binds as well to duplexes as to quadruplexes [79]. The classical intercalator ethidium (top of Fig. 6) and the carbazole derivative [80] shown at the bottom of the figure bind quite weakly to DNA quadruplexes, relative to the other compounds in the figure, and they illustrate some design problems for quadruplex targeted compounds. The two phenyl substituents of the carbazole system as well as the single phenyl substituent of ethidium are twisted out of the carbazole and phenanthridium ring planes. Placing these substituents in one of the quadruplex grooves apparently provides limited stabilizing interactions and at the same time may sterically reduce stacking of the heterocyclic ring systems with the large surface of the G-tetrad (Fig. 1).

Both TMPyP4 and the Se2SAP compounds also have out-of-plane phenyls but they bind strongly to quadruplex DNA. With these compounds the larger central ring system allows the phenyls to fit into quadruplex grooves and enhance affinity without disrupting stacking of the heterocyclic central ring. The size reference in Fig. 5 illustrates this point. The ethidium results in Fig. 6 agree with observations by Mergny et al. [81] that ethidium binds weakly to the human telomere quadruplex and more strongly to duplexes. They did show, however, that appropriately substituted ethidium analogs could bind strongly and selectively to quadruplexes. Their results also indicated that ethidium and the analogs bound on each end of the G-tetrad stack and not by intercalation or groove binding [81]. The compounds and results of Fig. 6 illustrate how sensitive quadruplex binding and selectivity are to compound shape, substituent type and position. They provide many directions for the design of new compounds to target quadruplexes by the end-stacking mechanism.

3.2. Compounds that stack in the grooves of DNA quadruplex structures

Since the grooves in quadruplex DNA structures have different geometries than with duplex DNAs as well as different patterns of donor–acceptor, hydrogen-bonding sites [44,82], targeting quadruplex structures through groove binding offers a very attractive strategy. There are, however, few known quadruplex groove-binding agents and this is clearly an under-developed area for G4 targeting. In order to investigate groove binding as a quadruplex binding mode, SPR experiments were conducted to quantify the binding of distamycin and DODC with the intramolecular human telomeric DNA sequence used for the studies in Fig. 6. It has previously been suggested that these two compounds may bind to the grooves of certain quadruplex DNA sequences [45–48,83], although more recent NMR results indicate that distamycin stacks on the ends of quadruplexes [84]. SPR results show that distamycin binds very weakly ($K_a < 10^5\ M^{-1}$) to the telomere DNA sequence and binding is likely to only be seen in high concentration experiments such as NMR. Screening experiments with other minor-groove binding polyamides (not shown) also did not reveal any significant quadruplex interactions. It seems unlikely that traditional polyamides will interact significantly with telomere, and probably other, DNA quadruplex structures. DODC binds with higher affinity ($K_a \sim 1–2 \times 10^5\ M^{-1}$) and is more promising as a quadruplex groove-binding model system. Since SPR is not capable of distinguishing between groove binding and intercalating-type end-stacking interactions, an alternate technique such as circular dichroism (CD) must be employed to evaluate the binding mode.

Circular dichroism pattern recognition has been used as a technique for determination of binding mode with duplex DNA-binding compounds [49]. Non-chiral molecules exhibit no CD signal in solution. However, when an achiral ligand binds tightly to a chiral host, such as DNA, a CD signal is induced in the wavelength region corresponding to the absorbance of the bound compound. Intercalating compounds usually produce negative induced CD signals or very small positive signals. Groove binding is generally indicated by the presence of a large positive induced CD signal upon titration of compound into duplex DNA. Cyanine derivatives, which form stacked complexes in the minor groove of duplex DNAs, exhibit strong exciton splitting in their induced CD spectra with duplexes [85–87]. Stacked species are of particular interest for recognition of quadruplexes since studies with duplex DNAs show that compounds that bind as stacked dimers have increased binding affinity and selectivity over similar compounds that bind as monomers [1–3,42,43,85,86]. Similar stacking in the grooves of quadruplex DNA structures would appear to be a favorable way to selectively recognize quadruplexes with optimum interactions, perhaps employing an induced fit component, between the stacked heterocycles and the G bases of the quadruplex tetrads.

