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Thiazole Orange as the Fluorescent Intercalator in a High Resolution FID Assay for Determining DNA Binding Affinity and Sequence Selectivity of Small Molecules

Dale L. Boger* and Winston C. Tse

Department of Chemistry and The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

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Abstract—The viability of using thiazole orange as an alternative to ethidium bromide in a fluorescent intercalator displacement (FID) assay is explored by profiling the DNA binding affinity and sequence selectivity of netropsin. Utilizing a library of hairpin deoxyoligonucleotides containing all possible four base-pair sequences, the method provides a high resolution profile of the DNA binding properties of small molecules in a high throughput format. © 2001 Elsevier Science Ltd. All rights reserved.

Introduction

The regulation of gene expression is based on the sequence selective recognition of nucleic acids by repressor, activator, and enhancer proteins. The prospect of selective disruption or control over such processes, in particular the use of cell permeable small molecules as therapeutics,[1] is a long standing goal of molecular biology, chemistry, and medicine.[2] In order to match the dexterity of regulatory proteins in sequence recognition, small molecules may need to target an ensemble of related sites that comprise a consensus binding sequence. As this recognition event is more complicated than targeting a single ideal sequence, the discovery and development of such small molecules have been slow. This is due to the complexity associated with understanding small molecule–DNA interactions, the iterative process of designing and synthesizing individual compounds targeted toward specific DNA sequences, and the technically demanding techniques involved in determining their binding affinity for any given sequence much less an ensemble of sequences.

Of the techniques commonly used to establish the DNA binding properties of small molecules,[3] notably footprinting and affinity cleavage,[4–6] most are technically challenging and time consuming and none are applicable to the high throughput screening required of large libraries of compounds generated by combinatorial syntheses. Moreover, the techniques are only capable of examining a limited set of DNA sites at one time, fitted within a custom piece of DNA, rendering the establishment of a full DNA binding profile nearly inaccessible.

In efforts which address some of the limitations of current techniques, we recently reported the development of a high throughput fluorescent intercalator displacement (FID) assay for establishing DNA binding affinity and sequence selectivity that is both technically nondemanding and non-destructive.[7,8] It relies on the fluorescence decrease derived from the displacement of DNA-bound ethidium bromide by a DNA binding compound. Full details of the method were disclosed along with the DNA binding profiles of several agents including distamycin A and netropsin against a 512-membered library of hairpin deoxyoligonucleotides containing all five base-pair (bp) sequences utilizing ethidium bromide as the fluorescent intercalator. In an extension of the assay, herein we report the scope and limitations of use of thiazole orange as an alternative to ethidium bromide defined in the course of establishing the DNA binding affinity and selectivity profile of netropsin against a 136-membered[9] library of hairpin deoxyoligonucleotides containing all possible four base-pair DNA sites (Fig. 1).

Thiazole orange is a member of a family of asymmetric cyanines that is the product of a rational design effort to develop an effective dye for reticulocyte analysis.[10] We

*Corresponding author. Tel.: +1-858-784-7522; fax: +1-858-784-7550; e-mail: boger@scripps.edu

