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Sequence and Length Dependent Thermodynamic Differences in Heterocyclic Diamidine Interactions at AT Base Pairs in the DNA Minor Groove

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Abstract

With the goal of developing a better understanding of the antiparasitic biological action of DB75, we have evaluated its interaction with duplex alternating and nonalternating sequence AT polymers and oligomers. These DNAs provide an important pair of sequences in a detailed thermodynamic analysis of variations in interaction of DB75 with AT sites. The results for DB75 binding to the alternating and nonalternating AT sequences are quite different at the fundamental thermodynamic level. Although the Gibbs energies are similar, the enthalpies for DB75 binding with poly(dA)-poly(dT) and poly(dA-dT)-poly(dA-dT) are +3.1 and -4.5 kcal/mole, respectively, while the binding entropies are 41.7 and 15.2 cal/mol-K, respectively. The underlying thermodynamics of binding to AT sites in the minor groove plays a key role in the recognition process. It was also observed that DB75 binding with poly(dA)-poly(dT) can induce T·A·T triplet formation and the compound binds strongly to the dT·dA·dT triplet.

Keywords

DB75; alternating and nonalternating AT DNAs; thermodynamics; differential scanning calorimetry; isothermal titration microcalorimetry; biosensor-surface plasmon resonance

1. Introduction

Synthetic organic cations that bind to the DNA minor groove provide important examples of clinically useful therapeutic agents, biotechnology reagents and compounds to probe the molecular basis of DNA recognition and control gene expression. Investigations on the molecular basis of DNA minor groove recognition by a variety of agents over the last several years have produced some interesting and thought-provoking results. As shown by DNA footprinting methods, well-characterized and structurally-diverse compounds, such as netropsin, Hoechst 33258 and berenil, which bind at AT sites in the DNA minor groove, can interact with a large variety of AT sequences [1,2]. For compounds of this size, a site of at least four base pairs in length is required but the affinities of the compounds for different AT sites can vary by a surprisingly large amount [3–5]. Early studies of netropsin binding to synthetic polydeoxyribonucleotides by Zimmer and coworkers[6,7] revealed sequence dependent differences in affinities and complex structures. The weak binding of the minor groove–

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targeted compounds listed above to GC containing sites has been explained by structural studies which show the compounds bound deep in the minor groove in AT sites and in close contact with the edges of AT base pairs at the floor of the groove [8–12]. This allows H-bonds to form with the TO2 and AN3 groups in AT base pairs but such close contact is sterically prevented by the 2-NH₂ group of G. The diversity of affinities for minor groove compounds with AT sequences, however, has not been extensively investigated.

The importance of the G-NH₂ group for binding specificity was verified by Bailly and Waring [13] who showed that replacement of G by inosine and substitution of 2,6-diaminopurine (DAP) for adenosine completely reversed the AT/GC binding specificity of minor groove binding agents [4]. Fox conducted a detailed footprinting study with netropsin, Hoechst 33258 and berenil with a designed DNA sequence that contained all possible sequences of four AT base pairs. The largest variations in affinity were found in comparison of sites such as AATT, which binds all three ligands quite strongly, and sites with a TA step such as TATA and TTAA, which bind the ligands significantly more weakly. With Hoechst 33258, for example, there is a fifty fold decrease in binding from the AATT to the TATA site [4]. Footprinting results with the AAAA and ATAT sequences, which are the same as in the synthetic polymers, poly(dA)·poly(dT) and poly(dA-dT)·poly(dA-dT), vary significantly among the compounds. Hoechst 33258 binds approximately ten fold better to the A₄ sequence, netropsin binds approximately three times better to that sequence but berenil shows no significant difference in affinity between the alternating and nonalternating sites. Berenil does bind at least ten fold more weakly to TTAA than to AATT, however, and it is clear that all of the minor groove binding compounds have different abilities to distinguish among equivalent length AT binding sequences [4].

Breusegem et al.[14], by using fluorescence changes on binding, found a 200 fold better affinity for Hoechst 33258 binding to AATT versus TTAA in DNA oligomers while a 30 fold difference was observed for the heterocyclic diamidine, DAPI, with the same sequences. It has been suggested that the difference among these sites is at least partly due to differences in groove width [15–17]. Sites such as AATT, for example, have a more narrow groove than TTAA and are in a pre-binding conformation that is better suited to complex formation with unfused heterocyclic compounds such as those discussed above. There are, no doubt, other contributing factors that result in individual compound differences, such as groove hydration and local sequence dependent helix axis bending [18].

Heterocyclic diamidines that target AT sequences and that have significant biological activity against specific DNA target sites in parasitic microorganisms [19–21] are of particular interest for drug development. A prodrug of the lead compound, DB75 (Figure 1), is currently in phase III clinical trials against trypanosome induced sleeping sickness and other compounds of this type are being tested against trypanosomes and other diseases in humans [19,22]. For detailed understanding of the biological mechanism of the diamidines as well as for drug design and development progress, it is important to develop a thorough understanding of the molecular recognition of different DNA sequences by these compounds. The underlying thermodynamics of binding to AT sites in the minor groove can be quite different and can play a key role in the recognition process. Leng and coworkers [23] have recently shown, for example, that the AT-hook transcription factor, HMGA, which targets AT sites in the DNA minor groove, has similar Gibbs energy for binding to alternating and nonalternating AT sites but quite different enthalpies of binding. Examples of enthalpy/entropy compensation have also been observed with minor groove binding drugs and different DNA AT sites. Binding of netropsin to poly(dA-dT)·poly(dA-dT), for example, is enthalpy driven but the binding to poly(dA)·poly(dT) is driven by entropy [24,25].

