A Simple, High-Resolution Method for Establishing DNA Binding Affinity and Sequence Selectivity

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Abstract: Full details of the development of a simple, nondestructive, and high-throughput method for establishing DNA binding affinity and sequence selectivity are described. The method is based on the loss of fluorescence derived from the displacement of ethidium bromide or thiazole orange from the DNA of interest or, in selected instances, the change in intrinsic fluorescence of a DNA binding agent itself and is applicable for assessing relative or absolute DNA binding affinities. Enlisting a library of hairpin deoxyoligonucleotides containing all five base pair (512 hairpins) or four base pair (136 hairpins) sequences displayed in a 96-well format, a compound's rank order binding to all possible sequences is generated, resulting in a high-resolution definition of its sequence selectivity using this fluorescent intercalator displacement (FID) assay. As such, the technique complements the use of footprinting or affinity cleavage for the establishment of DNA binding selectivity and provides the information at a higher resolution. The merged bar graphs generated by this rank order binding provide a qualitative way to compare, or profile, DNA binding affinity and selectivity. The 96-well format assay (512 hairpins) can be conducted at a minimal cost (presently ca. $100 for hairpin deoxyoligonucleotides/assay with ethiduim bromide or less with thiazole orange), with a rapid readout using a fluorescent plate reader (15 min), and is adaptable to automation (Tecan Genesis Workstation 100 robotic system). Its use in generating a profile of DNA binding selectivity for several agents including distamycin A, netropsin, DAPI, Hoechst 33258, and berenil is described. Techniques for establishing binding constants from quantitative titrations are compared, and recommendations are made for use of a Scatchard or curve fitting analysis of the titration binding curves as a reliable means to quantitate the binding affinity.

Introduction

The regulation of gene expression is based on the sequence selective recognition of nucleic acids by repressor, activator, and enhancer proteins. Selective control of such processes has been a long-standing goal, and small molecules that selectively bind DNA and activate (block a repressor) or inhibit (block an activator) gene expression hold significant promise as therapeutics.[1,2] Complicating such studies is the recognition that it is often not a single sequence that is ideally targeted but rather an ensemble of related sites that compose the consensus binding sequence of a nuclear receptor or transcription factor. Consequently, the discovery of such agents has been slow due to the complexity associated with understanding small molecule−DNA interactions, the iterative process of designing individual compounds targeted toward specific DNA sequences, and the technically demanding techniques involved in the determination of their binding affinity for any given sequence, much less an ensemble of related sequences. As part of a program aimed at identifying novel DNA binding compounds, we recently reported the combinatorial synthesis of prototypical libraries of known DNA binding agents.[3] Complementary to these efforts, we introduced a technically nondemanding assay for DNA binding affinity and selectivity applicable to the assessment of such libraries.[3] Herein, we report the development of this method, its capabilities for profiling a compound's DNA binding properties, and its use in the examination of a series of prototypical DNA binding compounds.

A variety of techniques are commonly used to establish the DNA binding properties of small molecules.[4] Most are technically challenging and require the knowledge of specialized biochemical procedures, and assay reproducibility comes only with experience.[5−7] None are applicable to high-throughput screening required for assaying a library of compounds against an ensemble or complete library of sequences. To date, the most widely used methods are footprinting or affinity cleavage and both have been used qualitatively and quantitatively to establish

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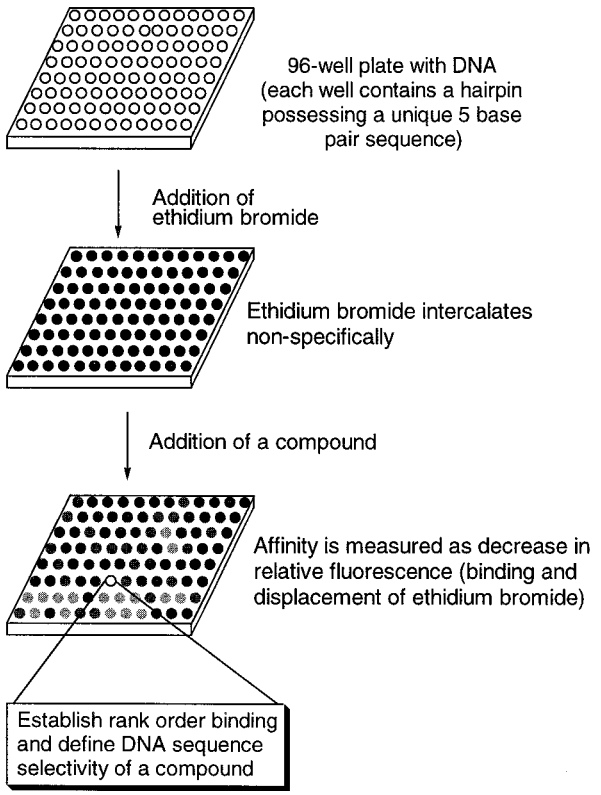

Figure 1. General procedure for determination of sequence selectivity for a DNA binding agent using the fluorescent intercalator displacement (FID) assay.

DNA binding selectivity or affinity.[6] Intrinsic in the methods employed in the destructive assays is the binding characterization at the highest affinity sites within an incomplete set of sites contained in a 100−200 bp segment of DNA, and modest or low affinity sites are not easily probed. As a consequence, the full DNA binding profile of candidate ligands is not easily assessed or compared using such techniques.

The nondestructive fluorescent intercalator displacement (FID) assay that we have examined, which addresses these issues of ease of use and information content, utilizes the displacement of ethidium bromide or thiazole orange from hairpin deoxyoligonucleotides (Figure 1).[3] Hairpins containing all four (136 hairpins) or five base pair (512 hairpins) sequences individually displayed in 96-well plates are treated with the intercalator, yielding a fluorescence increase upon DNA binding.[8] Addition of a DNA binding compound results in a decrease in fluorescence due to displacement of the bound intercalator. The % fluorescence decrease is directly related to the extent of DNA binding, providing relative binding affinities and a rank order binding to all possible five or four base pair (bp) sequences. The resulting profile defines the compound's sequence selectivity in high resolution, and subsequent quantitative titration of any given hairpin sequence provides reliable binding constants.[9−12]

Distamycin A. Perhaps the most extensively studied DNA binding compound is distamycin A, which binds to AT-rich sequences in the minor groove.[13] Its DNA binding has been studied in detail by footprinting,[6,14] calorimetry,[15] NMR,[16] and X-ray.[17] However, even for distamycin A, a detailed study of its binding to all possible five bp sequences has not been described and its relative affinity for nonoptimal binding sites is not easily assessed using available techniques. Consequently, it represented an ideal case with which the technique could be first examined and parameters optimized. Thus, a survey of distamycin A binding to all five bp sequences was conducted with a library of 512 hairpin deoxyoligonucleotides, each containing two of the possible five bp sequences in the general format 5′-CGXXXXXC-3′ with a 5-A loop (Figure 2). Although there are 1024 possible sequences containing five bp, two complementary sequences are contained in each hairpin, making, for example, the sequence 5′-ATGCA equivalent to the sequence

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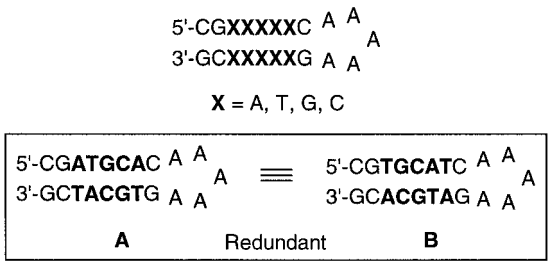

Figure 2. Structure of hairpin deoxyoligonucleotides representing all possible combinations of five base pairs. The position within the hairpin was not considered, making **A** and **B** redundant.

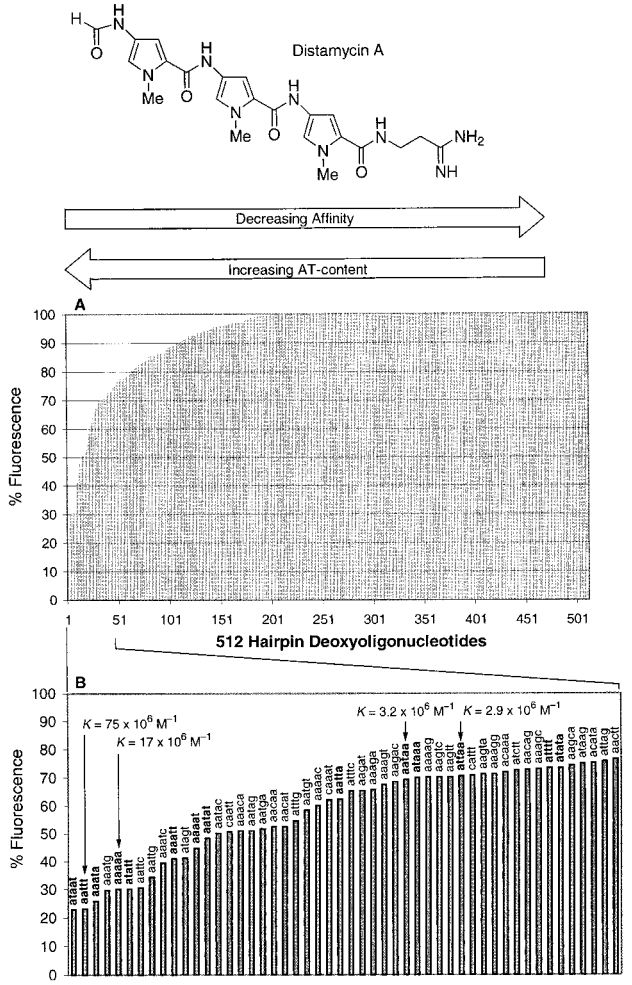

Figure 3. Screen of distamycin A (2 μM concentration) against a library of DNA hairpin deoxyoligonucleotides: (**A**) all 512 sequences, (**B**) top 50 sequences showing highest affinity.

5′-TGCAT (Figure 2). Such a library of binding sites is not limited to hairpins containing a variable five bp sequence. Smaller four bp sequences (5′-CGXXXXC-3′ with a 5-A loop, 136 hairpins) have also been used in our work, and larger sequences could also be employed.

