# Sequence and Structural Selectivity of Nucleic Acid Binding Ligands[†]

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ABSTRACT: The sequence and structural selectivity of 15 different DNA binding agents was explored using a novel, thermodynamically rigorous, competition dialysis procedure. In the competition dialysis method, 13 different nucleic acid structures were dialyzed against a common ligand solution. More ligand accumulated in the dialysis tube containing the structural form with the highest ligand binding affinity. DNA structural forms included in the assay ranged from single-stranded forms, through a variety of duplex forms, to multistranded triplex and tetraplex forms. Left-handed Z-DNA, RNA, and a DNA−RNA hybrid were also represented. Standard intercalators (ethidium, daunorubicin, and actinomycin D) served as control compounds and were found to show structural binding preferences fully consistent with their previously published behavior. Standard groove binding agents (DAPI, distamycin, and netropsin) showed a strong preference for AT-rich duplex DNA forms, along with apparently strong binding to the poly(dA)−[poly(dT)]$_2$ triplex. Thermal denaturation studies revealed the apparent triplex binding to be complex, and perhaps to result from displacement of the third strand. Putative triplex (BePI, coralyne, and berberine) and tetraplex [$H_2$TmPyP, 5,10,15,20-tetrakis[4-(trimethylammonio)phenyl]-21*H*,23*H*-porphine, and *N*-methyl mesoporphyrin IX] selective agents showed in many cases less dramatic binding selectivity than anticipated from published reports that compared their binding to only a few structural forms. Coralyne was found to bind strongly to single-stranded poly(dA), a novel and previously unreported interaction. Finally, three compounds (berenil, chromomycin A, and pyrenemethylamine) whose structural preferences are largely unknown were examined. Pyrenemethylamine exhibited an unexpected and unprecedented preference for duplex poly(dAdT).

Intense interest exists in the design and synthesis of small molecules that might selectively bind to defined sites in DNA or RNA (*1*). Targeting particular sequences within right-handed, B-form DNA is one approach to producing the desired selectivity (*2*). Sequence selectivity might exploit the unique, sequence-dependent patterns of hydrogen bond donors and acceptors within the major and minor grooves of DNA. The design of ligands capable of sequence-specific DNA binding was recently realized by the Dervan group with the development of the hairpin polyamides (*3*−*6*). The recognition code for the hairpin polyamides was elucidated (*4*), and the effectiveness of these molecules as selective inhibitors of gene expression in vivo was demonstrated (*7*).

Another possible approach to selective nucleic acid binding is to target unique nucleic acid structures. In this strategy, unique molecular shapes would be targeted. DNA and RNA are both polymorphic, and exist in a variety of structural forms that might provide unique binding sites for small molecules (*8*). Although DNA exists predominantly in a right-handed duplex form in the genome, specific regions of the genome can exist in single-stranded form, or can adopt multistranded structures such as triplexes or tetraplexes. In addition, duplex DNA can adopt a variety of secondary structures depending on its sequence and solution environment. These secondary structures include (among many possibilities) the normal B form, the A form, and the left-handed Z form. Alternate DNA structures may play a role in the control of gene expression and may represent attractive targets for small molecule therapeutics. As but one possible example, tetraplex DNA appears to be an integral part of telomeres and is a substrate for telomerases involved in chromosome replication. Compounds that stabilize tetraplex DNA within telomeres might effectively block telomerase activity by locking the nucleic acid substrate into an unfavorable conformation for its replication. Such small molecules may be potentially valuable as therapeutic agents (*9*).

One problem in the discovery of structurally selective small molecules is the lack of convenient methods for evaluating structural preferences. Typically, equilibrium binding assays or thermal denaturation studies are used. These methods are both labor intensive and comparatively slow, and are not well suited for screening large libraries of candidate compounds. In the case of thermal denaturation, the underlying physical basis of ligand stabilization is a complex function of many thermodynamic parameters (*10*). These include the ligand binding constant, binding enthalpy, and site size. Relating a ligand-induced shift in $T_m$ to binding affinity is not straightforward. Further, at less than saturating ligand concentrations, melting curves may become multiphasic due to ligand redistribution, further complicating data interpretation. A more suitable alternative for evaluating

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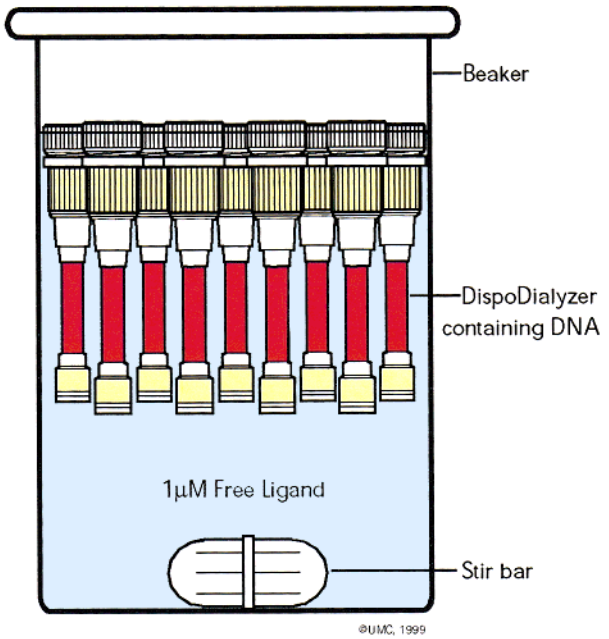


FIGURE 1: Schematic diagram of the competition dialysis experiment. Multiple disposable dialysis units, each containing 0.5 mL of a 75 $\mu$M solution of one of the DNA structures listed in Table 1, are placed in a beaker containing 200 mL of a 1 $\mu$M ligand solution. The system is allowed to equilibrate for 24 h with continuous stirring. The amount of ligand bound to each DNA structure is determined spectrophotometrically as described in Materials and Methods.

ligand structural preference is described and utilized here, a competition dialysis method that provides a rigorous, thermodynamically sound indication of structural selectivity.

Figure 1 shows a schematic diagram of the competition dialysis experiment. The method evolved from a technique first used by Muller and Crothers to explore the base specificity of DNA intercalation reactions (*11*, *12*). In the new application, we have devised a suitable buffer in which a variety of DNA and RNA structures are stable. Table 1 lists these structures, along with some of their physical properties. In the competition dialysis experiment, equal volumes of these DNA samples (at identical concentrations) are dialyzed against a common dialysate solution containing the ligand being studied. After equilibrium dialysis is attained (usually in $\leq$24 h), the amount of ligand bound to each DNA is measured by a simple absorbance or fluorescence assay. Since all of the DNA samples are in equilibrium with the same free ligand concentration, the amount of bound ligand is directly proportional to the association constant for ligand binding to a particular structure. Comparison of the amount of ligand bound to each DNA sample provides a rapid, thermodynamically rigorous indication of the structural selectivity of the ligand being studied. The advantages of the new method are many, including its speed, its sound thermodynamic basis, and its relative ease.