Diamidine compounds, such as DB75, have been shown to specifically target AT rich DNA sequences, such as those in the trypanosome mitochondrial kinetoplast DNA, and this can result

in differential biological activities or cell type targeting. The kinetoplast DNA (kDNA) has been shown by fluorescence microscopy studies to be the favored binding site of the compounds [22]. The kDNA is one of the most unusual nucleic acid structures found in any organism [26–28]. In *T. brucei*, the cause of sleeping sickness, the structure consists of approximately 50 DNA maxicircles of 20000–40000 base pairs. These are the coding DNAs of the mitochondria and are similar to the mitochondrial genomes of other organisms. There are approximately 10,000 minicircles of 1000 base pairs that are interlinked with the maxicircles to form a disk-shaped kinetoplast. The minicircles are AT rich, typically 70–75% AT base pairs, and contain phased A-tracts that lead to significant bending of the DNA helix. The extensive AT sequences in the minicircle DNAs form an attractive target for AT specific DNA binding compound. Even a small amount of interference with transcription and replication of minicircles can cause specific destruction of the complex interlocked kinetoplast structure and death of the trypanosome cell. A-tract bent DNA was discovered in early studies of minicircle DNAs from both trypanosome and *Leishmania* cells. DNA sequences for 25 minicircles have been reported and compared recently [29]. The minicircles contain nearly 100 AT sites of four or more contiguous AT base pairs that are potential binding receptor sites for diamidine drugs such as DB75 (Figure 1). Clearly, to better understand the mechanism of action of the diamidines against kinetoplastid parasites and to design a broader range of improved drugs, it is essential to investigate the interaction of diamidines, such as DB75, with DNAs of different AT sequences.

With the goal of developing a better understanding of the antiparasitic biological action of DB75 as well as to better understand the molecular recognition of DNA by this biologically important compound, we have evaluated its interaction with the duplex alternating and nonalternating sequence AT polymers and oligomers. These DNAs provide an important pair of sequences of an initial detailed analysis of variations in interaction of DB75 with AT sites of different sequence. Although the primary method used in the studies is isothermal titration calorimetry (ITC), results for ultraviolet thermal melting (T_m), circular dichroism (CD), biosensor–surface plasmon resonance (SPR) and differential scanning calorimetry (DSC) are also presented. The results for DB75 binding to the alternating and nonalternating AT sequences are quite different at the fundamental thermodynamic level. Because kinetoplast DNAs are closed circles with extended A-tracts, the possibility of fold back triple helical structures is significant and we have, therefore conducted a limited study of DB75 binding to the triple–helical polymer composed of T·A·T triplets. We observed that DB75 can induce and bind strongly to the triplex complementary to those results reported by Chaires et al.[30] for several diamidines.

2. Materials and methods

2.1. Compound, DNAs, and buffers

DB75 was synthesized as previous described [31]. Its purity was verified by NMR and elemental analysis. The double stranded polymers poly(dA)·poly(dT), poly(dA-dT)·poly(dA-dT) were purchased from Pharmacia (U. S. A.), and used for spectroscopic and calorimetric experiments. The hairpin oligomers used in this study were A_{15} [d(CCAAAAAAAAAAAGCCCCGCTTTTTTTTTTTTTTTGG)], $(AT)_7$ [d(CCATATATATATATATAGCCCCGCTATATATATATATATGG)], A_8 [d(CAAAAAAAAACCCCTTTTTTTTGG)] and $(AT)_4$ [d(CATATATATCCCCATATATATGG)], with the hairpin loop sequences underlined. In SPR experiments, three 5'-Biotin labeled hairpin DNA oligomers namely d(Biotin-CCA₁₅GCCCCGCT₁₅GG) (A_{15} hairpin), d(Biotin-CATATATATATATATACCCCTATATATATATATATGG) [$(AT)_7$ hairpin], d(Biotin-CGCGCGCGTTTTTCGCGCGCG) [$(CG)_4$ hairpin] were used. The above hairpin oligomers are all from Integrated DNA technologies, Inc. with reverse phased HPLC purification and

mass spectrometry characterization. The MES buffers used in these experiments contained 0.01 M [2-(N-morpholino) ethanesulfonic acid] (MES), 0.001 M EDTA, 0.1 M NaCl, pH 6.25.

2.2. UV melting studies

Ultraviolet melting curves were determined in 1 cm path length quartz cells using a Cary 300 UV–Visible spectrophotometer (Varian Inc., Palo Alto, CA), equipped with a thermoelectric temperature controller. Absorbance versus temperature profiles were measured at 260 nm with a heating rate of 0.5 °C/min. Experiments were generally conducted at a concentration of 4×10^{-5} M base pairs for polymeric DNA and 1.5×10^{-6} M oligomers for hairpin DNA. Thermal melting experiments for the DNA–compound complexes were conducted as a function of different ratios. Melting temperature (T_m) was taken as the temperature of half-dissociation of the DNA duplex or hairpin and was obtained from the maximum of the first derivative dA/dT plots (where A is absorbance and T is temperature).

2.3. CD titration and melting studies

CD spectra were recorded using a Jasco J–810 instrument with a 1cm cell and a scan speed of 50 nm/min with a response time of 1 s. The spectra from 500 to 220 nm were averaged over five scans. A buffer baseline scan was collected in the same cuvette and subtracted from the average scan for each sample. For isothermal titration studies, measurements were performed at 25 °C. The desired ratios of compound to DNA were obtained by adding DB75 to the cell containing a constant amount of DNA. For melting studies, CD spectra were collected at different temperatures from 10 °C to 95 °C. The compound to the polymeric DNA base pairs ratio was chosen as 0.25 and 0.5. Data processing and plotting were performed with Kaleidagraph software.