The results of screening the 512-member library of hairpins using distamycin A under a range of experimental parameters are summarized below, and those obtained under conditions recommended for use in a 96-well format are illustrated in Figure 3.[3] As expected, affinity increases with increasing AT content. All but two of the five bp AT sites were found in the top 45 sequences (1−45), essentially all four bp AT sites were observed in the top 151 sequences (4−151, 2 exceptions), and the three and two bp AT sites were observed in the range from

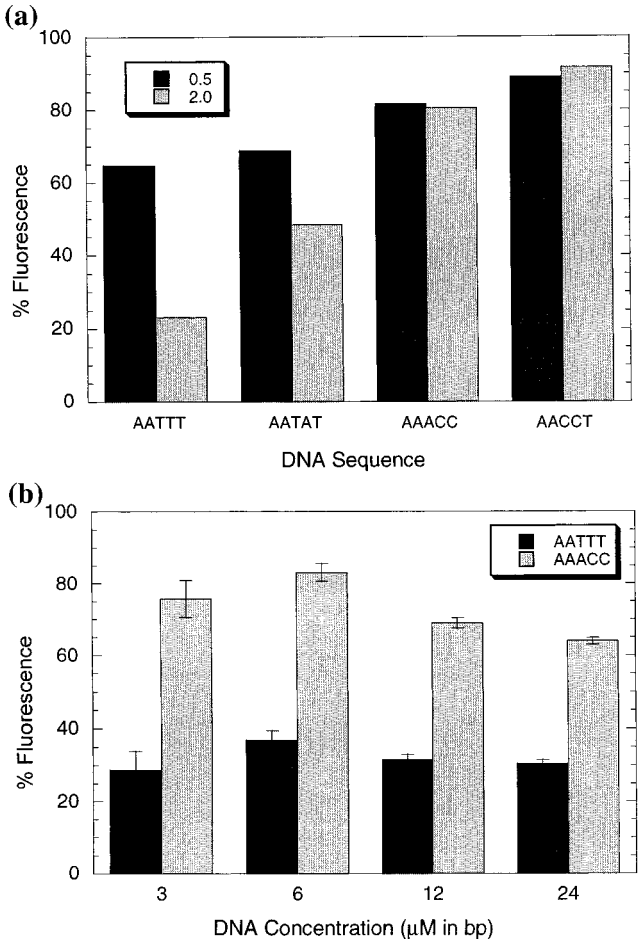

Figure 4. (a) Influence of compound concentration. Distamycin A (2.0 vs 0.5 μM), 1.5 μM hairpin (0.1 M Tris, 0.1 M NaCl, pH 8). (b) Influence of overall assay concentration. DNA concentration shown (μM in bp) with overall ratio (distamycin A:EtBr:DNA bp) held constant (1:2:4).

11 or 19 to 512, illustrating that distamycin A exhibits a clear selectivity for five and four bp AT sites over three and two bp AT sites. Thus, 14 of 16 five bp AT sites (88%), 15 of 32 four bp AT sites (47%), 10 of 80 three bp AT sites (13%), and a significant 11 of 176 two bp AT sites (6%) are found in the top 50 sequences. In contrast to the behavior of netropsin detailed later, 7 of the top 50 sequences (14%), but not the highest affinity sequences, contain a GC bp central to a five bp AT-rich site and this represents 7 of 16 such hairpin sites (44%). This distinct behavior has been noted in some footprinting studies, and the data summarized in Figure 3 provides a secure documentation of this observation and its relative importance.

Compound Concentration Dependence. Among the first parameters examined was the impact of the compound concentration. The assays were conducted at a single concentration of the hairpin (1.5 μM, 12 μM in bp) and under buffer conditions (0.1 M Tris, 0.1 M NaCl, pH 8) that represent relatively high salt concentrations which approximate physiologically relevant conditions and minimize simple electrostatic binding to DNA. This minimizes the lower affinity binding of ethidium bromide to the phosphate backbone that could complicate the assay. Optimally, the measured % fluorescence decrease should be sufficiently intense to provide a robust signal and the dynamic range sufficiently large that comparisons of compounds with different affinities could be made at a single concentration. For assays conducted with 1.5 μM hairpin, 1 or 2 μM compound concentrations met these criterion (Figure 4a). Use of lower

compound concentrations resulted in less discrimination between sequences (Figure 4a, 0.5 vs 2.0 μM), and higher compound concentrations similarly led to less distinction, especially among the higher affinity sequences. This use of a near 1:1 ratio of compound to DNA provided the desired robust intensity of measured % fluorescence decrease suitable for a high-throughput assay of compounds with varied affinities. Typically, the most useful concentration of compound proved to be either 1 or 2 μM, bracketing the concentration of DNA enlisted in the assay (1.5 μM).

Assay Concentration. In the initial studies, the concentration of DNA required to provide a robust assay reading was examined. The intention was to minimize the amount of DNA and reagents needed in a 96-well format (Costar black opaque, 100 μL assay volume) while maintaining a reliable measurement. The hairpin concentration for a high-affinity (AATTT) and lower affinity sequence (AAACC) was varied from 0.375 to 3.0 μM (3−24 μM bp), maintaining a 1:2 ethidium bromide: bp ratio and a constant ratio of distamycin A:hairpin (Figure 4b). To a first approximation, the % fluorescence decrease upon distamycin A binding should not vary with this change in concentration. Concentrations of 1.5 μM hairpin (12 μM bp) or higher in a 100 μL volume maintained an acceptably constant reading to be reliable for both hairpins whereas those below 1.5 μM suffered variations too large to be considered useful (Figure 4b). Consequently, a hairpin concentration of 1.5 μM was adopted for our work.

Assay Concentration Range. Several studies were conducted to establish an optimal assay concentration and single compound concentration that could be used in a high-throughput assay. This led to the identification of conditions that would discriminate among high and lower affinity sites for tight or even modest binding agents. One representative study is illustrated in Figure 5 with distamycin A and two sequences (AATTT and AAACC). With the high-affinity AATTT sequence, concentrations as low as 0.5 μM and optimally 1 or 2 μM distamycin A performed well across the range of hairpin concentrations (3−24 μM bp). With the lower affinity AAACC sequence, concentrations of 2−8 μM (2−4 μM optimally) distamycin A performed well across the range of hairpin concentrations whereas the lower concentrations of 0.5−1 μM were not useful regardless of the hairpin concentration. The optimal condition that accommodates both the high (AATTT) and modest (AAACC) affinity sites is the 2 μM distamycin A concentration and the midrange 12 μM bp hairpin (1.5 μM) concentration, the conditions adopted for the high-throughput assay.

Ratio of Ethidium Bromide to DNA. One special concern was the sensitivity of the method to the amount of ethidium bromide employed. The method was expected to perform best at a 1:2 ethidium bromide:base pair ratio (EtBr:bp) where all intercalation sites are occupied. However, it was not clear what impact deviations from this ratio would have. Under conditions where the salt concentration is modestly high and approximating that which is physiologically relevant, little impact was observed when this ratio was varied over a range of 1:4 to 2:1 EtBr:bp. Consistent with the conditions adopted in the assay, the greatest % fluorescence decrease was observed at the optimal 1:2 EtBr: bp ratio (Figure 6a). The slightly lower decrease in % fluorescence for the 1:4 EtBr:bp ratio underestimates the compound binding since not all available intercalation sites are occupied and compound binding can occur at sites where less or no intercalator is displaced. The % fluorescence decrease also diminished slightly as the ethidium bromide concentration was raised above the optimal 1:2 EtBr:bp ratio presumably due

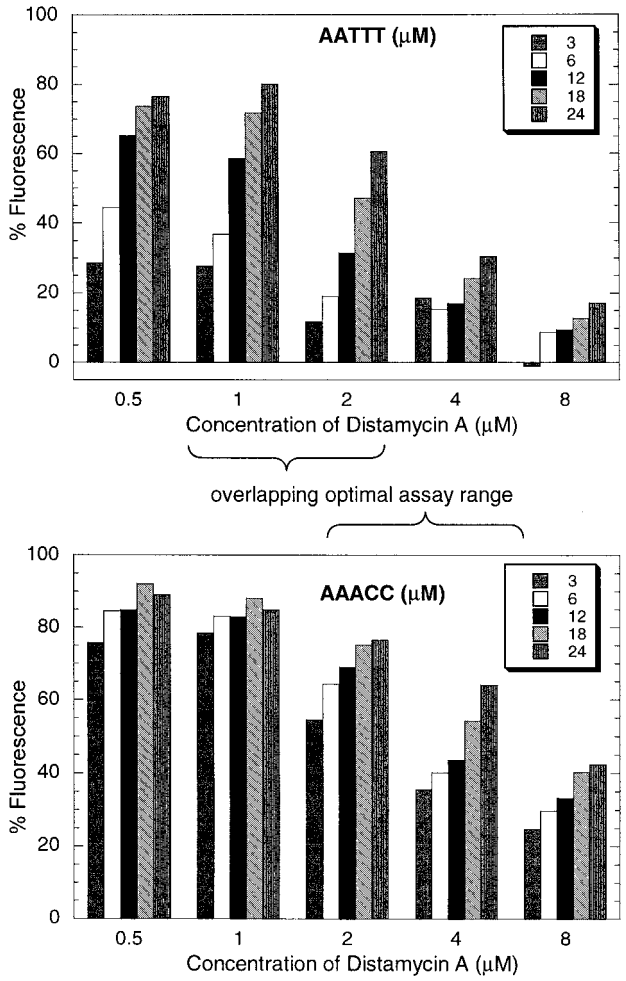

Figure 5. Survey of concentration range for distamycin A vs a tight binding sequence (AATTT) and a modest binding sequence (AAACC). Distamycin A concentration of 2 μM represents the overlapping optimal assay range.

to enhanced background fluorescence. However, the method proved surprisingly independent of this variable. Through repeated measurements on the same samples, the % fluorescence decrease variation was typically ±8% in this 1:4 to 2:1 EtBr: bp ratio range. Consequently, we recommend the use of the 1:2 ratio and note that variations may be expected if this ratio is not consistently maintained. However, this ratio does not appear to be critical and minor experimental variations are unlikely to have a significant impact.