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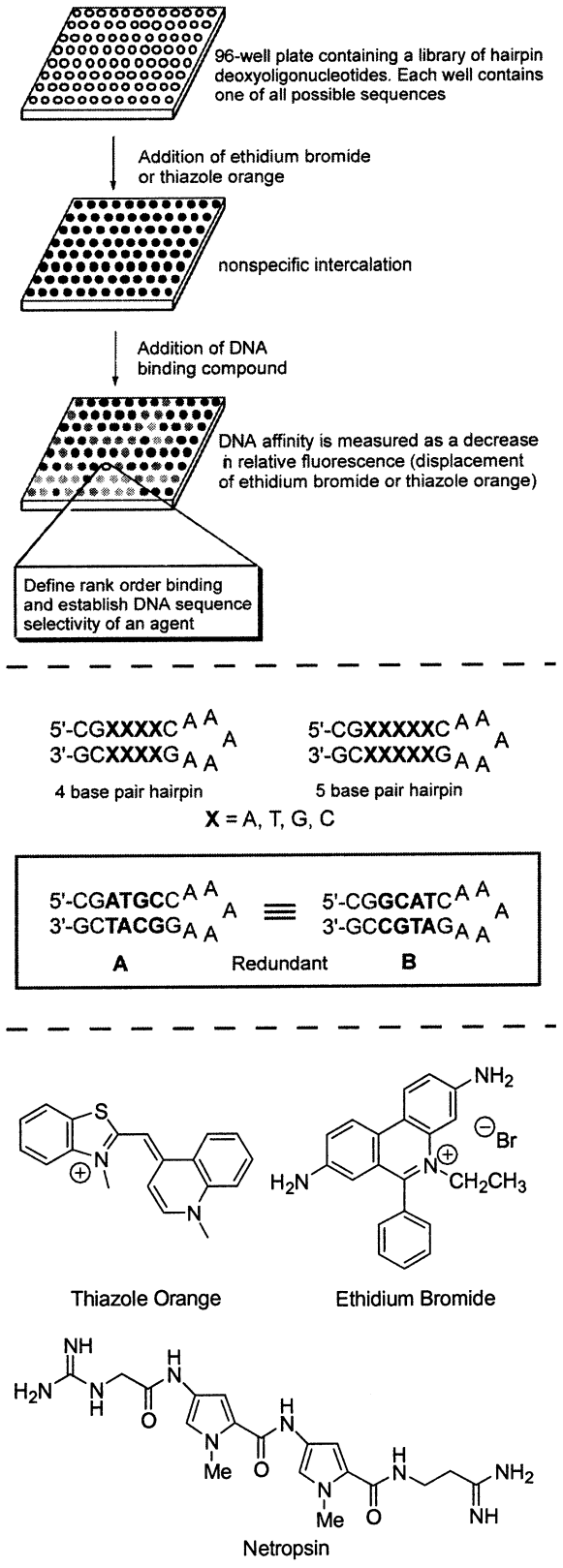


Figure 1. General procedure for the FID assay adaptable for the determination of the sequence selectivity of a DNA binding agent. The structures of the hairpin deoxyoligonucleotides for the four base-pair library and the five base-pair library is shown. Positioning within the hairpin was not considered, making A and B redundant.

chose to examine thiazole orange due to three aspects that are of particular interest to the FID assay: the excitation and emission maxima are distinct from ethidium bromide, the fluorescence enhancement upon DNA intercalation has been reported to far exceed that of ethidium bromide (ca. 3000-fold vs 20-fold), and thiazole orange is reported to display a comparable but less sequence dependent affinity for DNA.[11]

Results and Discussion

In order to examine the performance of thiazole orange, we conducted the screening of netropsin against a library of 136 hairpin deoxyoligonucleotides containing all possible four base-pair DNA binding sites displayed in a 96-well format (Fig. 1).[12] For comparison, we conducted the same assay in parallel with ethidium bromide. Employing the optimized conditions we reported for assay against a five base-pair library,[7] the final concentration of DNA in each well was set at 1.5 μM (10.5 μM in base-pairs) and the final concentration of thiazole orange or ethidium bromide was set at 4.5 μM (3:7 intercalator/base-pair), the expected stoichiometry of intercalator binding to the hairpin oligomer. For each fluorescent dye, three concentrations of netropsin (1.0, 1.5 and 2.0 μM) were screened against the library of hairpins.

Netropsin[13] has been extensively studied and well characterized to bind in the minor groove with AT selectivity by methods including footprinting,[14] NMR,[15] X-ray,[16] and calorimetry.[17] The results of our assay with both thiazole orange and ethidium bromide revealed the expected AT-rich binding sequence selectivity for netropsin against all possible four base-pair DNA sites. An examination of the rank order binding profile of the 136-membered library yields an overall portrait of the DNA binding affinity and a high resolution definition of the DNA binding sequence selectivity of netropsin (Figs. 2a and b). The observed decrease in percent fluorescence is directly related to the extent of DNA binding by netropsin, and the 96-well assay provides relative DNA binding affinities where the sequences with the lowest percent fluorescence possess the greatest affinity for netropsin. The merged bar graph of the rank order binding profiles provide some comparative measure of overall DNA binding selectivity. That is, the slope of the curve and the overall area under the curve may be useful ways to compare binding selectivity. Important to the evaluation of thiazole orange versus ethidium bromide, the overall profile remains similar with the use of the two different intercalators and under varying concentrations of netropsin.