2.4. DSC melting studies

The DSC experiments were performed on a Microcal VP–DSC microcalorimeter (MicroCal Inc., Northampton, MA, USA). The polymeric DNA [poly(dA)·poly(dT) or poly(dA–dT)·poly(dA–dT)] solution at a concentration of 0.04 mM basepairs in MES10 buffer was used for all experiments. Prior to scanning, the buffer and the samples were vacuum–degassed. The experiments over the temperature range from 10 to 110 °C and a heating rate of 60 °C/h was used. Primary data were corrected by subtraction of a buffer–buffer baseline, normalized to the concentration of DNA (in base pairs) and further base line corrected. Data acquisition and analysis were performed with the Origin graphics software. The calorimetric enthalpy, ΔH_{cal} , was determined by integration of the area enclosed by the transition curve and the pre/posttransition baseline, and the melting temperature, T_m , was determined as the midpoint of the melting transition.

The binding constants for the DB75 complexes with the poly(dA)·poly(dT) or poly(dA–dT)·poly(dA–dT) duplex could be calculated using a T_m shift method and the following equation:

$$\Delta H_m(1/T_m^0 - 1/T_m) = R \ln(1 + K_{T_m} L)^{1/n} \quad (1)$$

where T_m^0 is the melting temperature of the DNA alone, T_m is the melting temperature in the presence of saturating amounts of ligand, ΔH_m is the enthalpy of DNA melting (per mol bp) determined by DSC, R is the gas constant, K_{T_m} is the ligand binding constant at T_m , L is the free ligand concentration and n is the ligand site size [32–34].

The DNA binding constant of DB75 at lower temperatures was estimated by use of the van't Hoff equation:

$$R \ln(K/K_{T_m}) = -\Delta H_b(1/T - 1/T_m) \quad (2)$$

Where K is the DNA binding constant of DB75 at temperature T , and ΔH_b is the enthalpy of binding of DB75 to DNA determined by ITC at the corresponding temperature [35].

2.5. ITC thermodynamic studies

The ITC experiments were performed with a MicroCal VP-ITC (MicroCal Inc., Northampton, MA, USA) interfaced with a computer for instrument control and data collection with Origin 5.0 software. In a typical titration, 7 μ l of a 0.08 mM DB75 solution in MES10 buffer was added every 300 s to a total of 40 injections to polymeric DNA [poly(dA)-poly(dT) or poly(dA-dT)-poly(dA-dT)] solution in the sample cell at 0.04 mM basepairs. The observed heat for each injection was determined by integration of the power peak area with respect to time. Blank titrations were conducted by injecting the compound into the sample cell containing only MES10 buffer under the same conditions. The corrected interaction heat was determined by subtracting the blank heat from that for the compound/DNA titration. The number of binding sites, equilibrium constants, binding enthalpy and entropy were obtained by fitting the corrected data to an appropriate binding model.

Binding enthalpies for different length of DNA hairpins [A_{15} , A_8 , (AT) $_7$, (AT) $_4$] with DB75 were determined using the “model-free ITC” protocol to obtain multiple estimates of ΔH° and to avoid any possible fitting bias [35,36]. This protocol, which uses a high DNA concentration, ensures that all titrated drug is effectively bound after each addition. Specifically, a 0.03 mM hairpin DNA solution was loaded into the sample cell and a 0.15 mM DB75 solution in MES10 buffer was titrated into DNA. Usually 20 injections of 7 μ l were done with 300 s between injections to ensure equilibration. The heat of reaction (ΔH) was obtained by integration of the peaks after each injection. The dilution heats, determined by injecting drug solution into the same sample cell loaded with buffer alone, were subtracted from the ΔH value determined for titration into hairpin DNA to render a corrected value for the binding-induced enthalpy change.

2.6. Biosensor-SPR studies

SPR measurements were performed with a four-channel BIAcore 2000 optical biosensor system (BIAcore Inc.). 5'-biotin labeled DNA samples [A_{15} hairpin, (AT) $_4$ hairpin and (CG) $_4$ hairpin] were immobilized onto streptavidin-coated sensor chips (BIAcore SA) as previously described [37]. 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 in filtered, degassed MES10 buffer with 5×10^{-3} % v/v Surfactant P20. Steady state binding analysis was performed with multiple injections of different compound concentrations over the immobilized DNA surface at a flow rate of 25 μ l/min and 25 $^\circ$ C. 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. 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 [37,38]. The number of binding sites and the equilibrium constant were obtained from fitting plots of RU versus C_{free} . Binding results from the SPR experiments were fit with two- (K_3 , $K_4 = 0$), three- ($K_4 = 0$) or four-sites interaction models:

$$r = \frac{(K_1 * C_{free} + 2 * K_1 * K_2 * C_{free}^2 + 3 * K_1 * K_2 * K_3 * C_{free}^3 + 4 * K_1 * K_2 * K_3 * K_4 * C_{free}^4)}{(1 + K_1 * C_{free} + K_1 * K_2 * C_{free}^2 + K_1 * K_2 * K_3 * C_{free}^3 + K_1 * K_2 * K_3 * K_4 * C_{free}^4)}$$

where r represents the moles of bound compound per mole of DNA hairpin duplex, K_1 , K_2 , K_3 , K_4 are macroscopic binding constants, and C_{free} is the free compound concentration in equilibrium with the complex.

3. Results

3.1. UV melting studies

Relative stabilities of the compound–DNA complexes were evaluated by temperature-dependent UV absorbance measurements at 260 nm (Figure 2 and Figure S1 in supporting information). Figure 2A shows the profiles of thermally induced denaturation of the complexes formed with poly(dA)·poly(dT) and poly(dA-dT)·poly(dA-dT) in the absence and presence of DB75 at ratios of 0.25 and 0.5 compound per base pair. The similar observed large increases in melting temperature indicate that DB75 binds strongly to both polymeric DNA samples. As can be seen, the melting profiles for poly(dA)·poly(dT) and poly(dA-dT)·poly(dA-dT) in the absence and presence of DB75 were significantly different in the low temperature region. For the alternating copolymer duplex poly(dA-dT)·poly(dA-dT), the melting profiles in the absence and presence of DB75 were all monophasic, representing a single transition that corresponds to the well-known duplex to single strand denaturation of poly(dA-dT)·poly(dA-dT). In contrast, a biphasic melting profile was observed for poly(dA)·poly(dT) in the presence of DB75. The higher temperature transition corresponds to the melting of the expected Watson–Crick (WC) duplex, while the lower temperature transition is assigned to the dissociation of polydT from polydA·2polydT triplex [the Hoogsteen (HG) transition] [30]. Without DB75 only the high temperature WC transition for poly(dA)·poly(dT) was evident. The triplex formation suggests that the polymer sample contains a slight excess of the polydT strand that can form a DB75 induced dT·dA·dT triplex under these conditions. The triplex formation is DNA sequence dependent as expected.