Measurement Time, Number, and Assay Variability. We also examined the assay variability and the impact of repeated measurements using a 96-well format assay and a fluorescent plate reader. We employed black Costar 96-well plates with a flat bottom and a Spectra Max Gemini fluorescent plate reader from Molecular Devices that records and averages 30 fluorescent readings per well. With the exception of the initial reading, the % fluorescence decrease diminished slightly with time/reading, indicating some level of photobleaching with each reading (Figure 6a). Importantly, no alterations in the relative % fluorescence decrease readings were observed within each measurement grouping, indicating that the qualitative rankings are not affected. However, the absolute readings show considerable variation which are as large as ±10% fluorescence decrease (1:2 EtBr:bp ratio) to as small as ±5% (2:1 ratio). This suggests that variation in the assay may be expected from run to run but that the ranking within a given run may be considered

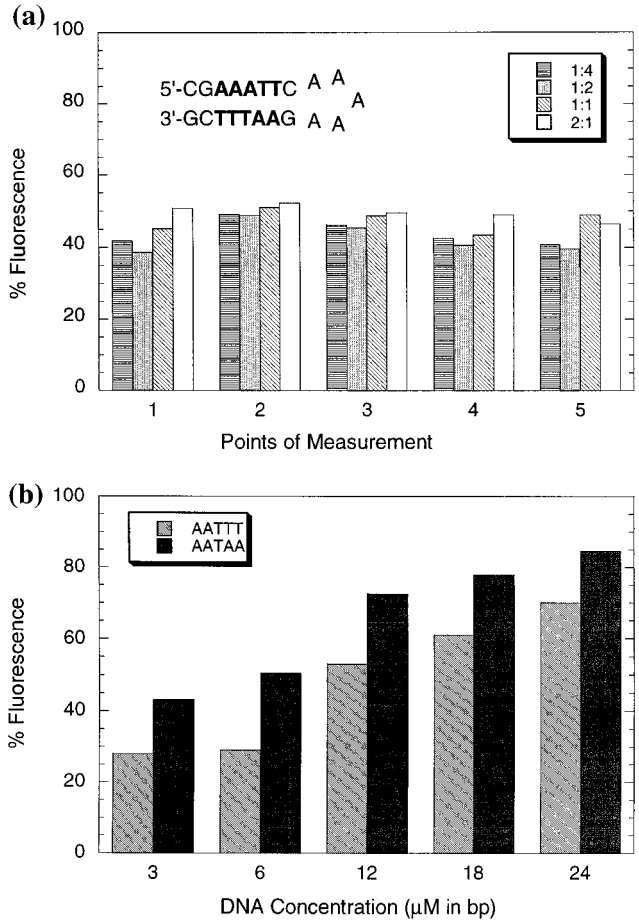

Figure 6. (a) Influence of ethidium bromide:DNA ratio. Distamycin A (2 μM) and DNA (AAATT hairpin deoxyoligonucleotide, 12 μM in bp) concentrations were kept constant, while the ethidium bromide concentration was varied. Measurements 1−4 were taken in succession after 40 min of incubation. Measurement 5 was taken after 2 h incubation. (b) Influence of DNA concentration on percent fluorescence, DNA varied from 3 to 24 μM in base pairs. Distamycin A (2.0 μM) concentration was kept constant.

reproducibly accurate. For more quantitative comparisons including titrations to establish binding constants or for careful comparison of side-by-side sequences in a ranked profile, we recommend against using the 96-well format and plate readers and recommend use of a 3 mL cuvette for the compound titrations.

Hairpin Deoxyoligonucleotide Quality. The variable that is critical to the success of the assay, and most likely to be responsible for avoidable errors, is the quality of the hairpins. In addition to the obvious concern of its constitution and purity, its concentration is critical and must be determined by measuring the UV absorption (260 nm) of the denatured, single-stranded DNA at 80−95 °C (0.01 M Na_2HPO_4/H_3PO_4, 0.1 M NaCl, pH 7). Given that each 21-mer in the library exists as a hairpin duplex at 25 °C, where this construct represents a combination of double- and single-stranded DNA, calculations of the single-strand oligo concentration based on UV absorption measurements at 25 °C underestimate the concentration by as much as 25%.[18] Consequently, the concentration of each hairpin in our library was determined by measuring the UV absorbance at 90 °C. In the course of making these measurements, all hairpins in the library displayed melting curves which indicated they adopt stable duplex structures at 25 °C. Unlike the use of simple deoxyoligonucleotides which may suffer from sequence-dependent single-strand/duplex equilibria under the conditions of our assay, the hairpins form stable duplex structures. An indication of the errors that might be introduced if the hairpin concentration is not addressed accurately is illustrated in Figure 6b. Measurements conducted with hairpin concentrations lower than expected (e.g., 3 and 6 vs 12 μM bp) yield much larger than expected % fluorescence decreases, falsely indicating higher affinity binding. Similarly, measurements conducted with hairpin concentrations higher than expected (18 and 24 vs 12 μM) result in lower than expected % fluorescence decreases and underestimate binding affinity. These alterations in the measured values are significant and indicate that this variable alone is the one most important to control. However, it is not the absolute concentration of the hairpin that is important, rather it is important that all hairpins are used at the same concentration for determination of a ranked binding profile.

Table 1. Distamycin Binding Constants

	DNA sequence	K ($\times 10^6$ M^{-1})[a]	K^{lit} ($\times 10^6$ M^{-1})
2	5'-AATTT-3'	75	31[b]
5	5'-AAAAA-3'	17	26[c]
31	5'-AATAA-3'	3.2	14[c]
36	5'-ATTAA-3'	2.9	1.9[c]

[a] Scatchard analysis of titration binding curve. [b] Calorimetry, ref 15. [c] Footprinting on a close analogue of distamycin A, ref 19.

Binding Constants. Although distamycin A has been studied in detail, few binding constants for short AT-rich sequences have been established. The comparison of those disclosed show the relative trend of 5′-AATTT > AAAAA > AATAA > ATTAA (Table 1).[15,19] The ethidium bromide displacement assay revealed the same general trend, and a quantitative titration with the hairpins containing these sequences afforded binding constants that are not only consistent with the relative trend (Figure 3) but also are within a factor of 2−4 of the binding constants determined through calorimetry or footprinting (Table 1). Given that the DNAs upon which the measurements were made are different, that the pH and buffer conditions are not identical, and that entries 2−4 in Table 1 were derived with a close analogue of distamycin A, all which may contribute to small discrepancies in the binding constant values, the method appears to be accurate at providing absolute binding constants. We compared three approaches to establishing the binding constants from a quantitative titration on the basis of the displacement of ethidium bromide. Two of these, involving the use of a noncompetitive[9b] or competitive[9a] binding model, were detailed and discussed in our earlier disclosure.[3] In the intervening time, we have found that a third method involving a Scatchard[20] or curve fitting analysis of the titration curves[12] provides more satisfactory results. The noncompetitive binding

(18) This range was determined using the absorbance value at 25 °C to calculate DNA concentration and compared with the same calculation using the absorbance determined at 80−95 °C. This procedure was conducted on a representative array of hairpins with varying GC content. **Note Added in Proof:** In the intervening time, we have found that the UV absorbance (A_{260}) at 25 °C may be measured and converted to an accurate concentration by adjusting the millimolar extinction coefficients for ss DNA. Excluding neighboring effects, the 512 hairpin library contains only six combinations of base composition corresponding to a 5 bp AT sequence, 4 bp AT/1 bp GC, 3 bp AT/2 bp GC, 2 bp AT/3 bp GC, 1 bp AT/4 bp GC, and 5 bp GC variable sequence. Utilizing the millimolar extinction coefficients published by Invitrogen, Life Technologies, correction factors relating A_{260} at 90 °C/A_{260} at 25 °C were determined to be 1.18, 1.13, 1.12, 1.12, 1.10, and 1.06 for the combinations listed above, respectively. Blind tests found that errors resulting from these measurements were consistently below 2% and always below 4%.

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model is experimentally difficult to implement (r value determination) but can provide reliable constants, whereas the often used competitive binding model fails to provide reliable estimates of K.[21]

This third method of establishing binding constants, which has been used extensively by Bruice,[12] provides the most reliable means of determining K and the stoichiometry of binding and is easily extended to analyzing higher order 2:1 or 3:1 complexes. In Bruice's work, this has entailed either the competitive displacement of Hoechst 33258 and a measurement of the resulting fluorescence decrease or a direct measurement of a fluorescent change in the DNA binding compound itself. We have found that the former works equally well in the assays measuring the displacement of prebound ethidium bromide or thiazole orange. For cases in which the binding stoichiometries are 1:1, binding constants may be established by Scatchard analysis[20] of the titration binding curves. Thus, eqs 1−3 are used to establish the free agent concentration employed to generate a Scatchard plot from which binding constants may be determined. In these equations, [free agent] = concentration of free agent, $[\mathrm{DNA}]_T$ = total concentration of DNA, X = molar equiv of agent vs DNA, ΔF_x = change in fluorescence, and ΔF_{sat} = change in fluorescence at the point where DNA is saturated with ligand.

$$\left(\frac{\Delta F_x}{\Delta F_{sat}}\right)\frac{1}{X} = \text{fraction of DNA−agent complex} \qquad (1)$$

$$\left[1 - \left(\frac{\Delta F_x}{\Delta F_{sat}}\right)\frac{1}{X}\right] = \text{fraction of free agent} \qquad (2)$$

$$[\mathrm{DNA}]_T\left[X - \frac{\Delta F_x}{\Delta F_{sat}}\right] = [\text{free agent}] \qquad (3)$$

A plot of the change in fluorescence vs equivalents of compound provides a titration curve from which the stoichiometry may be determined (Figure 7a). The mathematical intersection of the pre- and postsaturation portions of the curve provides ΔF_{sat} and allows for the determination of [free agent]. The plot of ΔF/[free agent] vs ΔF yields a linear portion of the Scatchard plot and provides $-K$ as the slope of this portion of the curve (Figure 7b).

Bruice has emphasized that such reciprocal plotting techniques are restrictive and in more complex systems (e.g., 2:1 or 3:1 binding) can lead to errors in interpreting the data.[12] In such cases, iterative curve fitting of the experimental points provides reliable results and can dissect multiple equilibria. For the analysis of 1:1 binding as illustrated for netropsin in Figure 7, this is unnecessary. Both the Scatchard and the curve fitting analyses provide nearly identical values for K. Given the relative ease of the use of this technique and the reliability with which visual inspection of the plotted data can confirm the assumption of 1:1 binding, we recommend use of the Scatchard analysis of the titration binding curves for determining K.