## MATERIALS AND METHODS

*Nucleic Acids.* *Clostridium perfringens* (lot 86H4010), *Micrococcus lysodeikticus* (lot 108H4017), and calf thymus (lot 95H9526) DNA samples were purchased from Sigma Chemical Co. (St. Louis, MO) and were sonicated, phenol extracted, and purified as previously described (*13*). Poly(dA) (lot 7067836021), poly(dT) (lot 8017834021), poly(dA)−poly(dT) (lot 8097860021), poly(dAdT) (lot 8067870021), and poly(dGdC) (lot 8107910021) were purchased from Pharmacia Biotech, Inc. (Piscataway, NJ). Poly(rA) (lot 97F-4070) and poly(A)−poly(U) (lot 10H4005) were purchased from Sigma Chemical Co. Synthetic polynucleotides were used without further purification. Solutions containing the poly(rA)−poly(dT) DNA−RNA hybrid and the poly(dA)−[poly(dT)]$_2$ triplex were prepared by mixing poly(rA) or poly(dA)−poly(dT) with poly dT in a 1:1 molar ratio, heating to 90 °C, and slowly cooling to room temperature. Tetraplex DNA [(5′-$T_2G_{20}T_2$)$_4$] was prepared by heating 5′-$T_2G_{20}T_2$ (from Research Genetics, Huntsville, AL) to 90 °C for 2 min, slowly cooling to room temperature, and then equilibrating for 48 h at 4 °C before use. Left-handed, Z-DNA was prepared by bromination of poly(dGdC) as previously described (*14*).

*Ligand Molecules.* Daunorubicin (lot 116H06752), ethidium bromide (lot 75F0228), coralyne chloride (lot 106C0362), chromomycin (lot 106C0362), distamycin (lot 93F0141), actinomycin D (lot 96H4005), berberine (lot 68H1028), berenil (lot 122F00161), and BePI[1] (lot 37H0494) were purchased from Sigma Chemical Co. and were used without further purification. 1-Pyrenemethylamine hydrochloride (lot 02724KS) and 5,10,15,20-tetrakis[4-(trimethylammonio)-phenyl]-21*H*,23*H*-porphine (lot 06631MR) were purchased from Aldrich Chemical Co. (Milwaukee, WI). DAPI (lot 8A) was obtained from Molecular Probes, Inc. (Eugene, OR). $H_2$TMPyP [meso-tetrakis(*N*-methyl-4-pyridyl)porphine] (lot 071498) and NMM (*N*-methyl mesoporphyrin IX) (lot 080797) were purchased from Porphyrin Products, Inc. (Logan, UT). Netropsin was purchased from Serva Feinbiochemica (Heidelberg, Germany).

Table 1: Nucleic Acid Conformation and Samples Used in Competition Dialysis Experiments[a]

| conformation | DNA or oligonucleotide | $\lambda$ (nm) | $\epsilon$ | $T_m$ (°C) | $H$ |
|---|---|---|---|---|---|
| single-stranded purine | poly(dA) | 257 | 8600 | − | 1.24 |
| single-stranded pyrimidine | poly(dT) | 264 | 8520 | − | − |
| duplex DNA | *C. perfringens* (31% GC) | 260 | 12476 | 82.5 | 1.28 |
| | calf thymus (42% GC) | 260 | 12824 | 85.5 | 1.36 |
| | *M. lysodeikticus* (72% GC) | 260 | 13846 | >100 | 1.16 |
| | poly(dA)−poly(dT) | 260 | 12000 | 75.2 | 1.58 |
| | [poly(dAdT)]$_2$ | 262 | 13200 | 67.8 | 1.53 |
| | [poly(dGdC)]$_2$ | 254 | 16800 | >100 | − |
| DNA−RNA hybrid | poly(rA)−poly(dT) | 260 | 12460 | 70.9 | 1.49 |
| duplex RNA | poly(rA)−poly(U) | 260 | 14280 | 62.5 | 1.54 |
| Z-DNA | Br[poly(dGdC)]$_2$ | 254 | 16060 | >100 | − |
| triplex DNA | poly(dA)−[poly(dT)]$_2$ | 260 | 17200 | 42.5 | 1.59 |
| tetraplex DNA | (5′-$T_2G_{20}T_2$)$_4$ | 260 | 39267 | 89.0 | 1.06 |

[a] Spectra and melting curves were obtained in a buffer consisting of 6 mM $Na_2HPO_4$, 2 mM $NaH_2PO_4$, 1 mM $Na_2EDTA$, and 185 mM NaCl (pH 7.0). $\lambda$ is the wavelength. $\epsilon$ is the molar extinction coefficient ($M^{-1}$ $cm^{-1}$). $T_m$ is the melting temperature. $H$ is the hyperchromicity ($H = A_{final}/A_0$). Concentration units of the extinction coefficient are expressed in terms of the monomeric unit that comprises the polymer, i.e., nucleotides, base pairs, triplets, and tetrads.

[1] Abbreviations: BePI, 7*H*-8-methylbenzo[*e*]pyrido[4,3-*b*]indole; $H_2$-TMPyP, meso-tetrakis(*N*-methyl-4-pyridyl)porphine; NMM, *N*-methyl mesoporphyrin IX; EDTA, ethylenediaminetetraacetic acid; DAPI, 4,6-diamidino-2-phenylindole.

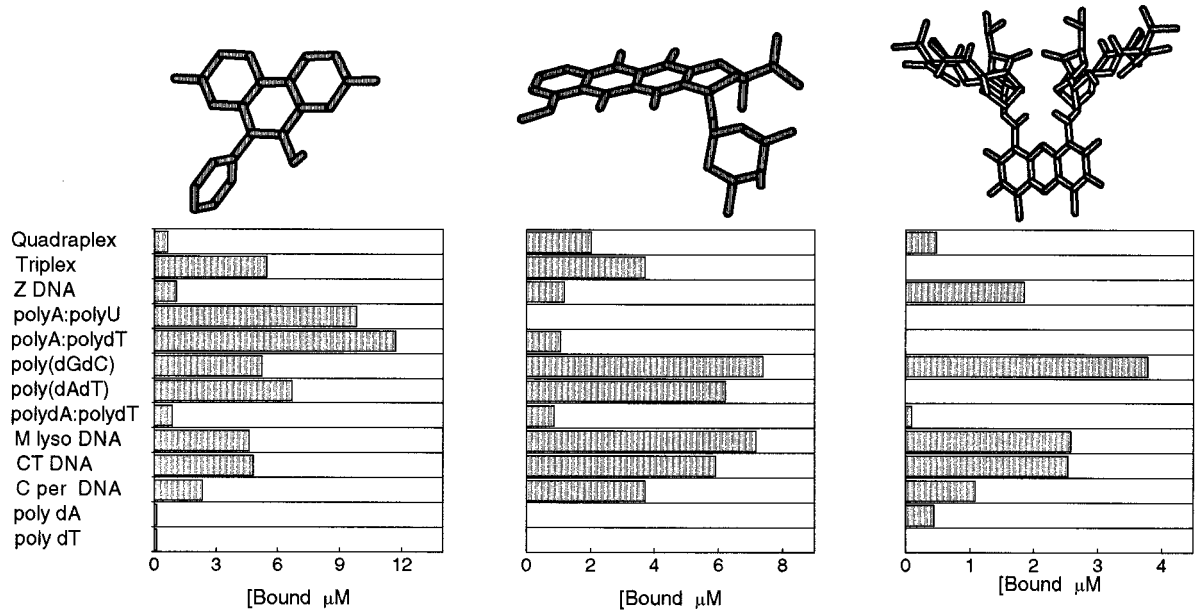


FIGURE 2: Results obtained by the competition dialysis method for common intercalators. The amount of ligand bound to each DNA structure is shown as a bar graph. The molecular structure of each intercalator is shown above each graph. The intercalators that were studied were ethidium (left), daunomycin (middle), and actinomycin D (right).