In addition, there is an associated vertical and horizontal displacement of the profiles as the concentration of netropsin increases for each intercalator. This shift is reflective of the assay being conducted under non-saturated and equilibrium conditions, as opposed to being conducted at agent saturation conditions, and is a vital component of the conditions optimized for the assay.[7] Conducting the assay at higher agent

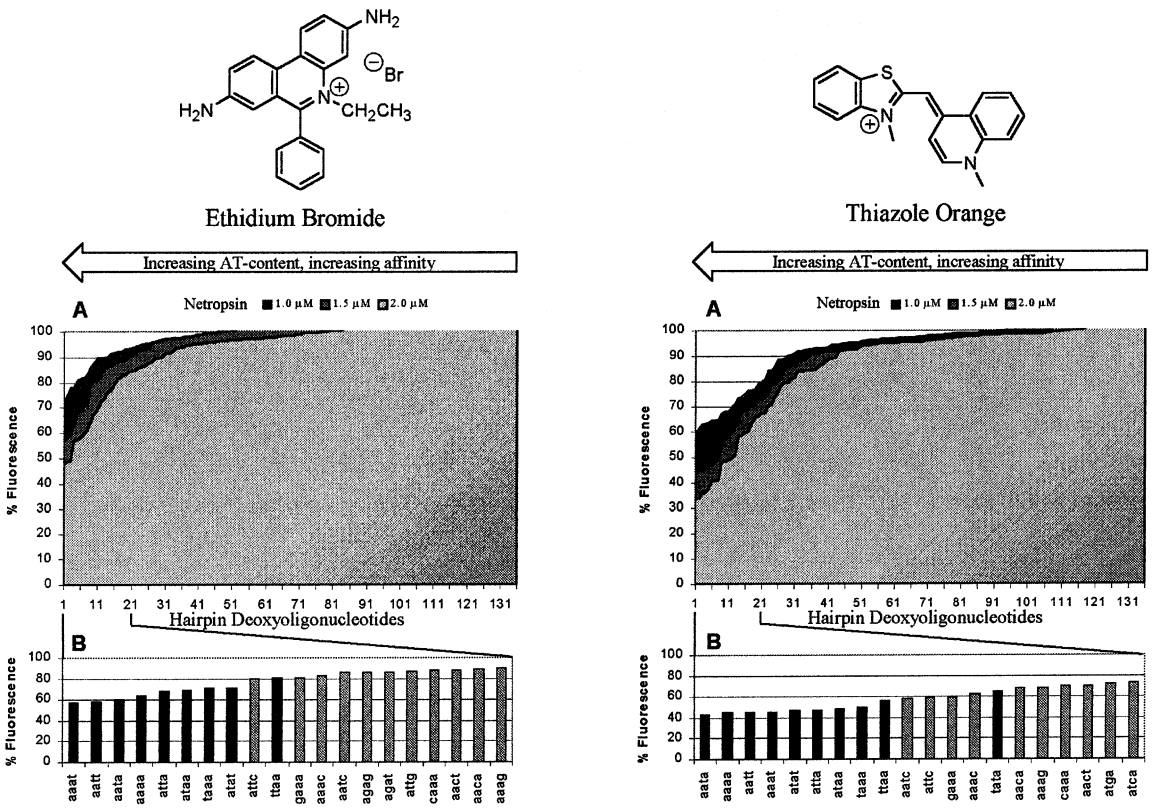


Figure 2. (a) FID assay of netropsin against a library of 136 hairpin deoxyoligonucleotides utilizing ethidium bromide: (A) merged bar graph overlay plot of netropsin at various concentrations; (B) enlargement of the first 20 sequences at 1.5 μM netropsin; (b) FID assay of netropsin against a library of 136 hairpin deoxyoligonucleotides utilizing thiazole orange. (A) merged bar graph overlay plot of netropsin at various concentrations; (B) enlargement of the first 20 sequences at 1.5 μM netropsin.

concentrations beyond the near 1:1 stoichiometry results in loss of resolution at the higher affinity sequences.[7]