In two of the examples which follow, binding constants could be assessed by this indirect technique involving the displacement of ethidium bromide or directly by measuring the fluorescent increase of the DNA binding compound itself, i.e., in the absence of a competitive binding agent. For the compounds examined to date, the direct and indirect methods provided comparable results that do not appear to be affected by the sequence-dependent affinity of ethidium bromide or the stoichiometry of binding displacement.

(21) A detailed discussion of the noncompetitive and competitive binding models is provided in the Supporting Information along with a discussion of limitations to their use.

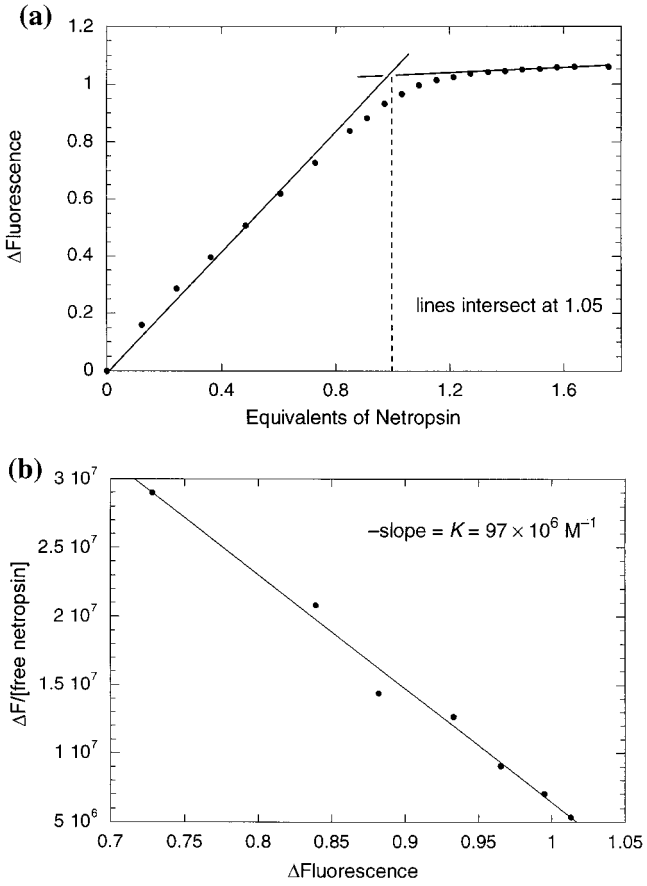

Figure 7. (a) Titration of netropsin vs the hairpin containing 5′-AATTT-3′ at 1.1 μM (8.8 μM bp) with ethidium bromide. (b) Scatchard plot for the titration of 5′-AATTT-3′ with ethidium bromide, slope = $-K$.

Netropsin. Early biophysical studies of Wells[22] and Zimmer[22] and more recent footprinting,[23] NMR,[24] X-ray,[25] and calorimetry studies[26] have characterized netropsin's minor groove AT binding selectivity. Its affinity matches or exceeds that of

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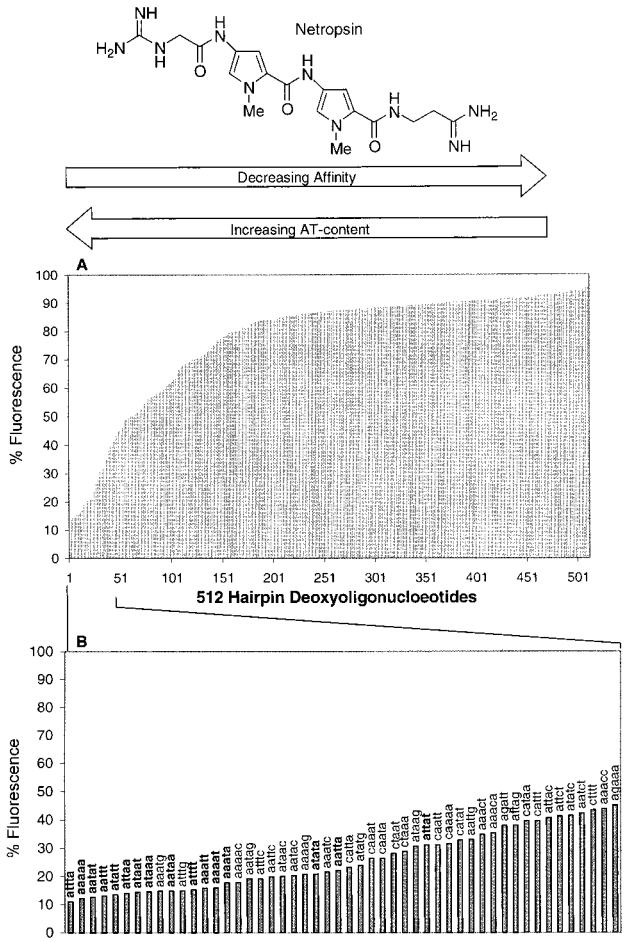

Figure 8. Screen of netropsin (2 μM concentration) against a library of DNA hairpin deoxyoligonucleotides: (**A**) all 512 sequences, (**B**) top 50 sequences showing highest affinity.

distamycin, and it has been characterized by a smaller four vs five bp AT selectivity. Netropsin behaved exceptionally well in the assay, so much so that it is recommended for use in its validation (Figure 8). Consistent with expectations, the affinity increases with increasing AT content. Even more smoothly than distamycin A, netropsin exhibited a five > four > three bp AT selectivity. The top 8 sequences and 13 of the top 15 sequences were five vs four bp AT sites. All five bp AT sequences were found in the top 33 sequences. Thus, 16/16 five bp AT sites (100%), 27/32 four bp AT sites (84%), but only 7/80 three bp AT sites were found in the top 50 sequences (9%). Unlike distamycin A, no AT-rich sequence interrupted by a central GC bp appears in the top 50 sequences and no three bp AT sites appear before hairpin 38. As such, the selectivity for a four or five bp AT site is more strictly adhered to by netropsin than distamycin.

Netropsin was also examined at 1.0 and 0.5 μM concentrations. Consistent with expectations, the assay performed similarly at 1.0 μM netropsin but exhibited greater variation in the sequence ordering at 0.5 μM due to greater intrinsic error in the measurements. In this regard, it is important to recognize that a minor reordering of the sequences is expected from run to run or upon altering the agent concentration since the difference in the % fluorescence decrease measured for most deoxyoligonucleotides adjacent to one another in the binding profiles is within the experimental variation of the assay. For example, ATTTA was identified as sequence nos. 1 and 3 at 2.0 and 1.0 μM netropsin, respectively.

Table 2. Netropsin Binding Constants

	DNA sequence	K ($\times 10^6$ M^{-1})[a]	K^{lit} ($\times 10^6$ M^{-1})
2	5′-AAAAA-3′	347 (0.99)	
4	5′-AATTT-3′	97 (1.05)	43,[b] 284[c]
6	5′-ATTAA-3′	78 (0.94)	
10	5′-AATAA-3′	71 (1.0)	

[a] Scatchard analysis of titration binding curve (experimental stoichiometry of binding). [b] Calorimetry, ref 15. [c] Calorimetry, ref 26.

The netropsin binding constants were established for several hairpin sequences by quantitative titration using the four sequences enlisted for distamycin A (Table 2). The binding clearly reflects a 1:1 stoichiometry (see Figure 7), and the Scatchard analysis of the titration binding curves was well behaved. In general, the affinities for netropsin were approximately 5−25-fold greater than those for distamycin A.

DAPI. 4′,6-Diamidine-2-phenylindole·2HCl (DAPI)[27] is widely used as a fluorescent dye for DNA and chromosomes.[28,29] Extensive studies have shown that DAPI binds selectively to AT-rich sites in the minor groove.[30] Early studies[31] postulated an intercalating binding mode, and Wilson later demonstrated that DAPI can interact with GC-rich regions by intercalation-albeit with binding that is 100−1000-fold weaker than its AT-rich minor groove binding.[32] Footprinting experiments revealed that DAPI requires three or more consecutive AT bp to bind in the minor groove,[33] X-ray[34] and NMR[35,36] studies have confimed a 1:1 binding stoichiometry, and the latter have also established a preference for a four > three > two bp AT binding site.[36]

DAPI was examined against the set of 512 hairpins at a 2 μM concentration and exhibited a high selectivity for a binding site containing at least three AT bp (Figure 9). All but one of the five bp AT sites were in the top 34 sequences (1−34), essentially all four bp AT sites were observed in the top 90 sequences (2−90, 4 exceptions), the three bp AT sites were observed in the range of 17−185 (2 exceptions), and the two bp AT sites were observed only in the higher range of >70. Thus, 16 of 16 five bp AT sites (100%), 23 of 32 four bp AT sites (72%), and 11 of 80 three bp AT sites (14%) are found in the top 50 sequences. Like netropsin, but unlike distamycin A, none of the top 50 sequences contain a GC bp central to a five bp AT-rich site. The weaker binding observed with non AT-rich hairpins (>150−512, % fluorescence decrease <20%), which was not observed with distamycin A, most likely represents the weaker DAPI intercalation first documented by Wilson.[32] The merged bar graph of the 512 sequences exhibits

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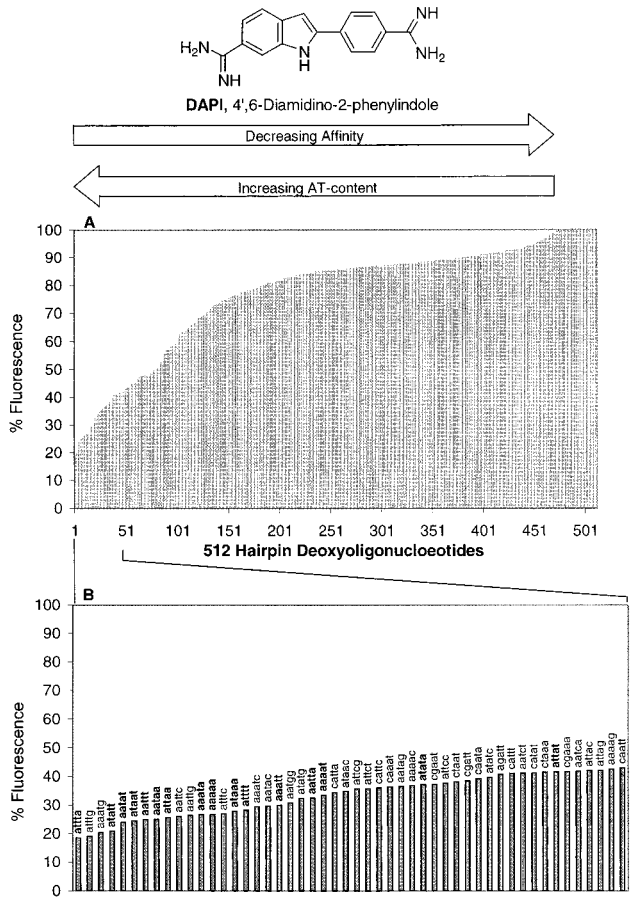

Figure 9. Screen of DAPI (2 μM concentration) against a library of DNA hairpin deoxyoligonucleotides: (**A**) all 512 sequences, (**B**) top 50 sequences showing highest affinity.