*Concentration Determinations*. Concentrations of nucleic acid samples were determined by UV absorbance measurements using the extinction coefficients and absorbance maxima listed in Table 1. Ligand concentrations were determined by visible absorbance measurements using extinction coefficients listed in the Supporting Information.

*Quality Control of Nucleic Acid Samples*. The quality of each nucleic acid sample was evaluated by recording their UV absorbance spectrum, their CD spectrum, and their thermal denaturation profile. Data from these measurements are shown in the Supporting Information for each nucleic acid structure included in the assay.

*Competition Dialysis Assay*. A buffer consisting of 6 mM $Na_2HPO_4$, 2 mM $NaH_2PO_4$, 1 mM $Na_2EDTA$, and 185 mM NaCl (pH 7.0) was used for all experiments. For each competition dialysis assay, 200 mL of the dialysate solution containing 1 $\mu$M ligand was placed into a beaker. A volume of 0.5 mL (at 75 $\mu$M monomeric unit) of each of the DNA samples listed in Table 1 was pipeted into a separate 0.5 mL Spectro/Por DispoDialyzer unit (Spectrum, Laguna Hills, CA). All 13 dialysis units were then placed in the beaker containing the dialysate solution. The beaker was covered with Parafilm and wrapped in foil, and its contents were allowed to equilibrate with continuous stirring for 24 h at room temperature (20−22 °C). At the end of the equilibration period, DNA samples were carefully removed to microfuge tubes, and were taken to a final concentration of 1% (w/v) sodium dodecyl sulfate (SDS) by the addition of appropriate volumes of a 10% (w/v) stock solution. The total concentration of drug ($C_t$) within each dialysis unit was then determined spectrophotometrically using wavelengths and extinction coefficients appropriate for each ligand (see Table S1 in the Supporting Information). An appropriate correction for the slight dilution of the sample resulting from the addition of the stock SDS solution was made. The free ligand concentration ($C_f$) was determined spectrophotometrically using an aliquot of the dialysate solution, although its concentration usually did not vary appreciably from the initial concentration of 1 $\mu$M. The amount of bound drug was determined by difference ($C_b = C_t - C_f$). Data were plotted as a bar graph using Orgin software (version 5.1, Microcal, Inc., Northampton, MA).

*UV Melting Studies.* A buffer consisting of 6 mM $Na_2HPO_4$, 2 mM $NaH_2PO_4$, 1 mM $Na_2EDTA$, and 185 mM NaCl (pH 7.0) was used for all melting experiments. Ultraviolet DNA melting curves were determined using a Cary 3E UV/visible spectrophotometer (Varian, Inc., Palo Alto, CA), equipped with a thermoelectric temperature controller. Solutions of DNA (final concentration of $2.0 \times 10^{-5}$ M) were prepared by direct mixing with aliquots from a ligand stock solution, followed by incubation for 12 h at 24 °C to ensure equilibration. Samples were heated at a rate of 1 °C/min, while the absorbance was being continuously monitored at 260 nm. Primary data were transferred to the graphics program Origin (Microcal, Inc.) for plotting and analysis.

*Fluorescence Titration Experiments*. Fluorescence titrations were conducted and analyzed as previously described (*15*).

## RESULTS AND DISCUSSION

We have utilized the competition dialysis assay to evaluate the structural selectivity of 15 ligands representing a variety of chemical classes. The results demonstrate the validity, utility, and value of the method as a rapid screening tool for structural selectivity. More importantly, the range and variety of small molecule structural selectivity will be clearly illustrated. Results obtained with common intercalators and groove binding agents with known binding preferences will first be described. Studies using compounds reported to be triplex selective will then be presented, followed by studies using several porphyrin compounds reported to be selective for tetraplex DNA. Finally, results from several compounds with previously unknown structural selectivities will be described.

Figure 2 shows results obtained for the common intercalating agents ethdium, daunorubicin, and actinomycin D.

Ethidium (Figure 2, left) is the prototypical intercalator, whose interactions with nucleic acids have been extensively investigated. Competition dialysis results obtained with ethidium will be described in detail, to introduce the method, the presentation of data, and data interpretation.

Competition dialysis results for ethidium are shown (Figure 2, left) as a bar graph in which the amount of ethidium bound is shown for each DNA structure included in the assay. These results were obtained after equilibration for 24 h, using 1 $\mu$M ethidium in the dialysate solution and a nucleic acid concentration of 75 $\mu$M in each sample dialysis tube. Nucleic acid concentrations are expressed in terms of the monomeric unit that comprises the polymer. This means nucleotide (nt) concentration for single-stranded forms, base pair (bp) concentrations for duplex forms, triplet concentrations for the triplex, and tetrad concentrations for the tetraplex. The data (Figure 2, left) show that there was no appreciable binding of ethidium to single-stranded forms [i.e., poly(dT) or poly(dA)]. Ethidium binds well to most duplex DNA forms, from both natural origin and synthetic deoxypolynucleotide duplexes. The exception is poly(dA)−poly(dT), to which ethidium binds poorly as do most intercalators, an effect that is well-known and which is due to the unusual structure adopted by the polymer in solution (*16*). To our surprise, ethidium bound most avidly to RNA and to the DNA−RNA hybrid, represented by poly(A)−poly(U) and poly(A)−poly(dT), respectively. A careful review of the literature, however, revealed equilibrium binding studies that supported this observation (*17*, *18*). Significant binding to the triplex poly(dA)−$[poly(dT)]_2$ was observed, fully consistent with literature reports that have characterized that interaction (*19*). Only slight binding to a parallel-stranded tetraplex form $[(T_2G_{20}T_2)_4]$ and to left-handed Z-DNA was observed (more discussion of the latter will be found below). In summary, ethidium prefers to bind to RNA and the DNA−RNA hybrid, but also interacts strongly with normal duplex DNA and with poly(dA)−$[poly(dT)]_2$ triplex DNA. It does not bind appreciably to single-stranded forms, left-handed DNA, or tetraplex DNA.