CD titrations were performed on the intramolecular human telomere DNA with DODC as well as distamycin, to determine if any unusual induced CD signature existed which could be used for pattern recognition in the screening of potential quadruplex DNA-binding compounds (Fig. 7). As expected, the CD spectra of human telomeric DNA titrated with distamycin show virtually no change in CD signal, consistent with a weak end-stacking binding mode. Spectra for titration of telomere DNA with DODC, however, show a strong induced CD signal in the DODC absorption region (Fig. 7). Exciton splitting in the induced spectra indicates that DODC binds to the quadruplex grooves as one or more stacked species. As a control for the CD analysis, a titration was also performed with a well-characterized quadruplex end-stacking compound, ethidium [81,88]. With this compound there are no significant induced signals (Supplementary Materials, Fig. S5).

The DODC results are quite exciting and indicate that compound stacking in the quadruplex grooves is a promising approach for the design of highly selective quadruplex-targeting

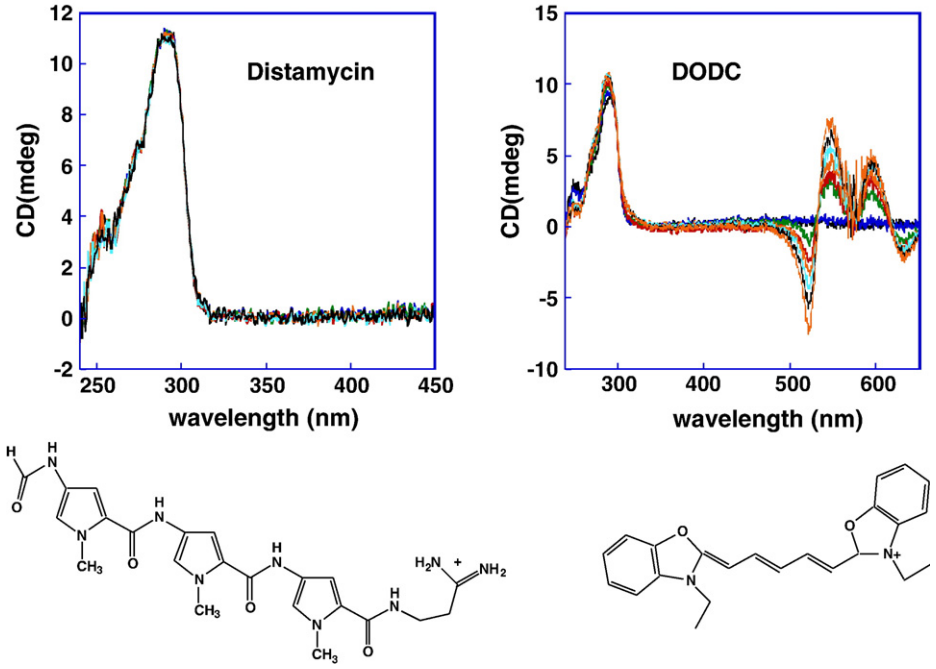

Fig. 7. CD spectra of distamycin and 3,3′-diethyloxadicarbocyanine (DODC) titrated into human telomeric DNA, d[$AG_3(T_2AG_3)_3$] in HEPES buffer containing 50 mM KCl are shown. The DNA concentration was 3.6 μM, with compound/DNA ratios ranging from 1:1 to 6:1 for distamycin. The DNA concentration was 3.1 μM, with compound/DNA ratios ranging from 0.5:1 to 3.5:1 for DODC. The spectra show no induced CD signal for distamycin, consistent with weak binding. A significant induced exciton signal is observed for DODC and the spectra are consistent with DODC binding to DNA as a stacked species.