To investigate the effects of DB75 on inducing the triplex formation, UV melting experiments with poly(dA)·poly(dT) in the presence of increasing molar ratios of added DB75 were carried out (Figure S1C, Supporting information). Upon addition of increasing molar ratios of DB75, two transitions were evident in melting profiles. These equilibrium positions are summarized with a triplex→duplex→single strand phase transition plot of thermal stability versus DB75 ratio in Figure 2C (left). We observed significant effects of the DB75 ratio on the T_m corresponding to the triplex to duplex transition, which increases from 32 °C to 51 °C, while that of duplex to single strand transition increases from 64 °C to 90 °C [Figure 2C (left)] as the ratio is increased. These studies clearly show that triplex formation induced by DB75 binding is compound–DNA ratio and temperature dependent.

The profiles of thermally induced denaturation of the complexes formed with DNA hairpins [A₁₅, (AT)₇, A₈ and (AT)₄], Figure 1] in the absence and presence of DB75 at different ratios are shown in Figure 2B and Figure S1(A,B) (Supporting information). As expected, only one transition, corresponding to the unfolding of hairpin DNA, is observed in these melting profiles. The effect of DB75 ratio on the T_m of DNA hairpins is shown in Figure 2C (right). As can be seen, saturation binding is observed at compound–DNA ratios of 4:1 for A₁₅ and (AT)₇ hairpins, and 3:1 for A₈ and (AT)₄ hairpins.

3.2. CD titration and melting Studies

CD titration experiments as a function of DB75 concentration were utilized to monitor the binding mode and saturation limit for DB75 complexes with poly(dA)·poly(dT) and poly(dA-dT)·poly(dA-dT) and DNA hairpins, A₁₅, (AT)₇, A₈ and (AT)₄ (Figure 1). Results for the polymers in Figure 3 show that the CD spectral titrations of DB75 had large positive induced CD signals above 300 nm for both AT sequences. In the 300–500 nm region, free DB75 and

DNA do not exhibit CD signals. DNA does not absorb in this wavelength range and free DB75 is optically inactive. Positive CD signals arise from the interaction of the DB75 chromophore with the dissymmetric environment of the DNA minor groove [39,40]. The large positive induced CD signal for the complex is a characteristic pattern for a minor groove binding mode in AT sequences. Similar CD patterns at 267 and 391 nm for poly(dA)·poly(dT) (Figure 3A) and A₁₅ (Figure 3C) as well as for poly(dA-dT)·poly(dA-dT) (Figure 3B) and (AT)₇ (Figure 3D) are also observed. The induced CD signals at 267 and 391 nm were also plotted against compound–DNA ratio (insert in Figure 3). For the polymeric DNAs, the break in the plot at the ratio of ~0.25 indicates a binding site size of 4~5 base pairs DNA per compound, which agrees with the ITC results shown below. The saturated compound–DNA ratio obtained as 4 DB75/hairpin is also consistent with the UV melting (Figure 2B) and SPR experiments (shown below).

To investigate the melting of the complexes formed by both polymeric DNAs in the absence and presence of DB75, the temperature–dependent CD spectra at various wavelengths were monitored (Figure 4, Figure S3, Supporting information). In the presence of DB75 the CD melting curves of poly(dA)·poly(dT) at either 267 nm (Figure 4A) or 391 nm (Figure 4C) are clearly biphasic. In accordance with UV melting experiments, the lower temperature transition reflects dissociation of the polydT third strand from the polydA·2polydT triplex (the HG transition), whereas the higher temperature transition corresponds to the expected WC dissociation reaction. These CD–detected transitions reaffirm that DB75 binding induced the formation of triple helices. As the DB75 ratio increases from 0.25 to 0.5, the T_m for the HG and WC transition also increases, consistent with the UV melting experiments (Figure 2A). In contrast to the biphasic melting profiles for poly(dA)·poly(dT), the CD melting curves of poly(dA-dT)·poly(dA-dT) were monophasic in the presence of DB75 at 267 nm (Figure 4B) and 391 nm (Figure 4D), indicating only a single WC transition.

3.3. DSC melting studies

DSC can be used to quantitatively evaluate thermally induced structural transitions in DNA. Binding of DB75 to poly(dA)·poly(dT) and poly(dA-dT)·poly(dA-dT) was evaluated from the DSC results by using a T_m shift method (Figure 5, Equation 1). The DSC enthalpy of DNA melting (ΔH_m) and T_m are shown in Table 1. Values for the T_m in the presence and absence of DB75 are consistent in both UV melting and DSC experiments. In the presence of saturating concentrations of DB75, the T_m increases 26 °C for poly(dA)·poly(dT) and 23 °C for poly(dA-dT)·poly(dA-dT), which agrees with strong binding for both polymeric DNAs. By application of equations 1 and 2, described in Materials and Methods, the binding constant (K) at 25 °C was calculated as $5.6 \times 10^6 \text{ M}^{-1}$ for poly(dA)·poly(dT) and $5.4 \times 10^6 \text{ M}^{-1}$ for poly(dA-dT)·poly(dA-dT), respectively. These equations require the enthalpy of binding of DB75 to the polymeric DNAs (ΔH_b) at 25 °C and the ligand site size (n) determined by ITC, described below (Table 2). These K values agree with the binding constants obtained from ITC experiments (Table 2). By using the above method, we could obtain K at any temperature without assuming that ΔH_b is independent of temperature.