Appropriate controls (not shown) using ethidium were carried out to investigate optimal conditions for the competition dialysis experiment. These controls included experiments in which both nucleic acid and ethidium concentrations were systematically varied, and others in which the time to reach dialysis equilibrium was carefully evaluated. On the basis of these studies, we set as standard conditions for our particular buffer and equilibrium dialysis apparatus a 24 h dialysis period and concentrations of 1 $\mu$M for ligand and 75 $\mu$M for nucleic acid. For all of the compounds described here, a careful mass balance computation was carried out, the results of which showed that no more than 5−10% of the ligand sample was lost to adsorption to the dialysis tubing or the surface of the container. From studies in which competition dialysis experiments were repeated three to five times for ethidium (and selected other ligands), we estimate that the error in estimates of the amount of ligand bound to each nucleic acid form is no more than 10%.

Results obtained for the anticancer agent daunorubicin are shown in the middle panel of Figure 2. The pattern of structural selectivity is distinct from that observed for ethidium. Daunorubicin binds preferentially to right-handed duplex forms. Within this category, it shows a clear preference for GC base pairs, as judged by its increased level of binding to *M. lysodeikticus* (71% GC) DNA relative to that to *C. perfringens* (31% GC) DNA. This result is fully consistent with its known sequence selectivity (*12*, *20*, *21*). In contrast to ethidium, daunorubicin binds poorly, if at all, to RNA and to the DNA−RNA hybrid. Daunorubicin interacts to some extent with triplex and tetraplex forms. Its apparent Z-DNA binding will be discussed below.

Actinomycin D (Figure 2, right) exhibits a selectivity pattern distinct from those of both ethidium and daunorubicin. It prefers binding to right-handed duplex forms, but shows a strong preference for GC base pairs, with no binding at all to duplex deoxypolynucleotides containing only AT base pairs. Actinomycin D shows some binding to poly(dA) and to tetraplex DNA. Actinomycin D shows appreciable binding to Z-DNA, but further investigation showed that this behavior is somewhat illusory. Ethidium, daunorubicin, and actinomycin D all exhibited some apparent binding to Z-DNA. Circular dichroism studies (not shown) revealed, however, that these compounds were in fact allosterically converting the left-handed form of the polymer to a right-handed form, with stable complexes formed only with the latter. Such behavior was fully investigated previously by this laboratory for daunorubicin (*22*) and by the Krugh laboratory for ethidium and actinomycin D (*23*, *24*).

The striking general features of the data presented in Figure 2 deserve emphasis at this point. The competition dialysis method allowed 13 structures and three different compounds to be examined rapidly, within a 24 h period. The pattern of structural selectivity of each compound was unique and characteristic. The method accurately reproduced the known binding preferences for these intercalators, and provided a thermodynamically sound screening procedure as intended.

Figure 3 shows results obtained for three common groove-binding agents, distamycin, netropsin, and DAPI. The selectivity patterns for these three compounds are similar, but not identical. The selectivity patterns found for these groove-binding agents are clearly distinct from those found for the intercalators (Figure 2). In general, all of these agents prefer right-handed duplex DNA forms, with a strong preference for AT base pairs, and virtually no binding to poly(dGdC). There is apparent binding to the triplex poly(dA)−$[poly(dT)]_2$, but this will be examined in more detail in the next paragraph. None of these compounds appears to bind to the RNA sample, but DAPI shows slight binding to the DNA−RNA hybrid. Slight binding of netropsin and DAPI to the tetraplex is observed.

The interaction of the groove-binding agents with poly(dA)−$[poly(dT)]_2$ triplex DNA was examined more fully by melting experiments, with the results shown in Figure 4. In the absence of ligand, melting of poly(dA)−$[poly(dT)]_2$ is biphasic, with the third strand dissociating at a $T_m$ of 42.5 °C and the remaining duplex melting at a $T_m$ of 75.2 °C. The melting curves shown in panels A and B of Figure 4 show that the addition of distamycin and netropsin results in a destabilization and eventual displacement of the third strand. These compounds bind to and stabilize the free duplex. DAPI (Figure 4C) apparently does not displace the third strand, but stabilizes only the duplex form. We conclude from these ancillary studies that the apparent binding of these agents to poly(dA)−$[poly(dT)]_2$ triplex DNA seen in Figure 3 may be illusory, and may result from displacement of the

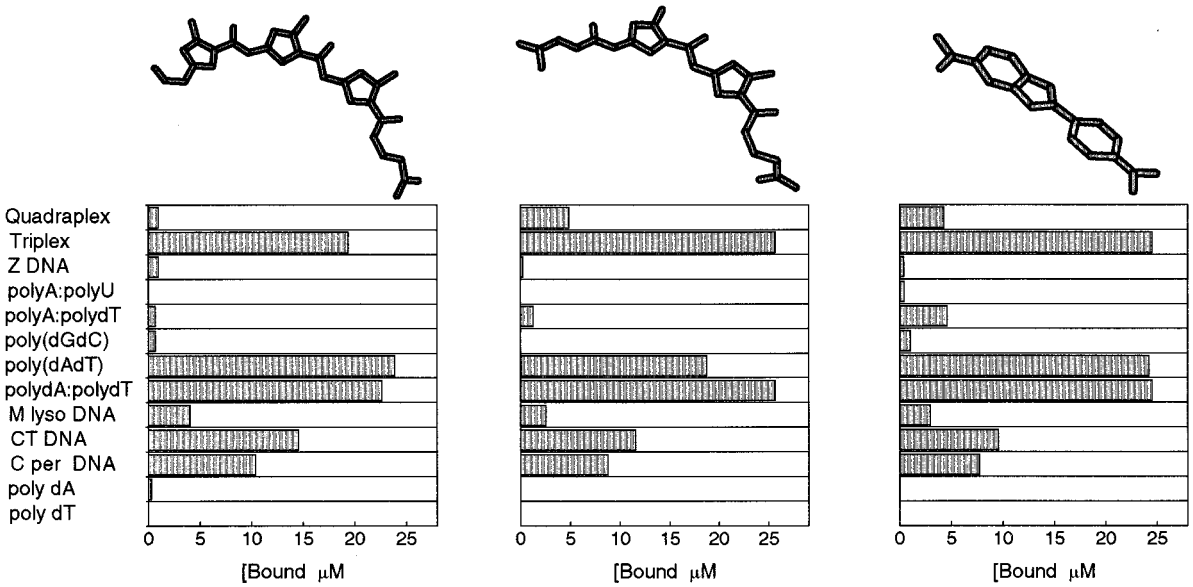


FIGURE 3: Results obtained by the competition dialysis method for known groove binding agents. The presentation is the same as described for Figure 2. The groove binders that were studied were distamycin (left), netropsin (middle), and DAPI (right).