Table 3. DAPI Binding Constants

	DNA sequence	K ($\times 10^6$ M^{-1})[a]	K ($\times 10^6$ M^{-1})[b]
7	5′-AATTT-3′	110 (0.94)	120 (1.1)
8	5′-AATAA-3′	59 (0.98)	87 (0.94)
9	5′-ATTAA-3′	52 (0.88)	77 (1.0)
13	5′-AAAAA-3′	50 (0.95)	65 (0.99)

[a] Scatchard analysis of titration binding curve (experimental stoichiometry of binding). [b] Direct titration using the fluorescence enhancement of DAPI (experimental stoichiometry of binding).

a slope that is shallower and an area under the curve that is smaller than those of distamycin A, indicating that DAPI binds effectively to more sequences and exhibits less sequence selectivity than distamycin A.

One key element that we wish to emphasize with this study is that the fluorescence *enhancement* characteristic of the DAPI binding does not interfere with the measurement of the fluorescent *decrease* derived from the ethidium bromide displacement. This is a consequence of the ethidium fluorescence measurement made with excitation at 545 nm and an emission at 595 nm that is unaffected by the DAPI absorption (372 nm) and emission (454 nm). Thus, the generality of the method extends even to compounds that themselves exhibit fluorescent properties. The binding of DAPI to the library of hairpins could also be conducted such that its own intrinsic fluorescence enhancement was monitored and relative binding established by a % fluorescence increase.

The binding constants for DAPI were determined for the four hairpins first examined with distamycin A (Table 3). Although no binding constants established by other methods were found

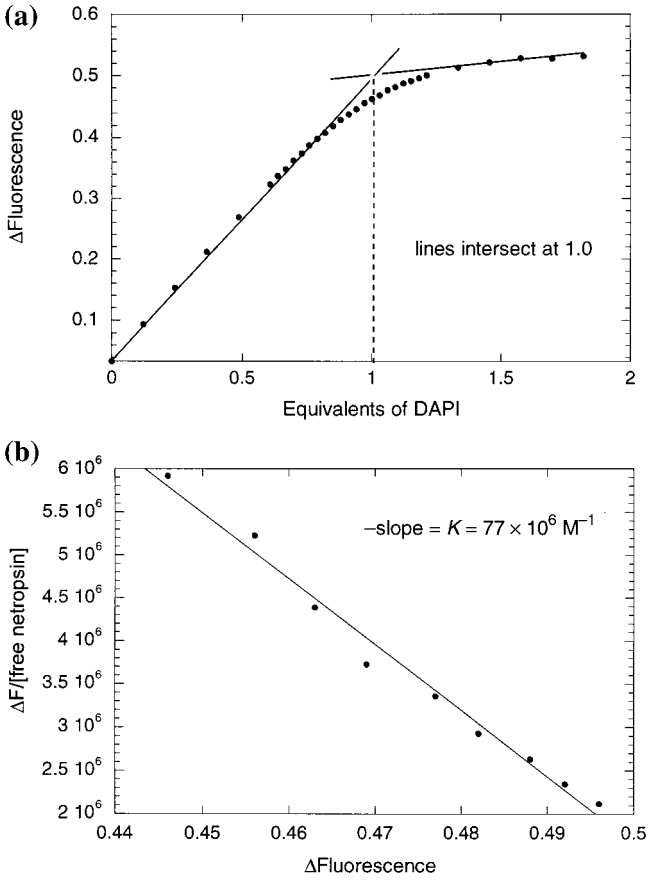

Figure 10. (a) Titration of DAPI vs the hairpin containing 5′-ATTAA-3′ at 1.1 μM (8.8 μM bp) utilizing the inherent fluorescence of the DNA:DAPI complex. (b) Scatchard plot for the titration of 5′-ATTAA-3′ utilizing the inherent fluorescence of the DNA:DAPI complex, slope $= -K$.

for these same sequences, closely related sequences have been studied by footprinting[37] or by direct fluorescence titration.[36] In addition, the characteristic fluorescence enhancement of DAPI upon DNA binding could be monitored in a direct titration in the absence of added ethidium bromide and used to independently establish binding constants and the 1:1 binding stoichiometry. The values obtained by this direct titration match closely but are slightly higher than those obtained in the ethidium bromide displacement titration, validating the accuracy of this latter technique (Table 3). Upon direct titration of the four hairpins, the resulting plot of fluorescence vs the number of equivalents of DAPI illustrated saturated binding upon addition of 1 equiv of DAPI (Figure 10).

Hoechst 33258. An additional fluorescent DNA stain, Hoechst 33258,[38] has been shown to selectively bind minor groove four and five bp AT sites.[39] Both NMR[40−46] and X-ray

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(44) Embrey, K. J.; Searle, M. S.; Craik, D. J. *Eur. J. Biochem.* **1993**, *211*, 437.

studies[47−50] have confirmed the AT-rich minor groove binding, and some of the latter X-ray studies, but not the former NMR studies, suggest an interaction of the *N*-methylpiperazine with a terminal GC bp. X-ray, NMR, and calorimetric studies revealed a 1:1 binding stoichiometry.[51] Footprinting studies[30,52] have further illustrated that Hoechst 33258 protects a five bp region within AT-rich sequences. Notably, these studies also corroborate the X-ray observation of a tolerance for AT-rich sequences containing isolated GC base pairs. Binding to GC-rich sequences has also been detected by a process that does not involve external or groove binding and has been attributed to a weaker intercalation binding.[53] Consistent with these observations, Hoechst 33258 exhibited AT-rich binding when assayed against the library of 512 hairpins (Figure 11). Its selectivity lies somewhere between that of DAPI and distamycin A, and this is clear from a comparison of the merged bar graphs of the 512 hairpin binding results. All three exhibit comparable binding to the best sequences, but the rise of the graph or slope of the plot lies between those of DAPI and distamycin A and the area under the curve indicates that the selectivity follows the order distamycin A > Hoechst 33258 > DAPI. Thus, 16 of 16 five bp AT sites (100%) and 19 of 32 four bp AT sites (59%) are found in the top 50 sequences. Although the top 20 sequences all contain five or four bp AT sites, 15 of the next 30 sequences (50%) or 15 in the top 50 (30%) contain a three bp AT site flanked by a GC bp. This binding is weaker than the four bp AT sites but significant enough to indicate that the X-ray observations of a GC interaction with the *N*-methylpiperazine represents the detection of this weaker binding to generic sequences represented by either GAAA or AAAG. Most prominent among these in the top 50 sequences of our assay are GATT (7, 14%) and GAAT (5, 10%). Unlike distamycin A, but like netropsin and DAPI, no site containing a GC bp central to a 5 bp AT-rich site is found in the top 50 sequences. Like the observations made with DAPI, the weaker binding of Hoechst 33258 with hairpins >100−150 (<20% fluorescent decrease) most likely represents that of the weaker intercalation. Most importantly, the intrinsic fluorescence enhancement upon Hoechst 33258 binding does not interfere with the assay.

The binding constants for Hoechst 33258 were established for the four hairpins first examined with distamycin A (Table 4). Although no binding constants for these sequences have been disclosed, those established by footprinting[37] or direct fluorescence titration[36,51] compare favorably with those established herein. Both the displacement of ethidium bromide and the measurement of a fluorescence *decrease* (excitation at 545 nm, emission at 595 nm) or the direct titration with Hoechst 33258 and the measurement of its fluorescence *increase* (excitation at 358 nm, emission at 454 nm) could be used to establish binding constants. Moreover, both titration binding curves confirmed the 1:1 stoichiometry of binding. Consistent with the trends observed with DAPI but slightly more pronounced with Hoechst 33258, the binding constants established by the ethidium bromide displacement were roughly and uniformly 2-fold weaker than those determined by direct titration. Thus, the former technique slightly underestimates the binding affinity.

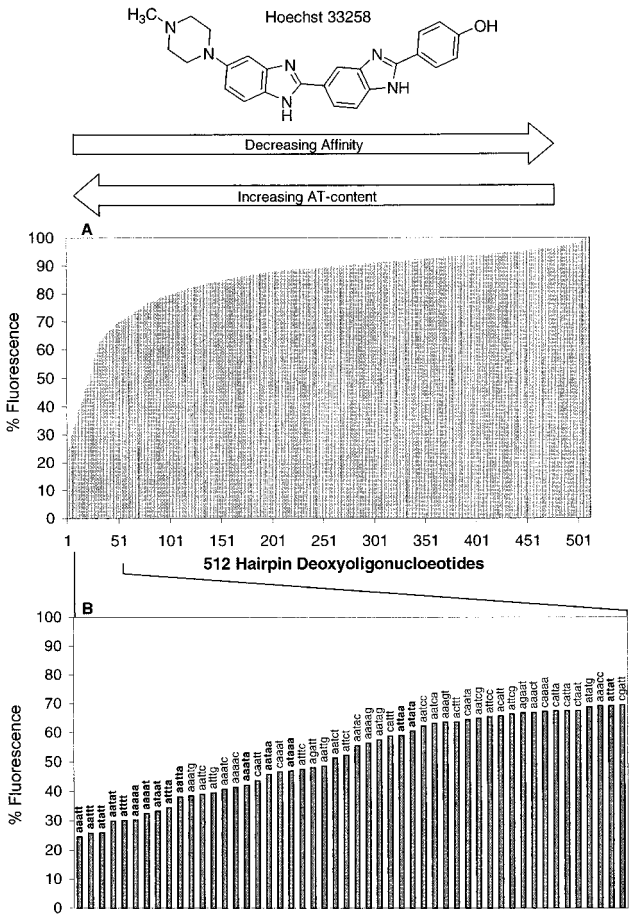

Figure 11. Screen of Hoechst 33258 (2 μM concentration) against a library of DNA hairpin deoxyoligonucleotides: (**A**) all 512 sequences, (**B**) top 50 sequences showing highest affinity.