Examination of the rank order binding for these higher affinity sites reveals that netropsin displays the expected strong preference for AT-rich sites.[18] In all assays (Figs. 3 and 4), nine of the total of 10 sequences within the library entirely comprised A or T were always among the best binders. At concentrations of 1.5 and 2.0 μM netropsin with ethidium bromide, the top eight sequences were four base-pair AT sequences, while for thiazole orange, the top nine sequences were four base-pair AT sequences. It is also interesting to note that with the use of thiazole orange, the percent fluorescence decrease is observed to be lower than that when the assay is conducted with ethidium bromide. The mean rank of the four base-pair AT sites was 6.9 whereas the mean positions for the 3 base-pair AT sites and 2 base-pair AT sites were 44 and 70, respectively. As such, netropsin exhibits the expected 4 bp > 3 bp > 2 bp AT selectivity and this is easily extracted from the data derived from the assay. The assays performed at 1 μM netropsin for either intercalator deviate more significantly from expectations and from the results observed at either 1.5 or 2.0 μM. We attribute this to the low agent to DNA ratio (1:1.5), the low assay concentration and the smaller percent fluorescence decrease which is measured, and the intrinsic errors associated with the 96-well assay format that places this concentration on the periphery of the optimal range of conditions. A similar effect was observed during the optimization of the assay utilizing a 512-membered five base-pair hairpin library.[7] Even with the increased variability at 1 μM netropsin, it is clear that netropsin exhibits a preference for a four base-pair AT binding site with either ethidium bromide or thiazole orange. That the assay rapidly and accurately identifies the ensemble of sequences favored by netropsin out of the full complement of possible sequences is a paramount conclusion of the assay.

As with all high throughput screens, the data generated

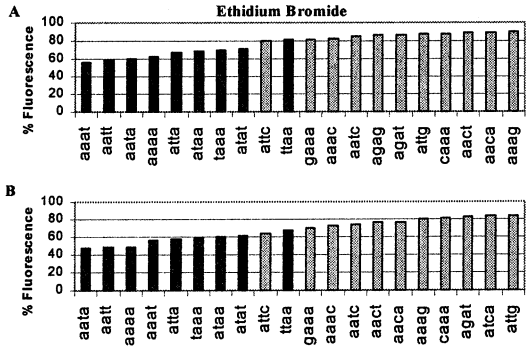


Figure 3. Binding affinity sequence rank order for netropsin against a library of four base-pair DNA hairpin deoxyoligonucleotides utilizing ethidium bromide as the fluorescent intercalator. Top 20 sequences shown with those comprised entirely of A and T in black, all others in gray: (A) 1.5 μM netropsin; (B) 2.0 μM netropsin.

by measurement at a single concentration in a 96-well format is not designed for absolute accuracy. The speed and breath of information for which such assays were developed often precludes absolute accuracy at some level. Nonetheless, the assay does exceptionally well at providing reproducible rank order bindings. A detailed inspection of the ten sequences comprised entirely of A or T reveals that only a slight reordering occurs in the rank order binding as intercalator and agent concentration is varied (Fig. 5). Arranged in the binding order established with 1.5 μM netropsin, the rank order binding comparison for ethidium bromide shows only a very minor reordering between 1.5 and 2.0 μM concentrations of netropsin (Fig. 5A). Thiazole orange similarly displays only minor variation in the sequence rank order between 1.5 and 2.0 μM netropsin (Fig. 5B). Direct comparison between the two intercalators at 2.0 μM netropsin, shown in Figure 6, exhibited a consistent rank order binding demonstrating that the performance of thiazole orange is comparable to ethidium bromide. However, it is important to realize that a minor reordering in rank order binding upon variation of intercalator and agent concentration is expected in the 96-well format assay since the differences between adjacent sequences are often within the experimental error of the assay. Nonetheless, the rank order binding across a full ensemble of sequences, even among the tightest binders, is consistent and reproducible. Notably, several independent footprinting studies have placed the sequences 5′-TTAA, 5′-TATA, 5′-TAAA, 5′-ATAT, and, to a lesser extent, 5′-ATAA among the lowest affinity four base-pair sites for netropsin[14] and these same trends are observed in our ranked order binding. Also consistent in these studies is the emergence of 5′-AAAA, 5′-AAAT, and 5′-AATT as the highest affinity sequences and identical trends are observed in the high throughput FID assay.[14]

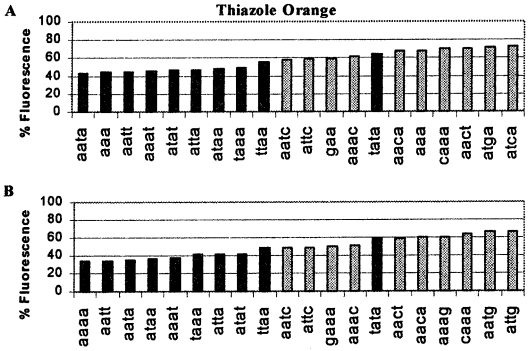


Figure 4. Binding affinity sequence rank order for netropsin against a library of four base-pair DNA hairpin deoxyoligonucleotides utilizing thiazole orange as the fluorescent intercalator. Top 20 sequences shown with those comprised entirely of A and T in black, all others in gray: (A) 1.5 μM netropsin; (B) 2.0 μM netropsin.