agents. The results also indicate that the presence of an induced exciton CD signal can be used as a method to distinguish among binding modes. Boykin and Tidwell have designed a series of unfused aromatic heterocycles with terminal amidine substituents that are excellent groove-binding agents with duplex DNA [89]. The compounds also get into cells and are promising antiparasitic drug development candidates [13]. A library of heterocyclic diamidines synthesized by Boykin and coworkers was screened against the intramolecular human telomeric sequence using induced CD signals to differentiate binding modes as described above for DODC. A bifuryl compound, DB832, was identified as a potential stacked quadruplex groove binding agent. DB 832 binds to the human telomere with strong induced exciton CD signals (Fig. 8), suggesting binding as a stacked complex or complexes. The large magnitude of the signal suggests that it probably forms complexes in more than one groove of the human telomeric quadruplex. The induced CD signals produced by DB832 are very sensitive to DNA and compound structure. DB832 does not exhibit exciton splitting with either AT- or GC-rich duplex DNA sequences (d[GCGAATTCGC] and d[$(GC)_7$], respectively) nor does it exhibit any induced CD signal with the c-MYC quadruplex sequence, d[$AG_3TG_4AG_3TG_4A$] (Fig. 9). Selectivity of a compound for its target quadruplex structure is important in order to reduce cytotoxicity from duplex binding as well as prevent drug loss from binding to non-targeted quadruplex sites.

The induced exciton CD signal is also sensitive to the relative placement of the heterocyclic rings on the compound. DB 1093 is an isomer of DB 832 in which the furan groups are not adjacent. This compound does not exhibit any change in CD signal when titrated into human telomeric DNA (Fig. 10). This suggests that recognition of the quadruplex grooves can occur via a bifurcated hydrogen bond between the two adjacent furan

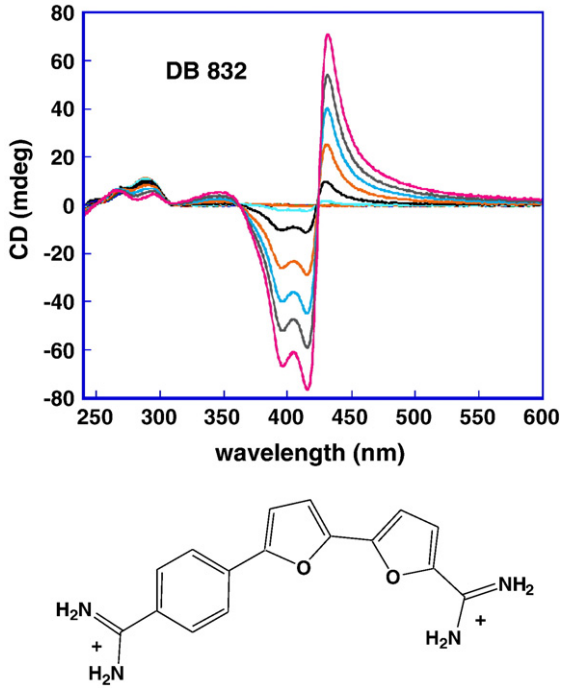

Fig. 8. CD spectra of DB832 titrated into a 3.3 μM solution of the human telomere quadruplex model system (d[$AG_3(T_2AG_3)_3$]) in HEPES buffer containing 50 mM KCl are shown. Compound/DNA ratios ranged from 0.5:1 to 5:1. The large magnitude of the induced exciton signal suggests binding as stacked species, perhaps in multiple grooves.