As observed in the UV and CD melting profiles (Figure 2A and Figure 4A), the heat capacity versus temperature curve for poly(dA)·poly(dT) binding with DB75 are also biphasic (Figure 5A), with the low–temperature transition centered around 50 °C due to the dissociation of the polydT third strand from the polydA·2polydT triplex (the HG transition) and the high–temperature transition at 90 °C due to the dissociation of the duplex strands (the WC transition). By contrast, a clear monophasic transition corresponding to WC transition appeared in the thermal denaturation profile for poly(dA-dT)·poly(dA-dT) binding with DB75 (Figure 5B). The UV and CD melting profiles and the DSC thermograms for poly(dA)·poly(dT) all reveal triplex formation induced by DB75 binding.

3.4. ITC thermodynamic studies

To probe the detailed energetic basis of the DB75 sequence dependent interaction differences in more detail and at a constant temperature, ITC experiments were conducted with the AT sequence DNAs (Figure 1). Calorimetric titration curves of poly(dA)-poly(dT) and poly(dA-dT)-poly(dA-dT) with DB75 at 25 °C (Figure 6) are completely different. A large positive enthalpy is observed in the ITC titration of DB75 into poly(dA)-poly(dT), while a large negative enthalpy is obtained on titration of DB75 into alternating poly(dA-dT)-poly(dA-dT) at the same conditions. The titration heat was converted to heat per mole as a function of total molar ratio with subtraction of the heat for a blank titration of DB75 into buffer with no DNA. The corrected isotherm was fitted to a 1:1 model to obtain a full set of thermodynamic parameters (Table 2). The binding stoichiometry of 4~5 base pairs of DNA per compound by ITC experiments is consistent with that obtained from CD titration experiments. At 25 °C DB75 binding to poly(dA)-poly(dT) is overwhelmingly entropy driven, whereas DB75 binding to poly(dA-dT)-poly(dA-dT) is enthalpy driven. The similarity in the DB75 binding free energies for both polymeric DNAs results from impressive enthalpy-entropy compensation, a common signature of minor groove binders [41,42].

Binding enthalpies for different length DNA hairpins (Figure 1) with DB75 were determined using a “model-free ITC” protocol at low binding ratios to avoid any fitting bias and influence by end effects for binding to oligomers [36]. Figure 6C and 6D show representative primary data from the titration of DB75 into A₁₅ and (AT)₇ hairpin DNA solution at 25 °C, respectively. As with polymeric DNAs, a large positive enthalpy was observed in ITC titration of DB75 into the A₁₅ hairpin and a large negative enthalpy for titration of DB75 into the alternating (AT)₇ hairpin. Integration of the peaks followed by normalization for the number of moles of added DB75 and subtraction of the blank heat provides a direct estimate of the binding enthalpy. The average binding enthalpy values are 2.9 kcal/mol for the A₁₅ hairpin and -4.6 kcal/mol for the (AT)₇ hairpin (Table 3). Interestingly, these values with the oligomer duplexes, closely match the endothermic and exothermic values for poly(dA)-poly(dT) and poly(dA-dT)-poly(dA-dT) (Table 2). The binding enthalpies for the still shorter A₈ and (AT)₄ hairpins with DB75, however, decreased relative to the polymers and 15mer hairpin sequences (Supporting Material, Figure S4). The average binding enthalpies are 0.064 kcal/mol for A₈ and -6.0 kcal/mol for (AT)₄, both substantially more negative than with the longer sequences. These results suggest that the A₁₅ and (AT)₇ hairpins are good models to mimic results with longer DNA binding sequences.

To investigate the sequence dependent binding differences for DB75 in more detail, the corrected binding isotherms for DB75 titrations into the two polymeric DNAs at different temperatures from 5 °C to 50°C were obtained (Supporting Material, Figure S5). Representative ITC titration and integrated heat results for the titration of DB75 into poly(dA)-poly(dT) and poly(dA-dT)-poly(dA-dT) at four different temperatures are plotted in Figure 7. Inspection of Figure S5A reveals that the binding enthalpies for poly(dA)-poly(dT) from 5 °C to 30 °C are strongly temperature dependent and decrease regularly (becoming more exothermic) as the temperature is increased. At temperatures above 30 °C, however, we observed abnormal binding enthalpy drops, indicating that an unusual DNA conformation transition occurred. These drops at higher temperature are clearly DNA-compound ratio dependent. Previous UV melting experiments for poly(dA)-poly(dT) (Figure 2C and Figure S1C in Supporting Material) showed that triplex formation induced by DB75 binding was DNA-compound ratio and temperature dependent. The phase diagram in Figure 2 suggests that we are observing a duplex-triplex transition at higher temperatures as DB75 is added to the duplex DNA.

Further inspection of Figure S5B revealed that the binding enthalpies for poly(dA-dT)-poly(dA-dT) at all studied temperatures were negative and their magnitude decreased regularly

with increasing temperature from 5 °C to 40 °C. When the temperature was above 45 °C, however, a larger, nonlinear decrease in binding enthalpy was observed. Since the T_m for poly(dA-dT)·poly(dA-dT) itself was around 55 °C (Figure 2A), it is likely that some pre-melted single strand DNA fractions fold back to poly(dA-dT)·poly(dA-dT) duplex on titration of DB75 into DNA solution at higher temperatures. In this case, the observed binding enthalpy is the sum of heat of binding interaction and heat of refolding DNA to give the abnormal large values relative to lower temperature results.