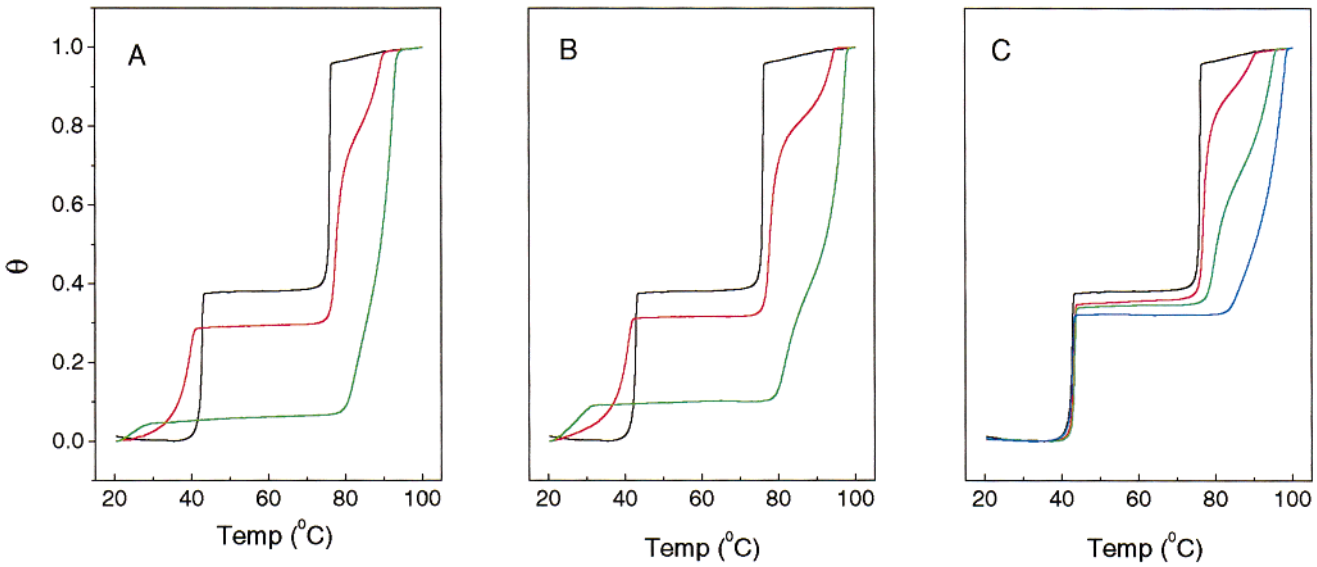


FIGURE 4: Results of UV melting experiments with poly(dA)−[poly(dT)]$_2$ triplex DNA and the ligands shown in Figure 3. Poly(dA)−[poly(dT)]$_2$ in BPES buffer was melted alone (black curves) or in the presence of increasing molar ratios (moles of ligand per mole of triplet) of added ligand: 0.033 (red), 0.1 (green), and 0.2 (blue). The ligands that were studied were (A) distamycin, (B) netropsin, and (C) DAPI.

third strand and binding to the duplex form. Literature reports are generally consistent with this conclusion (*25*−*28*).

Figure 5 shows results obtained for agents reported to be selective for triplexes. BePI and coralyne were both reported to be triplex selective binding agents (*29*−*31*). Competition dialysis reveals that BePI does indeed show a preference for poly(dA)−[poly(dT)]$_2$ triplex DNA, but that it also interacts with almost all other structures in the assay except for single-stranded forms (Figure 5, left). Coralyne shows a more dramatic preference for poly(dA)−[poly(dT)]$_2$ triplex DNA relative to BePI under our solution conditions (Figure 5, middle). Surprisingly, coralyne also shows strong binding to single-stranded poly(dA), a characteristic unique among all of the compounds studied by the competition dialysis assay thus far. In contrast to BePI, coralyne shows more variety in its binding to duplex forms, and appears to interact more strongly with RNA and the DNA−RNA hybrid.

The binding of coralyne to poly(dA) was examined by absorbance titration experiments (not shown). These studies showed that the binding constant for the association of coralyne with poly(dA) was $(1.05 \pm 0.1) \times 10^5$ M$^{-1}$. For comparison, coralyne binding to calf thymus DNA was determined to be 1 order of magnitude weaker, with a $K$ of $(1.25 \pm 0.1) \times 10^4$ M$^{-1}$. These independent binding studies verified the most surprising result to emerge from the competition dialysis experiment with coralyne.

Figure 5 (right) shows results obtained with berberine, a compound with heretofore poorly characterized structural selectivity. Berberine was chosen for study by inspection of its structure, which appeared to be similar in shape to BePI and coralyne. Berberine clearly prefers poly(dA)−[poly(dT)]$_2$ triplex DNA, and seems to bind to that structure as well as BePI and coralyne. In contrast to those compounds, the level of berberine binding to all other structural forms is greatly

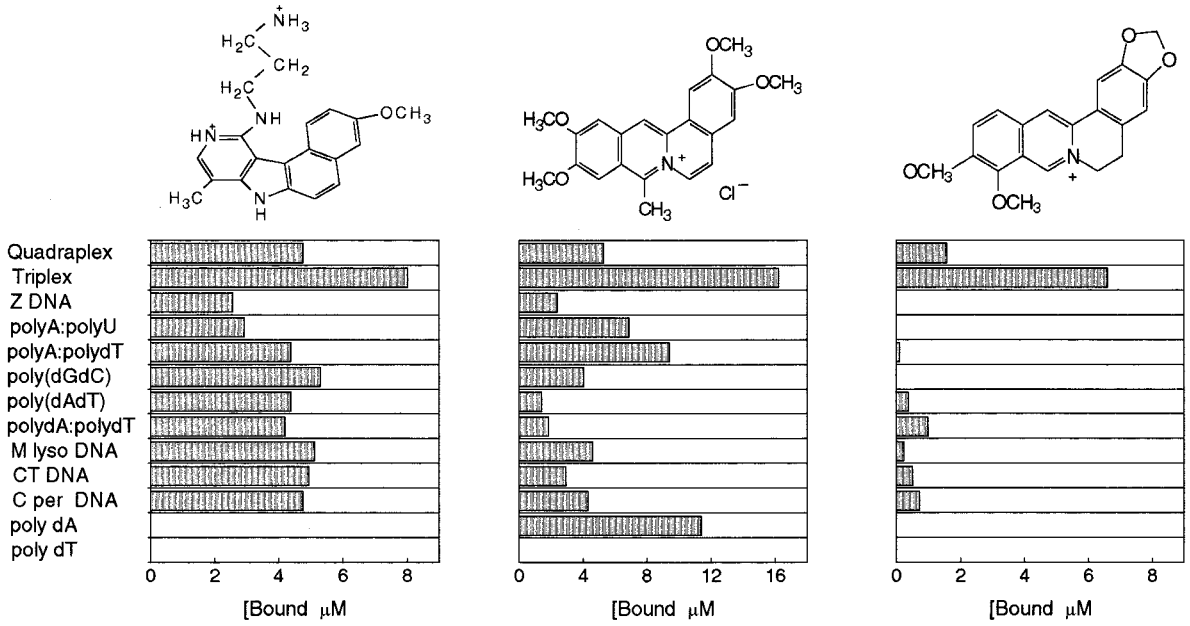


FIGURE 5: Results obtained by the competition dialysis method for compounds reported or discovered to be selective for triplex DNA. Chemical structures are shown for the compounds that were studied, along with bar graphs showing the results of the competition dialysis experiments. The compounds that were studied were BePI (left), coralyne (middle), and berberine (right).