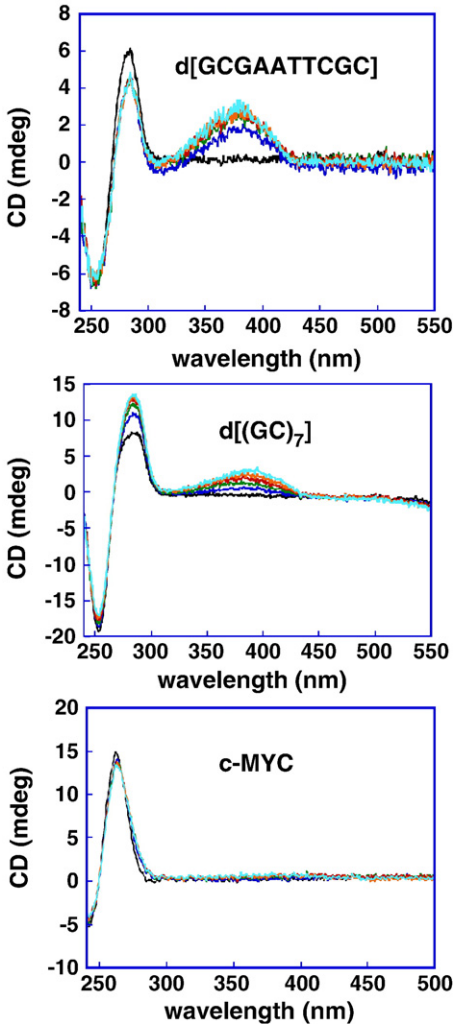

Fig. 9. CD spectra of DB832 titrated into a series of non-telomeric DNA sequences in HEPES buffer containing 50 mM KCl are shown. The top graph shows the spectra for DB 832 titrated into a 4.6 μM AT-rich duplex sequence, d[GCGAATTCGC]. The middle graph shows DB 832 titrated into a 5.6 μM GC-rich duplex sequence, d[$(GC)_7$]. The lower graph shows DB 832 titrated into a 2.30 μM promoter region quadruplex, d[$AG_3TG_4AG_3TG_4A$], from the c-MYC oncogene. Compound/DNA ratios ranged from 1:1 to 5:1 for each of the titrations. None of the spectra indicate induced exciton splitting, suggesting that DB 832 binds quite selectively as a stacked species in the grooves of the human telomeric DNA.

oxygens and a G-NH_2 amino group hydrogen in the quadruplex grooves. DB 914, a derivative of DB 832 with an additional phenyl ring, exhibits a similar, but somewhat weaker, CD pattern than DB 832 when titrated into human telomeric DNA (Fig. 10). This observation further suggests that the adjacent furans are involved in H-bonding interactions for recognition of quadruplex grooves. Additional recognition sites between DB 832 and DB 914 and the quadruplex groove may arise from hydrogen bonding between N3 of the guanine and an amidine hydrogen from the DB 832 molecule, as well as electrostatic interaction between amidines and phosphate groups on the DNA backbone. NMR studies to determine the orientation of the DB 832 molecules relative to quadruplex groove are currently in progress. These exciting preliminary results with potential quadruplex groove-binding compounds provide new directions for design of compounds for structure-specific targeting of DNA quadruplexes.

4. Summary and conclusions

- Recognition of specific structures of nucleic acids is a promising method to reduce the number of bases/base pairs required to achieve selectivity and affinity in the binding of synthetic compounds. Four-stranded quadruplexes formed from certain G-rich DNA sequences, such as the chromosomal telomere, are one such structure.
- G-tetrads are an important structural feature of quadruplex DNA and provide large, planar end surfaces onto which small molecules can bind. SPR results for binding of a number of synthetic aromatic cations with large planar surfaces to the ends of telomere DNA indicate that some compounds bind strongly and specifically with equilibrium constants near 5×10^7 M^{-1}. Both the magnitude of the binding constant for quadruplexes and the specificity over duplex interactions depend strongly on the size, charge and shape of the stacking ring system.
- Substituents on the ring system also have a major effect on the affinity and specificity. Moving from a 3,6-substituted acridine system, for example, to a 3,6,9-trisubstituted compound gives approximately a 10-fold increase in quadruplex affinity, a significant decease in dissociation rate constant, and an increase in selectivity over the duplex. ITC results with the trisubstituted acridine indicated that binding is enthalpy driven with a smaller favorable entropy of binding.
- The currently observed upper limit on the end-stacking equilibrium constant is approximately 10^8 M^{-1} with a specificity level over duplexes approaching 100. This affinity probably represents the limit of Gibbs energy that can be obtained with end stacking and some quadruplex groove contacts. Additional improvements on end stacking affinity and specificity will probably require systems with substituents that provide optimum contacts with as many quadruplex grooves as possible, in addition to favorable interactions with the G-tetrads.
- Since the grooves of quadruplex DNA structures have different groove geometries than duplex DNA, as well as different patterns of donor–acceptor sites, compounds that bind only in the grooves should be able to achieve excellent structure-specific recognition affinity and specificity. Investigation of a library of heterocyclic diamidine dications by using CD spectroscopy has identified a group of structurally related compounds that target quadruplex grooves as stacked species with characteristic strong exciton induced CD signals.
- Results show that these compounds bind very selectively as a stacked species to human telomeric DNA over duplex DNA as well as other quadruplex DNAs. The binding mode of these compounds is very sensitive to the relative placement and orientation of the heterocyclic systems. CD results