The corrected isotherms for poly(dA)·poly(dT) (from 5 °C to 30 °C) and poly(dA-dT)·poly(dA-dT) (from 5 °C to 40 °C) were fitted with 1:1 binding model to obtain the binding free energy, enthalpy and entropy (Figure 8). Because the free energy values change much less with temperature than the enthalpies, there was significant compensation in the enthalpy and entropy values as can be seen in Figure 8. No curvature in the plots of the thermodynamic constants *versus* temperature is apparent over the corresponding temperature range and all of the plots were fitted with linear functions. The experimental heat capacity changes for formation of the complexes were obtained from linear fitting of binding enthalpy as a function of temperature (Table 2). The calculated ΔC_p values for DB75 binding are all large and negative: for poly(dA)·poly(dT), $-174 \pm 7.3 \text{ cal mol}^{-1} \text{ K}^{-1}$; for poly(dA-dT)·poly(dA-dT), $-127 \pm 3.1 \text{ cal mol}^{-1} \text{ K}^{-1}$.

3.5. Biosensor–SPR studies

Because large K values are difficult to determine accurately at the concentrations generally required in diamidine–DNA ITC studies, the DB75–DNA binding constants were also evaluated by biosensor–SPR methods. Since ITC results show that the 15mer hairpins are good mimics for longer DNAs binding with DB75, 5′-Biotin labeled A_{15} , $(AT)_7$ and $(CG)_4$ hairpin sequences (Figure 1) were immobilized on three flow cells of a Biacore sensor chip and the fourth flow cell was left blank for reference subtraction. Sensorgrams for the interaction of A_{15} and $(AT)_7$ sequences with DB75 are compared in Figure 9 (a). The differences in interaction strength for all three sequences binding with DB75 could be more easily visualized in the plot of r (moles of compound bound/mole of hairpin DNA) versus C_{free} , the free compound concentration [Figure 9 (b)]. The data for DB75 binding with A_{15} and $(AT)_7$ hairpin are best fit with a four–site binding model and the $(CG)_4$ results were well fit using a two–site binding model (Table 3). As expected, DB75 binds strongly to A_{15} and $(AT)_7$ hairpins with similar interaction strength, but it binds quite weakly to the $(CG)_4$ sequence. From T_m melting (Figure 2B) and CD titration experiments (Figure 3C and D) of A_{15} and $(AT)_7$ hairpins binding with DB75, the compound–DNA binding ratio of 4:1 was obtained. These observations are in good agreement with the SPR fitting results, suggesting that one A_{15} or $(AT)_7$ sequence could bind four DB75 molecules. As described above, the average binding enthalpies for A_{15} and $(AT)_7$ hairpins have been obtained using the model–free ITC protocol (Table 3). From the experimental binding free energy and enthalpy values, the entropies for A_{15} and $(AT)_7$ hairpins could be calculated (Table 3).

4. Discussion

As described in the introduction, AT site specific minor groove binding diamidines are important for development of new antiparasitic therapeutics and for biotechnology reagents. A prodrug of DB75, for example is in Phase III clinical trials against sleeping sickness[43] and a derivative of DB75 is the basis of a highly sensitive fluorescence assay for trypanosomes resistant to melarsoprol, the primary drug currently used to treat late stage sleeping sickness [44]. These compounds target the AT rich sequences of kDNA minicircles with destruction of the trypanosome kinetoplast and cell death [22].

In spite of extensive studies of diamidines as well as other AT specific minor groove agents, there is little quantitative information on the energetic basis for their ability to distinguish among different sequences in AT binding sites such as those in kDNA. Available results for a small number of AT specific minor groove binding compounds indicate that binding affinities vary quite significantly among different sequences of all AT sites [4]. We have also discovered that similar variations can occur with sequence specific minor groove binding polyamides which recognize mixed sequences of DNA [45,46]. The polyamides have affinities for their cognate DNA binding sites, for example, that vary by over a factor of 1000. There are few detailed thermodynamic studies of the basis for the different affinities among minor groove binding sites. Given the increasing importance of diamidines as DNA-targeted therapeutics and biotechnology reagents, it is important to establish the variations in thermodynamic quantities that characterize their interactions at different sequence AT binding sites. Since a thermodynamic analysis of DB75 has been previously conducted with d (CGCGAATTCGCG)₂ [47] that compound has been used with the alternating and nonalternating DNA sequences of different length in the research reported here to probe the sequence dependent thermodynamics of binding. Since extended AT sequences in parasitic mitochondrial kinetoplast DNA have been shown to be the biological target of diamidines [22], these studies are part of the essential information needed for detailed understanding of the therapeutic activity of DB75 and for design of new drugs.

DB75 and a number of other diamidines have been crystallized with the self-complementary DNA duplex d(CGCGAATTCGCG)₂ [48]. The compounds bind in the minor groove at the -AATT- site and the complex is stabilized by H-bonding, van der Waals and electrostatic interactions. The curvature of DB75 lets it slide deeply into the minor groove and the twist of the diamidines allows them to H-bond with terminal T bases in the AATT sequences on opposite strands of the duplex [48]. In agreement with this binding mode, CD studies indicate that DB75 binds in a similar manner to the minor groove of the alternating and nonalternating AT sequences of different length (Figure 3). Melting experiments by UV, CD and DSC methods all show that the alternating and nonalternating AT sites have similar affinities for DB75. Triple-helical dT·dA·dT is not stable under the conditions of these experiments but the melting studies also show that DB75 can induce formation of the triplex in the nonalternating sequence as the ratio of DB75 to duplex is increased. By using competition dialysis and melting experiments, Chaires et al.[30] previously have shown that DB75 is a triplex stabilizing agent.

SPR and ITC experiments confirm that DB75 has similar affinities for the alternating and nonalternating AT sequences, but ITC results illustrate striking differences in DB75 interactions with these two types of sites. At 25 °C, for example, the nonalternating sequence has $\Delta H = +3.1$ kcal/mole while the alternating sequence binds with an exothermic enthalpy of -4.5 kcal/mole. At the same temperature $\Delta H = -2.2$ kcal/mole for the AATT oligomer[47]. The three AT sequences have very similar binding Gibbs energies but they obtain the similar affinities with a large variation in the underlying thermodynamics of binding. Similar results with the AT polymers have been seen with other AT specific minor groove binding compounds such as netropsin [24,49].