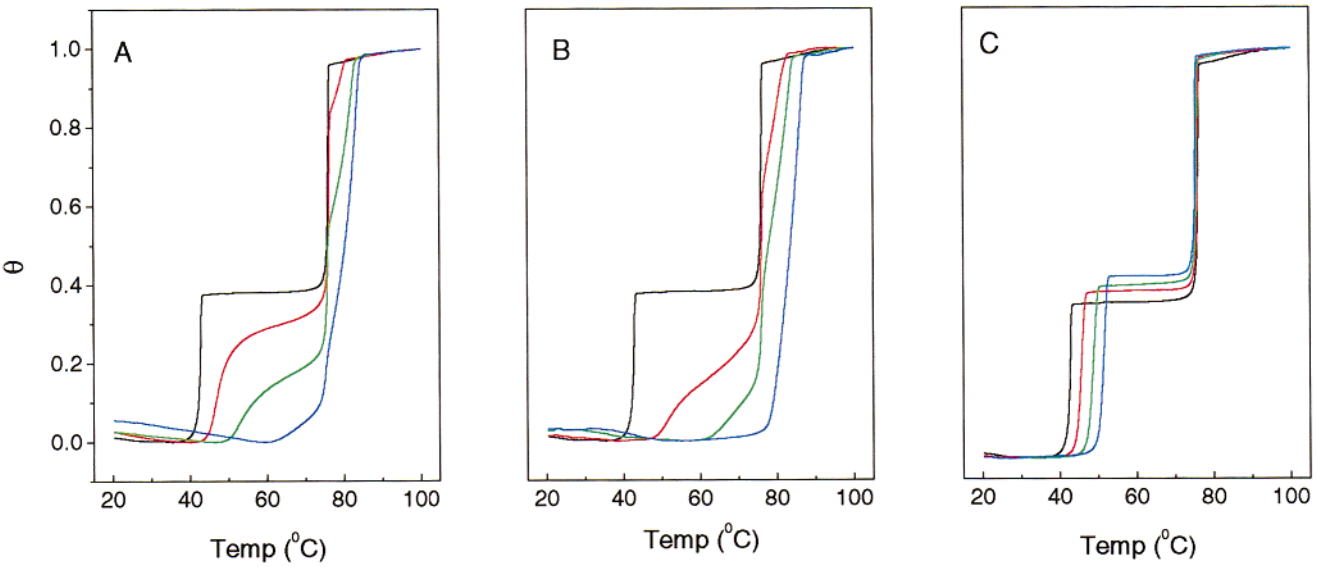


FIGURE 6: Results of UV melting experiments with poly(dA)−[poly(dT)]$_2$ triplex DNA and BePI (A), coralyne (B), and berberine (C). Black curves show melting of the poly(dA)−[poly(dT)]$_2$ triplex alone. Colored curves show melting of the triplex in the presence of increasing molar ratios (moles of ligand per mole of triplex) of added ligand: 0.033 (red), 0.1 (green), and 0.2 (blue).

reduced or absent. Competition dialysis reveals that berberine preferentially binds to poly(dA)−[poly(dT)]$_2$ triplex DNA with apparently greater selectivity than BePI or coralyne.

Figure 6 shows the results of triplex DNA melting experiments using BePI, coralyne, and berberine. In contrast to the results shown in Figure 4, these agents all selectively stabilize the triplex form, and elevate the $T_m$ for the melting of the third strand. The behavior shown in panels A and B of Figure 6 is consistent with published reports about the effects of BePI and coralyne on triplex melting (*29*, *30*). The results in Figure 6C show that berberine, in contrast to BePI and coralyne, appears not to stabilize duplex DNA at all under these reaction conditions, but selectively stabilizes poly(dA)−[poly(dT)]$_2$ triplex DNA. That result is consistent with the pronounced selectivity shown in the competition dialysis study (Figure 5, right). We note that Lee et al. (*29*) concluded that berberine bound more weakly to triplex DNA than did coralyne, on the basis of the results of melting experiments carried out under different ionic conditions. Our results indicate that such is not the case, and we ascribe the different conclusions in part to the difficulty of properly interpreting multiphasic melting curves. Such difficulties in fact motivated us to design the more direct and less ambiguous competition dialysis system that more clearly shows structural preferences.

Figure 7 shows the results obtained for three porphyrin compounds. The large planar surface of porphyrins might result in favorable interactions with tetraplex DNA, and for this reason, the interaction of these compounds with tetraplex DNA has been studied by a variety of experimental

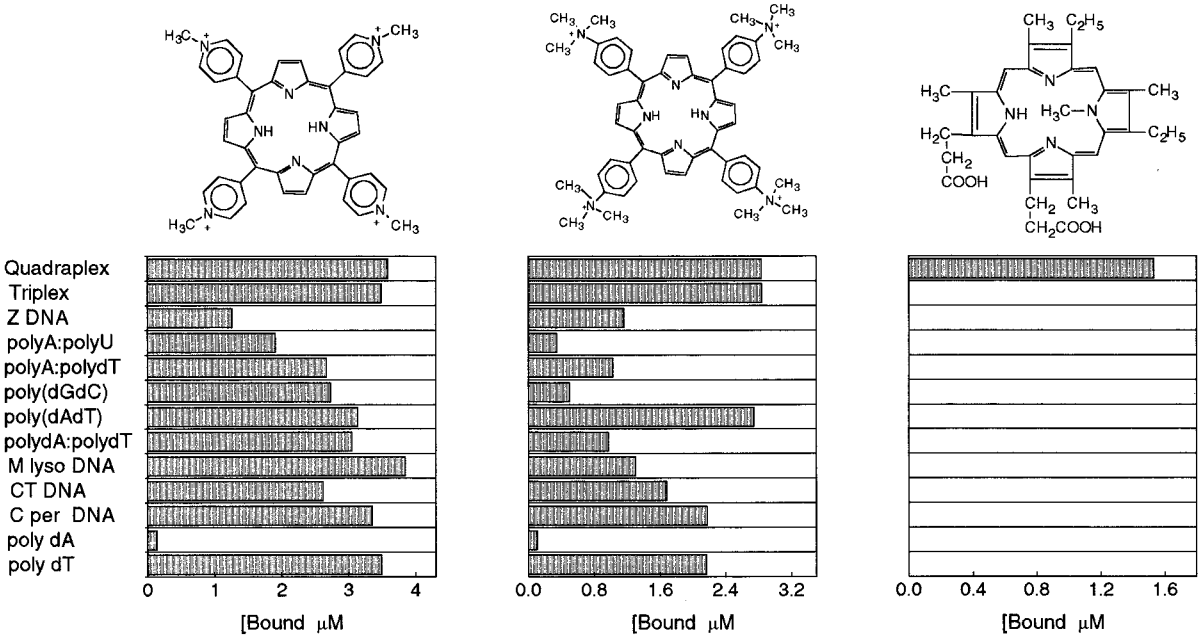


FIGURE 7: Results obtained by the competition dialysis method for porphyrin compounds. Chemical structures of the compounds that were studied are shown above bar graphs that summarize the results. The compounds that were studied were $H_2$TmPyP [meso-tetrakis(*N*-methyl-4-pyridyl)porphine] (left), 5,10,15,20-tetrakis[4-(trimethylammonio)phenyl]-21*H*,23*H*-porphine (middle), and NMM (*N*-methyl mesoporphyrin IX) (right).