Table 4. Hoechst 33258 Binding Constants

	DNA sequence	K ($\times 10^6$ M^{-1})[a]	K ($\times 10^6$ M^{-1})[b]
2	5′-AATTT-3′	88 (0.96)	177 (0.98)
6	5′-AAAAA-3′	72 (0.98)	145 (0.92)
18	5′-AATAA-3′	34 (1.06)	83 (1.01)
30	5′-ATTAA-3′	19 (0.91)	47 (1.05)

[a] Scatchard analysis of titration binding curve (experimental stoichiometry of binding). [b] Direct titration using the fluorescence enhancement of Hoechst 33258 (experimental stoichiometry of binding).

Berenil. A member of the aromatic diamidine class of DNA binding agents, berenil, reversibly binds AT-rich duplex DNA. X-ray[54] and NMR[55] structures of the complex between berenil and d(CGCGAATTCGCG)$_2$ illustrate a 1:1 stoichiometry of

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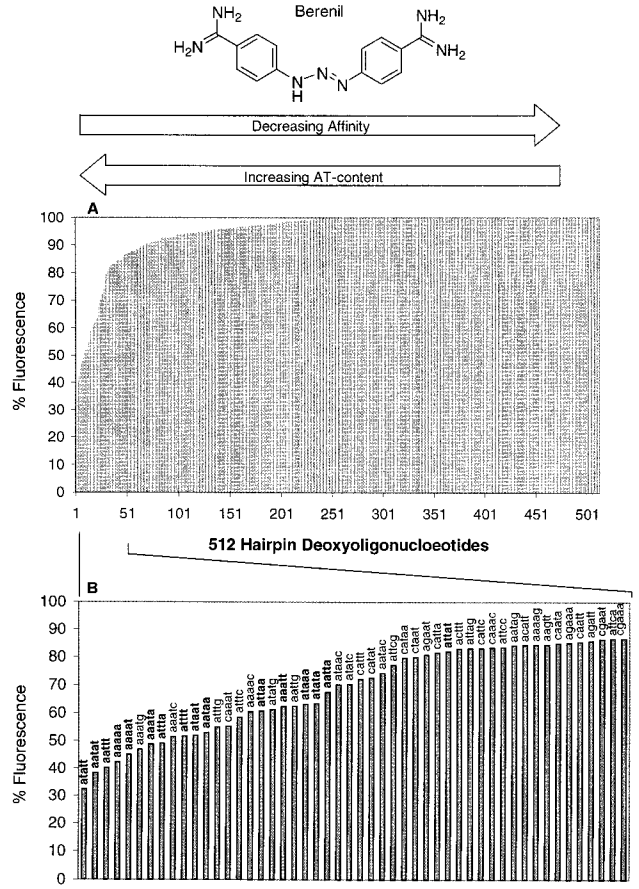

Figure 12. Screen of berenil (2 μM concentration) against a library of DNA hairpin deoxyoligonucleotides: (**A**) all 512 sequences, (**B**) top 50 sequences showing highest affinity.

binding with berenil residing in the minor groove of the AT tract. The terminal amidines were shown to engage in H-bonding with the terminal adenine N3 or thymine C2 carbonyls. The aromatic rings adopt an isohelical conformation and align parallel to the walls of the minor groove making close contacts with adenine. Footprinting studies established a sequence selectivity similar to that of netropsin and distamycin A with the highest affinity sites containing at least three contiguous AT bp.[56] In addition to binding in the minor groove and at higher concentrations, intercalative binding has also been observed.[57]

Its examination against the library of 512 hairpins revealed it to be a weaker DNA binding agent than the other compounds examined and that it exhibited the expected AT-rich binding selectivity (Figure 12). Despite its small size, it still exhibited a five > four > three AT bp site selectivity and the tightest binding sequences all contained five, and to a lesser extent four, AT bp. In addition, 16/16 five bp AT sites (100%), 21/32 four bp AT sites (66%), and 12/80 three bp (15%) sites were found in the top 50 sequences. The binding is modest, even to thebest sequences, and falls off rapidly such that only the top 20−30 sequences exhibit substantial binding at 2 μM.

Enlisting the four hairpins first examined with distamycin A, the binding constants for berenil were established, Table 5. All the binding constants followed the expected trends from the ranked binding profile and all were lower than those of distamycin A or the remaining minor groove binders. The experimentally measured saturation of binding for berenil

(56) Laughton, C. A.; Jenkins, T. C.; Fox, K. R.; Neidle, S. *Nucleic Acids Res.* **1990**, *18*, 4479.

(57) Pilch, D. S.; Kirolos, M. A.; Liu, X.; Plum, G. E.; Breslauer, K. J. *Biochemistry* **1995**, *34*, 9962.

Table 5. Berenil Binding Constants

	DNA sequence	K ($\times 10^6$ M^{-1})[a]
3	5′-AATTT-3′	13 (1.11)
4	5′-AAAAA-3′	6.4 (1.14)
12	5′-AATAA-3′	4.2 (1.12)
17	5′-ATTAA-3′	2.9 (1.11)

[a] Scatchard analysis of titration binding curve (experimental stoichiometry of binding).

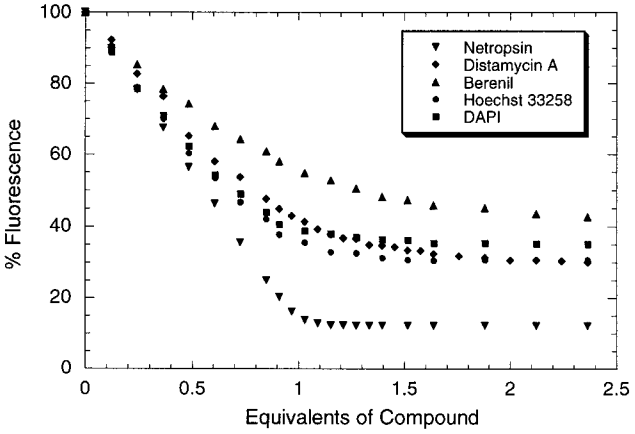

Figure 13. Titration curves of compounds vs the hairpin containing 5′-AAAAA utilizing ethidium bromide displacement.

confirmed the expected 1:1 stoichiometry of binding to the selected hairpins.

Displacement Stoichiometry. Initially, we expected that the % fluorescence decrease at saturation binding would not only provide information on the stoichiometry of ethidium bromide displacement but also on the binding site size of the compound. To a certain extent, this was observed with the saturation % fluorescence decrease following the expected order of distamycin A, Hoechst 33258 ≥ DAPI > berenil corresponding roughly to the displacement of 3 and 2 equiv of ethidium bromide, respectively (Figure 13). Inconsistent with expectations, netropsin displaced essentially all the ethidium bromide including that which would be expected to intercalate at the capping 5′-CGXXXXXC site. Although we do not presently have an explanation for this behavior of netropsin, it does indicate that caution should be taken in comparing affinities when examining the bar graph profiles of two different compounds measured at a single concentration. That is, the % fluorescence decrease can reflect not only relative affinity but also a different stiochiometry of ethidium bromide displacement. This limitation does not affect the comparisons made upon quantitative titration. Reflective of the titration curves, the binding constants for the hairpin containing AAAAA were established as follows: netropsin = 347 $\times$ 10^6 M^{-1}, Hoechst 33258 = 72 $\times$ 10^6 M^{-1}, DAPI = 50 $\times$ 10^6 M^{-1}, distamycin A = 17 $\times$ 10^6 M^{-1}, and berenil = 6.4 $\times$ 10^6 M^{-1}.

In addition, the visual inspection of the titration binding curves for the five compounds indicate that netropsin, Hoechst 33258, and DAPI exhibit clean 1:1 binding with no evidence of a second binding event. By contrast, both distamycin A and berenil exhibit a weaker second or continued fluorescence decrease through the addition of 2 equiv of compound. For distamycin A, this most certainly represents the additional 2:1 side-by-side binding, whereas this may represent a second, lower affinity end-to-end binding for berenil.

Selectivity of the Minor Groove Binding Agents. The intention of the studies detailed herein was not to redefine the

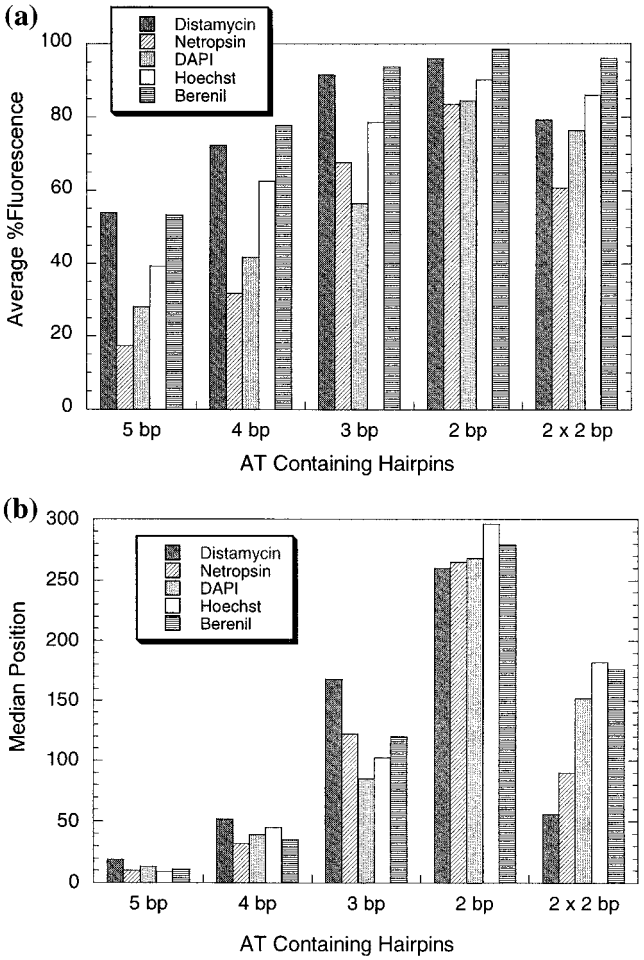

Figure 14. (a) Average % fluorescence of hairpins containing five, four, three, two, and two × two contiguous AT bp for all five compounds surveyed. (b) Median position of hairpins containing five, four, three, two, and two × two contiguous AT bp for all five compounds surveyed.