To better understand the thermodynamics for DB75 binding with poly(dA)·poly(dT) and poly(dA-dT)·poly(dA-dT), the observed binding free energy (ΔG_{obs}) at 25 °C for DB75 binding to DNA was divided into two primary contributions[50,51]:

$$\Delta G_{\text{obs}} = \Delta G_{\text{pe}} + \Delta G_{\text{t}} \quad (1)$$

where ΔG_{pe} is the electrostatic (polyelectrolyte) contribution, due mainly to coupled polyelectrolyte effects, the most important of which is the release of condensed counterions from the DNA helix upon ligand binding. ΔG_{t} is the nonpolyelectrolyte contribution, which arises from all other molecular interaction factors such as hydrophobic contacts, van der Waals

forces, H-bonding, etc. The polyelectrolyte term may be calculated using the relationship [52]:

$$\Delta G_{pe} = Z * \phi * RT \ln(M^+) \quad (2)$$

where Z is the charge on the ligand (for DB75, Z is equal to 2 for the two amidines charged, Figure 1), and ϕ is the proportion of total counterions associated with each DNA phosphate (about 0.88 for B-DNA duplex). (M^+) is salt concentration. For both polymeric DNAs binding with DB75:

$$\Delta G_{pe} = 2 * 0.88 * RT \ln(0.1) = -2.4 \text{ kcal mol}^{-1},$$

and ΔG_t is $-6.9 \text{ kcal mol}^{-1}$ and $-6.7 \text{ kcal mol}^{-1}$ respectively for poly(dA)·poly(dT) and poly(dA-dT)·poly(dA-dT).

Similarly, the observed binding enthalpy can be parsed into two contributions:

$$\Delta H_{obs} = \Delta H_{pe} + \Delta H_t \quad (3)$$

where ΔH_{pe} is assumed to be essentially zero [53], and ΔH_t equal to ΔH_{obs} . For poly(dA)·poly(dT) binding with DB75, ΔH_t was obtained as $3.1 \text{ kcal mol}^{-1}$, while for poly(dA-dT)·poly(dA-dT) binding with DB75, ΔH_t was obtained as $-4.5 \text{ kcal mol}^{-1}$. From the binding free energy and enthalpy values determined above, the enthalpy (ΔS) of complex formation could be calculated from equation (4):

$$\Delta G = \Delta H - T \Delta S \quad (4)$$

The obtained thermodynamics parameters are summarized in Table S1. To visualize the differences of thermodynamic forces more easily, the overall thermodynamic profiles for the interaction of poly(dA)·poly(dT) and poly(dA-dT)·poly(dA-dT) with DB75 are compared in Figure 10. As can be seen, DB75 binding to the non-alternating and alternating AT-tracts results in distinctive, sequence dependent, thermodynamic profiles. The minor groove of nonalternating A_nT_n sequences has been shown to be more narrow than that of alternating $(AT)_n$ by solution and x-ray studies [4,54–57]. The results also show a much more ordered hydration array in A_nT_n sequences and this feature can explain, at least quantitatively most observations in Figure 10. Displacement of water from AT sites on binding of DB75 results in a positive entropy contribution for both alternating and nonalternating AT sequence. The much larger $T \cdot \Delta S$ value for A_nT_n is expected from the more highly ordered water in its minor groove. Release of the minor groove water results in the breaking of a highly favorable H-bonded network with a positive enthalpy, however, DB75–DNA interactions for A_nT_n do not add enough negative enthalpy to overcome the positive term so that the binding enthalpy is positive until high temperature. The molecular interaction term adds more enthalpy than the breaking of the water network costs for $(AT)_n$ binding and the overall enthalpy for binding is exothermic. The release of counterions on binding of the dicationic DB75 also results in a positive entropy contribution, while the bimolecular association adds a negative term. These two values are essentially the same, however, for A_nT_n and $(AT)_n$ and the difference in binding enthalpy for the sequences must be dominated by hydration effects.

The ΔC_p value for DB75 binding to both AT polymers is negative in agreement with a binding mechanism involving extensive water release. The ΔC_p for the alternating polymer is close to the value observed for DB75 binding to the –AATT– site in a duplex oligomer[47]. The value is also close to the predicted value of ΔC_p based on changes in solvent accessible surface area on binding and calculated from an equation from Chaires and coworkers[36] based on small molecule–DNA experiments. The larger value of ΔC_p for DB75 binding to poly(dA)·poly(dT) again agrees with a more extensive and highly ordered water network in the minor groove of

this polymer. DB75 binding would disrupt the network over a larger region than the binding site and such extended disruption would result in a large experimental binding ΔC_p .

In summary, unlike minor groove binding, organic cations in general, DB75 has similar binding constants for binding to alternating and nonalternating AT sequences. As shown in Figure 10, however, the thermodynamics underlying the ΔG value for binding are completely different. The differences of ΔH , ΔS and ΔC_p on binding to the two DNAs, can be explained by models that involve differences in amount and order of water release from the minor groove with DB75 binding.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

ITC	isothermal titration microcalorimetry
DSC	differential scanning calorimetry
SPR	surface plasmon resonance
CD	circular dichroism
T_m	thermal melting
kDNA	kinetoplast DNA
MES	2-(N-morpholino) ethanesulfonic acid
RU	response units

poly(dA)·poly(dT): dA_n·dT_n

poly(dA-dT)·poly(dA-dT): d(AT)_n·d(AT)_n

Fig. 1.

Structure of the compound and the DNA sequences used in this study.

Fig. 2.