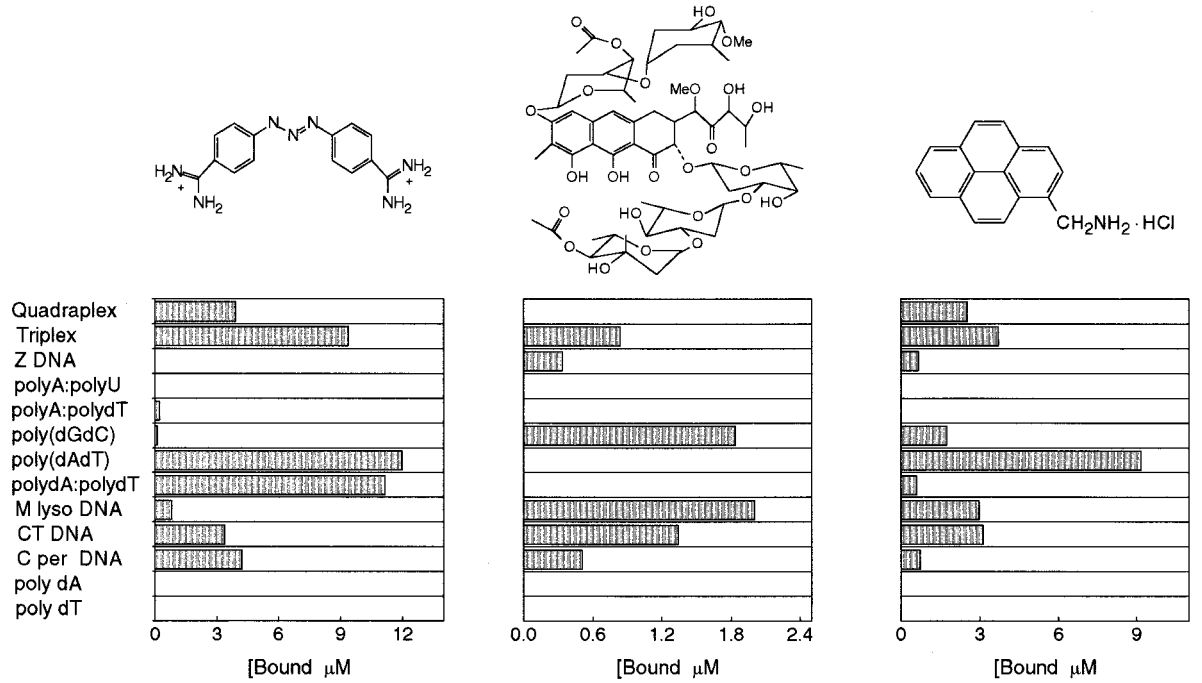


FIGURE 8: Results obtained by the competition dialysis method for berenil (left), chromomycin (middle), and 1-pyrenemethylamine (right).

approaches (*32*−*35*). $H_2$TMPyP (Figure 7, left) and 5,10,-15,20-tetrakis[4-(trimethylammonio)phenyl]-21*H*,23*H*-porphine (Figure 7, middle) bind to all DNA structures except poly(dA). These two compounds are the first that we encountered in our assay that bind to an appreciable extent to poly(dT). While both of these compounds indeed bind to multistranded triplex and tertraplex structures, the preference over the other structures in the assay is only marginal at best.

In contrast, NMM (Figure 7, right) appears to bind only to tetraplex DNA. Note that its level of absolute binding is low, but that it does not bind to any form other than tetraplex in amounts that can be detected by our assay. Binding affinity in this case was apparently sacrificed for selectivity. Bolton's laboratory found by fluorescence spectroscopy that NMM bound to tetraplex DNA but not to duplex forms (*32*). Our assay confirms their observation, and shows in addition that other nucleic acid conformations apparently do not bind NMM either.

Figure 8 shows results obtained for a miscellaneous but interesting series of compounds. Berenil (Figure 8, left) is a known groove binding agent (*36*). Its behavior is similar to that shown by the compounds in Figure 4, except that its absolute binding is somewhat weaker. In contrast to the groove binders shown in Figure 3, berenil is reported to

stabilize triplex DNA (*37*−*39*). The apparent binding to poly(dA)−[poly(dT)]$_2$ triplex evident in the left panel of Figure 8 is consistent with that report.

Chromomycin (Figure 8, middle) is thought to bind in the minor groove as a dimer to runs of GC base pairs (*40*−*42*). Competition dialysis shows that it indeed has a strong preference for GC rich duplex DNA forms. Chromomycin does not bind at all to duplexes containing all AT base pairs, to RNA, to the DNA−RNA hybrid, to single-stranded forms, or to tetraplex DNA.

Finally, results for pyrene methylamine are shown (Figure 8, right). This compound was selected for study because we thought that its planar aromatic ring structure might render it selective for either triplex or tetraplex forms. Instead, we found a unique and unprecedented selectivity for duplex poly(dAdT). The origin of this strong preference is by no means clear, but shows that the competition dialysis method can reveal unexpected examples of selectivity on which to base more detailed explorations. The preference of pyrene methylamine for poly(dAdT) was verified by independent fluorescence titration experiments (data not shown). Binding constants of $(6.4 \pm 0.4) \times 10^5$, $(1.3 \pm 0.1) \times 10^5$, and $(0.7 \pm 0.1) \times 10^5$ M$^{-1}$ were determined for pyrene methylamine binding to poly(dAdT), to calf thymus DNA, and to poly(dGdC), respectively.

In summary, the competition dialysis method allowed the structural selectivity of these 15 compounds to be examined quickly and efficiently. The methods provided results that were fully consistent with the known structural preferences of test intercalators and groove binding agents. Each compound that was studied appeared to have a unique, characteristic pattern of structural selectivity, with distinct differences evident among compounds. Novel, previously unknown structural preferences were revealed by the assay, most notably the strong triplex selectivity shown by berberine, poly(dA) binding by coralyne, and a striking preference for duplex poly(dAdT) shown by pyrene methylamine.

The competition dialysis method clearly provides a reliable, thermodynamically sound assay for the rapid screening of structurally selective compounds. While at present we have included only 13 structures in the assay, there is no reason a greater variety of structures could not be included. The only limitations are that all included structures must be stable under the solution conditions of the assay, and must be of an appropriate size to be retained by the dialysis tubing selected for use. While the current version of the assay is relatively rapid, its efficiency could be further improved by adapting it to a microplate format to reduce the manual manipulations of the samples and to automate the spectrophotometric quantitation of bound ligand.