well established selectivity of the minor groove binding agents. However, the results of the comparisons provide a few new insights that were not previously easily recognized. Analyzing either the average % fluorescence decrease for related sites (Figure 14) or evaluating the statistical site frequency occurrence (Table 6) in the top 50 sequences offer approaches to comparing selectivities or recognizing distinctions. Netropsin is the most AT selective of the compounds examined and, by some accounts, distamycin A is the least AT selective. All exhibit tight binding to five > four > three bp AT sites. Presumably, this five > four > three bp AT site preference is related in part to the conformational characteristics of DNA where the longer AT-rich sites possess the narrower, deeper minor groove known to contribute substantially to the selective binding affinity. Arbitrarily selecting the top 50 sequences and analyzing the statistical site frequency, little distinction is observed for five bp AT sites although the frequency decreases slightly in the order netropsin, DAPI, Hoechst-33258, berenil (100%) > distamycin A (88%), Table 6. More significant was the frequency decrease observed for the four bp AT sites, netropsin (84%) > DAPI (72%) > berenil (66%) > Hoechst-33258 (59%) > distamycin A (47%), and the corresponding increase in three or two bp AT sites, distamycin A (27%) > Hoechst 33258 (19%) > berenil (15%) > DAPI (14%) > netropsin (9%). This represents the unique inclusion of AT-rich five bp sites containing a central GC base pair for distamycin A and the ability of Hoechst 33258 to bind three bp AT sites capped with a GC bp. We suggest that this reflects (1) the larger five bp binding site requirement for distamycin A vs a four bp site for netropsin and the compensating ability for distamycin A to bind selected GC bp interrupted five bp AT-rich sites, (2) the ability for Hoechst 33258 to uniquely bind GATT/GAAT sites presumably via a G interaction with the terminal *N*-methylpiperazine, and (3) the smaller size of DAPI and berenil which more easily accommodates a three vs four bp AT site.

The preferences for a five > four > three > two bp AT site for all compounds studied and the ability for distamycin to bind a GC bp interrupted five bp AT site (two × two bp) is also clear from the average % fluorescence decrease for the grouped sites (Figure 14). Thus, distamycin exhibits an affinity for the two × two bp AT sites that exceeds that of the three bp ATsites and is only slightly weaker than that of the four bp AT sites. Netropsin shows a weaker preference for such two × two bp AT sites, none of which appear in the top 50 sequences (Table 6), whereas DAPI, Hoechst 33258, and berenil do not exhibit a similar unique affinity. An alternative way of examining the same data is to establish a median position in the 512 hairpin library for the grouped sequences (Figure 14b). This avoids some confusion in comparing the data due to the differences in the stoichiometry of ethidium bromide displacement (different % fluorescence decrease). Clearer in this plot of the data is the preference for distamycin binding to a two × two bp AT-rich site vs three bp AT site and the former is nearly as effective as binding to a four bp AT site. Netropsin shows a weaker ability to bind a two × two bp AT site which is comparable with a three bp AT site but much less effective than binding a four bp AT site. In contrast, DAPI, Hoechst 33258, and berenil exhibit a clear preference for a three bp AT site over a two × two bp AT site.

Thiazole Orange as an Alternative to Ethidium Bromide. The enhancement of fluorescence for the binding of ethidium bromide to duplex DNA is roughly 20-fold and varies from sequence to sequence. For the excitation and emission wavelengths used (545 and 595 nm, respectively), not only is the enhancement small and the sensitivity of the assay modest but the fluorescence of some DNA binding agents may interfere at these wavelengths. As a consequence, we examined an additional DNA intercalating dye, thiazole orange (TO),[58] that exhibits a different excitation and emission fluorescence (509 and 527 nm, respectively),[59] displays a higher, but less sequence dependent affinity for DNA,[59] and produces a more intense fluorescence enhancement upon binding to duplex DNA (50−2000 vs 20-fold for ethidium bromide).[59] The less sequence dependent DNA affinity suggests that thiazole orange should behave in a manner analogous to ethidium bromide but that intrinsic errors derived from individual sequence variance may be further minimized. However, its greater affinity introduces errors potentially attributable to more effective competitive binding. More importantly, the sensitivity enhancement derived from the 10−100 fold increase in the fluorescence enhancement means the assay can be conducted with a more robust measurement signal and/or at even lower concentrations. The former increases the accuracy of the comparisons whereas the latter potentially reduces the reagent expense for conducting the assay. Presently, the ethidium bromide assay requires amounts of the 512 hairpins that cost $100/assay (20 cents/hairpin) that can be further reduced with the use of thiazole orange.

(58) Lee, L. G.; Chen, C.-H.; Chiu, L. A. *Cytometry* **1986**, *7*, 508.
(59) Nygren, J.; Svanvik, N.; Kubista, M. *Biopolymers* **1998**, *46*, 39.

Table 6. Statistical Comparison of Top 50 Sequences

DNA sequence	distamycin A	netropsin	DAPI	Hoechst 33258	berenil
five-base pair AT sites	14/16 (88%)	16/16 (100%)	16/16 (100%)	16/16 (100%)	16/16 (100%)
four-base pair AT sites	15/32 (47%)	27/32 (84%)	23/32 (72%)	19/32 (59%)	21/32 (66%)
three-base pair AT sites	10/80 (13%)	7/80 (9%)	11/80 (14%)	15/80 (19%)	12/80 (15%)
two-base pair AT sites	4/160 (3%)	0/160 (0%)	0/160 (0%)	0/160 (0%)	0/160 (0%)
two × two base pair AT sites	7/16 (44%)	0/16 (0%)	0/16 (0%)	0/16 (0%)	1/16 (6%)

Table 7.

A. Ethidium Bromide and Thiazole Orange Binding Constants

hairpin	K_{EB} ($\times 10^6$ M^{-1})[a]	K_{TO} ($\times 10^6$ M^{-1})[a]
5′-AAAAA-3′	0.27 (4.5)[b]	1.2 (3.5)[b]
5′-AATTT-3′	0.42 (3.6)	1.3 (3.3)
5′-ATTAA-3′	0.87 (3.3)	1.0 (3.3)
5′-AATAA-3′	0.73 (3.3)	1.1 (3.3)

B. Netropsin Binding Constants

	DNA sequence	K ($\times 10^6$ M^{-1})[c]	K ($\times 10^6$ M^{-1})[d]
2	5′-AAAAA-3′	238 (0.98)	347 (0.99)
4	5′-AATTT-3′	85 (1.03)	97 (1.05)
6	5′-ATTAA-3′	67 (0.95)	78 (0.94)
10	5′-AATAA-3′	35 (1.1)	71 (1.0)

C. DAPI Binding Constants

	DNA sequence	K ($\times 10^6$ M^{-1})[c]	K ($\times 10^6$ M^{-1})[d]	K ($\times 10^6$ M^{-1})[e]
7	5′-AATTT-3′	58 (0.91)	110 (0.94)	120 (1.1)
8	5′-AATAA-3′	39 (0.83)	59 (0.98)	87 (0.94)
9	5′-ATTAA-3′	24 (0.83)	52 (0.88)	77 (1.0)
13	5′-AAAAA-3′	22 (0.94)	50 (0.95)	65 (0.99)

D. Hoechst 33258 Binding Constants

	DNA sequence	K ($\times 10^6$ M^{-1})[c]	K ($\times 10^6$ M^{-1})[d]	K ($\times 10^6$ M^{-1})[e]
2	5′-AATTT-3′	75 (0.90)	88 (0.96)	177 (0.98)
6	5′-AAAAA-3′	36 (0.86)	72 (0.98)	145 (0.92)
18	5′-AATAA-3′	28 (0.77)	34 (1.06)	83 (1.01)
30	5′-ATTAA-3′	13 (0.89)	19 (0.91)	47 (1.05)

[a] Established herein for the hairpin deoxyoligonucleotides containing the indicated sequence by direct titration. [b] Stoichiometry of binding established experimentally. [c] Thiazole orange displacement (experimental stoichiometry of binding). [d] Ethidium bromide displacement (experimental stoichiometry of binding). [e] Direct fluorescence titration (experimental stoichiometry of binding).

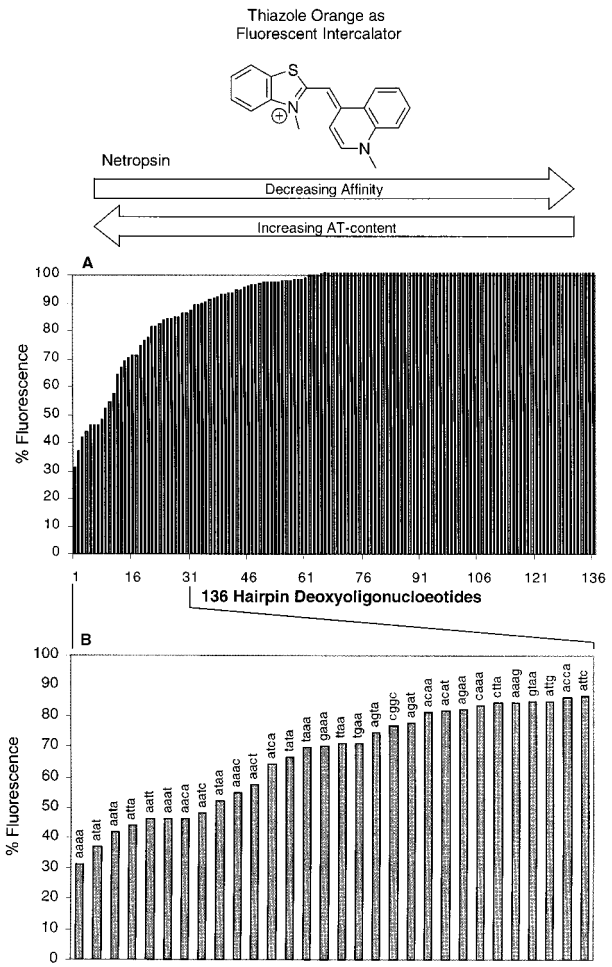

Figure 15. Screen of netropsin (2 μM concentration) against a library of hairpin deoxyoligonucleotides containing a four bp variable region and utilizing thiazole orange as the fluorescent intercalator: (**A**) all 136 sequences, (**B**) top 30 sequences showing highest affinity.