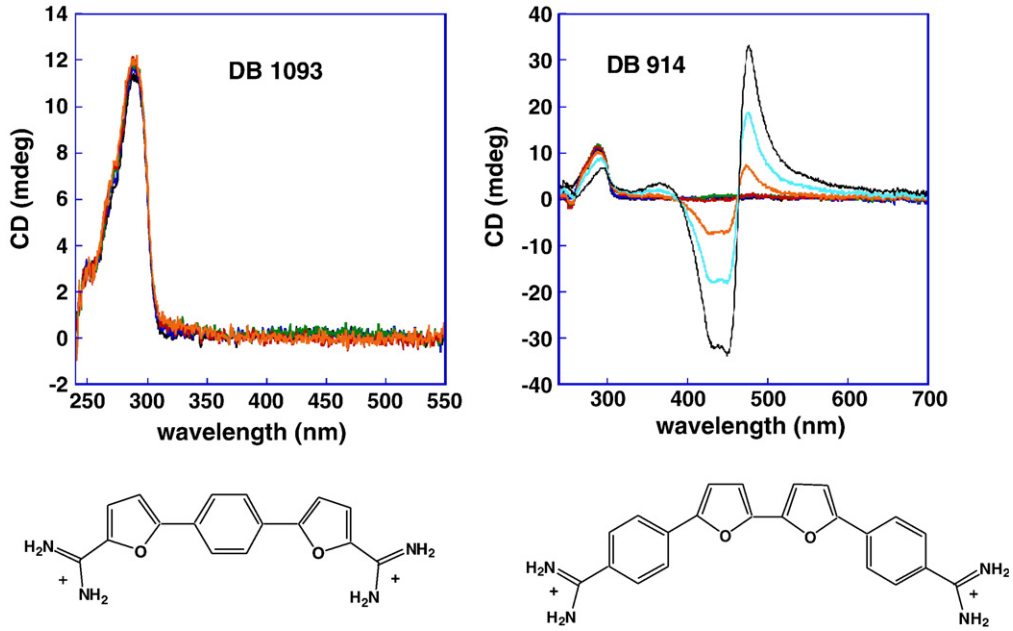

Fig. 10. CD spectra of DB 832 derivatives titrated into the telomere in HEPES buffer containing 50 mM KCl are shown. DB 1093 was titrated into 3.8 μM DNA, with compound/DNA ratios ranging from 1:1 to 4:1. DB 914 was titrated into 3.5 μM DNA with compound/DNA ratios ranging from 1:1 to 6:1. DB 914 exhibits exciton splitting, which suggests that adjacent furan groups may be involved in the recognition of the quadruplex groove.

suggest that this series of compounds must contain two adjacent furan groups, possibly for H-bonding recognition of the grooves as a stacked species.

- This group of compounds may serve as the starting point for the design of a new class of highly selective groove-binding molecules. Structure-specific recognition of quadruplex ends as well as grooves offers a very attractive strategy for the discovery and design of highly selective quadruplex DNA-binding ligands with attractive biological properties.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bpc.2006.06.006.

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