UV melting profiles at 260nm of the polymeric DNAs and DNA hairpins in the absence and presence of DB75. (A) Thermal melting curves of poly(dA)·poly(dT) and poly(dA-dT)·poly(dA-dT) at the indicated ratio. (B) Thermal melting curves of A15 and AT7 hairpins at the indicated ratio. (C) A phase diagram for T_m versus molar ratio for the thermal transitions of PolydA·PolydT (left) and DNA hairpins (right). The regions of triplex–duplex–single strand stability are labelled for the polymer. The oligomers have only duplex and single strand phase.

Fig. 3. CD titration spectra of the polymeric DNAs and DNA hairpins binding with DB75 at various mixing ratios. Insert: increase in CD magnitude at 391nm (○) and decrease in CD magnitude at 267nm (●).

Fig. 4. Temperature-dependent CD magnitude (relative to the CD at 10 °C, $CD_T/CD_{10^\circ C}$) of the polymeric DNAs and their DB75 complexes at 267 and 391 nm.

Fig. 5. DSC excess heat capacity (ΔC_p) versus temperature profiles for polymeric DNAs binding with DB75. Melting curves for poly(dA)·poly(dT) in the absence and presence of DB75 (A), and poly(dA-dT)·poly(dA-dT) in the absence and presence of DB75 (B).

Fig. 6. ITC curves for the binding of DB75 to the AT DNA polymers and hairpins in MES10 at 25 °C. Every peak represents the instrument response for injection of DB75 into DNA during the course of the titration (top). A binding isotherm from integration with respect to time, with appropriate dilution correction (bottom). For poly(dA).poly(dT) (A) and poly(dA-dT).poly(dA-dT) (B), the smooth lines show the fit to the results and best fit ΔH (Table 2) values for binding. For A₁₅ (C) and (AT)₇ (D) hairpins, a 'model-free ITC' protocol was used to obtain ΔH .

Fig. 7. Representative ITC titration and integrated heat data for DB75 binding to poly(dA)·poly(dT) (top) and poly(dA-dT)·poly(dA-dT) (bottom) at 10, 20, 30, 40 °C.

Fig. 8. Complete thermodynamic results for binding of DB75 to poly(dA)-poly(dT) (open) and poly(dA-dT)-poly(dA-dT) (solid) are shown as a function of temperature. Because the free energy changes much less with temperature than the enthalpy, there is significant enthalpy-entropy compensation as can be seen in the Figure. The ΔC_p values in Table 2 are the slopes of the ΔH° plots and no curvature is apparent over this temperature range.

Fig. 9. SPR binding affinity: (a) SPR sensorgrams for the interaction of A_{15} hairpin and $(AT)_7$ hairpin with DB75. The compound concentrations for DB75 from bottom to top are 0 to 1 μ M. (b) RU values from the steady-state region of SPR sensorgrams were converted to r ($r = RU/RU_{max}$) and are plotted against the unbound compound concentration (flow solution) for A_{15} (diamonds), $(AT)_7$ (circles) and $(CG)_4$ (squares) hairpins binding with DB75. The lines are the best fit values using appropriate binding models.

Fig. 10. Parsing the free energy (ΔG_{obs}) of polymeric DNAs binding with DB75 into the contributor energy terms. The two bars on the right represent $\Delta C_p^* = \Delta C_p/10$ in unit of $\text{cal mol}^{-1}\text{K}^{-1}$.

Table 1
DSC determined binding parameters for the interaction of DB75 with poly(dA)·poly(dT) and poly(dA-dT)·poly(dA-dT).^a

	T_m^0 (°C)	T_m (°C)	ΔH_m (kcal/mole)	K_{Tm} (M ⁻¹)	ΔH_b (kcal/mole)	K (M ⁻¹)
Poly(dA)·poly(dT)	66.1	92.2	9.84	1.82×10^7	3.11	6.94×10^6
Poly(dA-dT) ₂	59.5	82.4	7.16	1.57×10^6	-4.52	5.35×10^6

^aExperiments were carried out in MES10 buffer at 25°C. The ΔH_m values are from DSC and ΔH_b values are from ITC. The ΔH values have an experimental error of approximately ±5%.

Table 2
ITC determined binding parameters for the interaction of DB75 with poly(dA)-poly(dT) and poly(dA-dT)-poly(dA-dT).^a

	<i>n</i> (bp/drug)	<i>K</i> (M ⁻¹)	Δ <i>H</i> (kcal/mole)	TΔ <i>S</i> (kcal/mole)	Δ <i>G</i> (kcal/mole)	Δ <i>C_p</i> (cal mol ⁻¹ K ⁻¹)
Poly(dA)-poly(dT)	4.71	7.06×10 ⁶	3.11	12.4	-9.34	-174
Poly(dA-dT) ₂	4.01	4.51×10 ⁶	-4.52	4.54	-9.06	-127

^a Experimental error limits for ITC experiments are approximately ±5%.

Table 3
SPR determined binding parameters for the interaction of DB75 with A₁₅, (AT)₇ and (CG)₄ sequences.

	K_1 (M ⁻¹)	K_2 (M ⁻¹)	K_3 (M ⁻¹)	K_4 (M ⁻¹)	K_{mean}^a (M ⁻¹)	ΔG (kcal/mole)	ΔH^b (kcal/mole)	$T\Delta S$ (kcal/mole)
A ₁₅	2.99×10 ⁷	2.95×10 ⁷	2.34×10 ⁶	1.91×10 ⁶	1.59×10 ⁷	-9.81	2.94	12.8
(AT) ₇	5.09×10 ⁷	4.61×10 ⁷	3.79×10 ⁶	8.40×10 ⁵	2.54×10 ⁷	-10.08	-4.56	5.52
(CG) ₄	2.14×10 ⁶	2.63×10 ⁵	—	—	1.20×10 ⁶	—	—	—

^a $K_{\text{mean}} = (K_1 + K_2 + K_3 + K_4)/4$.

^b ΔH values are from “model-free ITC” experiments as described in Materials and Methods. Experimental error limits for SPR K values and “model-free ITC” ΔH values are both approximately ±10%.