While the competition dialysis method offers a powerful new approach for the study of ligand structural selectivity, we freely acknowledge several possible limitations of the assay. First, the current assay uses relatively high salt (0.185 M NaCl) which tends to decrease the binding affinity of charged ligands by decreasing the polyelectrolyte contribution to the binding free energy. Second, the ionic conditions for the assay were selected to be appropriate to maintain the stability of the particular nucleic acid structures chosen for study. These ionic conditions may not be optimal for all types of structures of interest, certain triplex forms, for example. Different ionic conditions could most certainly be used for the assay, but the structure and stability of all nucleic acids forms used would need to be reestablished under the new conditions. Third, different sizes of nucleic acids are used in the assay (of necessity), which might result in a disproportionately higher concentration of ends in some samples, like the tetraplex form. One must be aware of a potential bias that could result if a ligand preferentially bound to end residues for some reason. Finally, one must be aware of what might be considered "false positive" results. For example, the assay registered apparently significant intercalator binding to "Z-DNA", but further studies revealed the allosteric conversion of the polymer to the preferred right-handed form. In addition, groove binders were observed to bind to "triplex" DNA, but further melting studies revealed complex underlying interactions. The competition dialysis method was designed to provide a rapid screening procedure that would reveal interesting ligand binding behavior and which would guide more detailed physical or biological studies. The apparent false positives just described and the followup studies that were carried out to investigate the behavior illustrate how the competition dialysis method was in fact intended to function, and emphasize the need to exercise appropriate caution in the interpretation of initial results.

## SUPPORTING INFORMATION AVAILABLE

Graphs of the CD and UV spectra and the thermal denaturation curve of each nucleic acid structure used in the competition dialysis assay and a table of extinction coefficients for all of the ligands used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

## REFERENCES

1. Chaires, J. B. (1998) *Curr. Opin. Struct. Biol. 8*, 314−20.
2. Dervan, P. B. (1986) *Science 232*, 464−71.
3. Wemmer, D. E., and Dervan, P. B. (1997) *Curr. Opin. Struct. Biol. 7*, 355−61.
4. White, S., Szewczyk, J. W., Turner, J. M., Baird, E. E., and Dervan, P. B. (1998) *Nature 391*, 468−71.
5. White, S., Baird, E. E., and Dervan, P. B. (1997) *Chem. Biol. 4*, 569−78.
6. Trauger, K. W., Baird, E. E., and Dervan, P. B. (1996) *Nature 382*, 559−61.
7. Gottesfeld, J. M., Neely, L., Trauger, J. W., Baird, E. E., and Dervan, P. B. (1997) *Nature 387*, 202−5.
8. Sinden, R. R. (1994) *DNA Structure and Function*, Academic Press, San Diego, CA.
9. Mergny, J. L., and Helene, C. (1998) *Nat. Med. 4*, 1366−7.
10. Crothers, D. M. (1971) *Biopolymers 10*, 2147−60.
11. Muller, W., and Crothers, D. M. (1975) *Eur. J. Biochem. 54*, 267−77.
12. Chaires, J. B. (1992) in *Advances in DNA Sequence Specific Agents* (Hurley, L. H., Ed.) pp 3−23, JAI Press, Inc., Greenwich, CT.
13. Chaires, J. B., Dattagupta, N., and Crothers, D. M. (1982) *Biochemistry 21*, 3933−40.
14. Moller, A., Nordheim, A., Kozlowski, S. A., Patel, D. J., and Rich, A. (1984) *Biochemistry 23*, 54−62.
15. Qu, X., and Chaires, J. B. (1999) in *Methods in Enzymology* (Johnson, M. L., and Brand, L., Eds.) Academic Press, San Diego, CA.
16. Herrera, J. E., and Chaires, J. B. (1989) *Biochemistry 28*, 1993−2000.
17. Bresloff, J. L., and Crothers, D. M. (1981) *Biochemistry 20*, 3547−53.
18. Waring, M. J. (1966) *Biochim. Biophys. Acta 114*, 234−44.

19. Scaria, P. V., and Shafer, R. H. (1991) *J. Biol. Chem. 266*, 5417−23.
20. Chaires, J. B., Fox, K. R., Herrera, J. E., Britt, M., and Waring, M. J. (1987) *Biochemistry 26*, 8227−36.
21. Chaires, J. B., Herrera, J. E., and Waring, M. J. (1990) *Biochemistry 29*, 6145−53.
22. Chaires, J. B. (1986) *J. Biol. Chem. 261*, 8899−907.
23. Walker, G. T., Stone, M. P., and Krugh, T. R. (1985) *Biochemistry 24*, 7462−71.
24. Walker, G. T., Stone, M. P., and Krugh, T. R. (1985) *Biochemistry 24*, 7471−9.
25. Gondeau, C., Maurizot, J. C., and Durand, M. (1998) *J. Biomol. Struct. Dyn. 15*, 1133−45.
26. Durand, M., and Maurizot, J. C. (1996) *Biochemistry 35*, 9133−9.
27. Durand, M., Thuong, N. T., and Maurizot, J. C. (1992) *J Biol Chem 267*, 24394−9.
28. Park, Y. W., and Breslauer, K. J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6653−7.
29. Lee, J. S., Latimer, L. J., and Hampel, K. J. (1993) *Biochemistry 32*, 5591−7.
30. Mergny, J. L., Duval-Valentin, G., Nguyen, C. H., Perrouault, L., Faucon, B., Rougee, M., Montenay-Garestier, T., Bisagni, E., and Helene, C. (1992) *Science 256*, 1681−4.
31. Marchand, C., Bailly, C., Nguyen, C. H., Bisagni, E., Garestier, T., Helene, C., and Waring, M. J. (1996) *Biochemistry 35*, 5022−32.
32. Arthanari, H., Basu, S., Kawano, T. L., and Bolton, P. H. (1998) *Nucleic Acids Res. 26*, 3724−8.
33. Anantha, N. V., Azam, M., and Sheardy, R. D. (1998) *Biochemistry 37*, 2709−14.
34. Han, F. X., Wheelhouse, R. T., and Hurley, L. H. (1999) *J. Am. Chem. Soc. 121*, 3561−70.
35. Haq, I., Trent, J. O., Chowdhry, B. Z., and Jenkins, T. C. (1999) *J. Am. Chem. Soc. 121*, 1768−79.
36. Pilch, D. S., Kirolos, M. A., Liu, X., Plum, G. E., and Breslauer, K. J. (1995) *Biochemistry 34*, 9962−76.
37. Pilch, D. S., Kirolos, M. A., and Breslauer, K. J. (1995) *Biochemistry 34*, 16107−24.
38. Durand, M., Thuong, N. T., and Maurizot, J. C. (1994) *J. Biomol. Struct. Dyn. 11*, 1191−202.
39. Pilch, D. S., and Breslauer, K. J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 9332−6.
40. Gao, X. L., and Patel, D. J. (1989) *Biochemistry 28*, 751−62.
41. Gao, X. L., and Patel, D. J. (1990) *Biochemistry 29*, 10940−56.
42. Gao, X. L., Mirau, P., and Patel, D. J. (1992) *J. Mol. Biol. 223*, 259−79.

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