We conducted a preliminary examination of thiazole orange, assessing the assay concentration range, establishing its affinity and stoichiometry of binding with representative hairpins (Table 7), and utilizing it in both the 96-well format assay and in quantitative titrations for determining binding constants (Table 7). These preliminary studies indicate that thiazole orange permits the assay concentration to be reduced at least 2−4-fold, reducing the amount and cost of the required hairpins accordingly. A representative example of the use of thiazole orange in the 96-well format assay is illustrated in Figure 15 with the assay of netropsin against a 136-membered hairpin library containing all possible four bp sites. Netropsin exhibited the expected four > three > two bp AT selectivity. The mean rank of the 10 available four bp AT sites was 7.3, whereas the mean positions for the three and two bp sites were 41.6 and 67.7, respectively. Moreover, the rank order binding across the full ensemble of four bp AT sites is consistent with that established in footprinting studies.[14,23,60] Notably, the highest affinity site is 5′-AAAA and the sequences 5′-TTAA, 5′-TATA, 3′-TAAA, and to a lesser extent 5′-ATAA, are among the lowest affinity four bp AT sites for netropsin, and these same trends are observed in our rank order binding profile: AAAA > ATAT, AATA, ATTA, AATT, AAAT > ATAA > TAAA, TATA, TTAA. Thus, although is has been less widely studied, the comparisons suggest thiazole orange is a superb screening alternative to ethidium bromide.

Consistent with past observations, the stoichiometry of binding and the K_{app} for thiazole orange for the four hairpins examined proved less sequence dependent than ethidium bromide, but it displayed higher binding constants (Table 7). The netropsin binding constants and stoichiometry of binding established using the displacement of thiazole orange proved to be in good agreement with those established with ethidium bromide (Table 7), albeit lower. The additional comparisons of binding constants established for DAPI and Hoechst 33258 illustrate these similarities and distinctions in the use of ethidium bromide and thiazole orange (Table 7). Binding constants for both DAPI and Hoechst 33258 were established by direct

(60) Ward, B.; Rehfuss, R.; Goodisman, J.; Dabrowiak, J. C. *Biochemistry* **1988**, *27*, 1198. Fish, E. L.; Lane, M. J.; Vourakis, J. N. *Biochemistry* **1988**, *27*, 6026. Kittler, L.; Baguley, B. C.; Lober, G.; Waring, M. J. *J. Mol. Recognit.* **1999**, *12*, 121.

titration, the results of which can be used to assess the accuracy of the ethidium bromide or thiazole orange displacement titration results. In each case, the direct and indirect titration methods accurately reflected the trends observed in the 96-well format assay (rank order affinity). However, the intercalator displacement titrations systematically underestimated the binding constants. The constants established with ethidium bromide typically were within an acceptable factor of 2 or less of those established by direct titration, whereas the systematic derivation with thiazole orange was noticeably larger. Presumably, this reflects the greater competitive binding of thiazole orange, reducing the measured apparent binding constant. Interestingly, the sequence-dependent variance in the binding affinity of ethidium bromide did not introduce errors of significance that were detectable in our comparisons. As such, the studies indicate that the binding constants established with ethidium bromide are preferred and, for most practical purposes, are comparable in both the trends and absolute magnitudes established by direct titration.

Conclusions

A technically nondemanding method for establishing DNA binding selectivity and affinity was disclosed. The technique entails the measurement of the loss of fluorescence derived from the displacement of ethidium bromide or thiazole orange from hairpin deoxyoligonucleotides containing all possible five (512 hairpins) or four bp sequences (136 hairpins) displayed in a 96-well format. This provides a rank order binding to all possible five or four bp sequences resulting in a high-resolution definition of a compound's sequence selectivity. In addition to the high-resolution definition of a compound's sequence selectivity, we anticipate that such binding profiles will prove instrumental in selecting or distinguishing candidate ligands that display selectivity for an ensemble of related sequences characteristic of nuclear hormone receptors or transcription factors. Technical issues associated with the implementation of the assay were investigated, parameters affecting the accuracy of the results were defined, experimental conditions suitable for conducting assays applicable to multiple compounds of varying affinities were determined, and its use in comparing the DNA binding properties of distamycin A, netropsin, DAPI, Hoechst 33258, and berenil were presented. Under the conditions disclosed, the present cost of the 512 hairpin deoxyoligonucleotides enlisted for a single assay is ca. $100 (ethidium bromide) or less (2−4-fold, thiazole orange).[61] Paramount to the success of the approach is the establishment of the hairpin concentration as single-strand DNA (80−95 °C, UV). Notable observations include the fact that intrinsic fluorescence properties of the DNA binding agents themselves do not interfere with the assay, and if they do, alternative fluorescent intercalators are available for use (TO vs EtBr).

Just as importantly, the assay may be utilized to establish DNA binding constants for a given sequence through quantitative titration. Several methods of establishing binding constants were examined, and the most reliable entails a Scatchard or curve fitting analysis of the titration binding curve which also determines the stoichiometry of binding.[12]

Finally, the technique is nondestructive, suggesting that immobilization of the hairpins (chips, beads, glass slides) would permit their rinsing and reuse in subsequent assays. This hairpin immobilization and reuse would remove the barrier to examining complete libraries of longer sequences (>five base pairs) and extend the use of the technology to the characterization of binding site sizes typical of proteins.

(61) The full set of 512 hairpin deoxyoligonucleotides may be purchased from GenBase, 6450 Lusk Blvd., Suite E 107, San Diego, CA 92121. Telephone: 858-453-8879. E-mail genbase@aol.com.

Experimental Section

Ethidium Bromide Assay. 512-Member Deoxyoligonucleotide Library. Hairpin deoxyoligonucleotides were purchased from Genbase Inc.[61] as 1200 μM (bp) solutions in water and stored as stock solutions at −80 °C. Prior to use, each deoxyoligonucleotide was diluted to 120 μM in water and stored at 0 °C for no longer than 2 days. Each well of a Costar black 96-well plate was loaded with Tris buffer containing ethidium bromide (88 μL of a 0.68×10^{-5} M solution in buffer (0.1 M Tris, 0.1 M NaCl, pH 8), 0.60×10^{-5} M ethidium bromide final concentration). To each well was added one hairpin deoxyoligonucleotide (10 μL, 1.2×10^{-5} M in DNA bp final concentration) followed by agent (2 μL of a 0.1 mM solution in water, 2.0×10^{-6} M final concentration). After incubation at 25 °C for 30 min, each well was read (average of 30 readings) on a Molecular Devices Spectra Max Gemini fluorescent plate reader (ex. 545 nm, em. 595 nm, cutoff filter at 590 nm) in duplicate with two control wells (no agent = 100% fluorescence, no DNA = 0% fluorescence). Fluorescence readings are reported as % fluorescence relative to the control wells. In our experience, fluorescence plate readers show a variability of ±10%, but surface effects (i.e., bubbles, dust) may contribute to larger variations requiring a second set of measurements.

All other experiments described (i.e., compound concentration, overall assay concentration, ethidium bromide:DNA ratio, time variability, etc.) utilized this general procedure in a 96-well plate format.

Determination of Binding Constants. Ethidium Bromide/Thiazole Orange Displacement. A 3 mL quartz cuvette was loaded with Tris buffer (0.1 M Tris, 0.1 M, NaCl, pH 8) and ethidium bromide or thiazole orange (0.44×10^{-5} M final concentration). The fluorescence was measured (excitation 545 nm, emission 595 nm, EtBr) and normalized to 0% relative fluorescence. The hairpin deoxyoligonucleotide of interest was added (1.1 μM, 8.8 μM in bp final concentration), and the fluorescence was measured again and normalized to 100% relative fluorescence. A solution of compound (2 μL, 0.1 mM in DMSO) was added, and the fluorescence was measured following 5 min of incubation at 23 °C. The addition of 2 μL aliquots was continued until the system reached saturation and the fluorescence remained constant with subsequent compound additions.

Direct Fluorescence Titration of DAPI, Hoechst 33258, Ethidium Bromide or Thiazole Orange. A 3 mL quartz cuvette was loaded with Tris buffer (0.1 M Tris, 0.1 M, NaCl, pH 8) and hairpin deoxyoligonucleotide (1.1 μM, 8.8 μM in bp final concentration). A solution of DAPI (excitation 372 nm; emission 454 nm; 2 μL, 0.1 mM in DMSO), Hoechst 33258 (excitation 358 nm; emission 454 nm; 2 μL, 0.1 mM in DMSO), ethidium bromide (excitation 545 nm; emission 595 nm; 2 μL, 0.5 mM in H_2O) or thiazole orange (excitation 509 nm; emission 527 nm; 2 μL, 0.5 mM in DMSO) was added, and the fluorescence was measured following 5 min of incubation at 23 °C. The addition of 2 μL aliquots was continued until the system reached saturation and the fluorescence remained constant with subsequent additions.

Scatchard Analysis of the Titration Curve. The ΔF was plotted vs molar equiv of agent and the ΔF_{sat} was determined mathematically by solving the simultaneous equations representing the pre- and postsaturation regions of the titration curve. Utilizing eqs 1−3, a Scatchard plot was generated where ΔF/[free agent] was plotted vs ΔF. The slope of the region immediately preceding complete saturation of the system provided $-K$.

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Supporting Information Available: Expanded versions of Figures 3, 8, 9, 11, and 12 showing the rank order binding of distamycin A, netropsin, DAPI, Hoechst 33258, and berenil to all 512 hairpins, an expanded version of Figure 15 showing the rank order binding of netropsin to all 136 hairpins, and a discussion of the noncompetitive and competitive binding models for binding constant determination. This material is available free of charge via the Internet at http://pubs.acs.org.

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