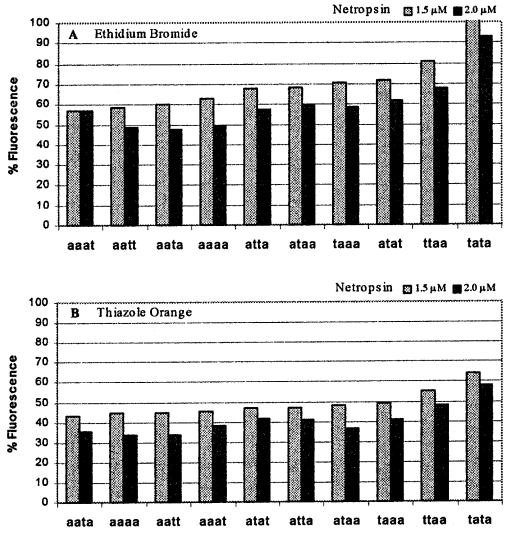


Figure 5. Sequence rank order comparison at varying concentrations of netropsin: (A) use of ethidium bromide as the fluorescent intercalator; (B) use of thiazole orange as the fluorescent intercalator.

The 96-well format fluorescence intercalator displacement (FID) assay provides a rapid and comprehensive survey of all possible binding sequences, providing comparison data that exceeds that of existing methods. However, the 96-well format FID assay is not quantitative nor is it designed to distinguish among adjacent sequences. The issues of careful comparisons of side-by-side sequences or minor reorderings in the rank order binding profiles can be addressed by quantitative titrations of chosen sequences. Binding constants are best established by Scatchard analysis of the titration binding curves and the stoichiometry of binding best established from the method employed by Bruice.[7,19,20]

Before examining the titration of netropsin against the hairpin DNAs, the binding characteristics of ethidium bromide and thiazole orange were examined. Direct titration of ethidium bromide and thiazole orange with a selected DNA hairpin measuring the fluorescence increase provided both an absolute binding constant and the stoichiometry of binding. The four base-pair hairpin library contains a stretch of seven base-pairs (four variable and three capping base-pairs) and is expected to accommodate three sites of intercalation at saturation. Direct titration of the hairpin 5′-AATT with each intercalator resulted in an observed binding stoichiometry of 2.92 for ethidium bromide and 3.05 for thiazole orange. Binding constants were within the range of those reported in the literature, and as established in our prior study,[7] and the binding constant for thiazole orange was found to be greater than that of ethidium bromide (Table 1).

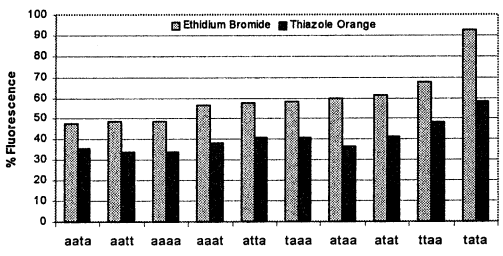


Figure 6. Sequence rank order comparison between ethidium bromide and thiazole orange at 1.5 μM netropsin.

In order to establish quantitative binding constants for individual hairpin deoxyoligonucleotides in the library, we developed a titration method based on the same principles as the FID assay, namely the displacement of DNA bound ethidium bromide or thiazole orange and monitoring the ensuing loss of fluorescence. We chose to examine the complete set of 10 DNA hairpins that were comprised entirely of A and T utilizing both intercalators. The quantitative titrations unambiguously established the sequence preference of netropsin to these DNA hairpin deoxyoligonucleotides by producing reliable apparent associative binding constants (Fig. 7). These binding constants are not only consistent with those sequences previously determined within a five base-pair library context[7] but also are consistent with those published in literature (Table 1).[14,17] The binding constants obtained by use of thiazole orange are observed to be comparable, albeit slightly lower, than those obtained by use of ethidium bromide, reflective of the greater competitive binding of thiazole orange. Prior comparisons with direct titration results[7] indicate that those established with ethidium bromide are thus preferred. The stoichiometry of binding in each case was found to be the expected one molar equivalent of agent per hairpin deoxyoligonucleotide.

Table 1. Comparative binding constants

Titration binding constants			
Titrant	DNA Sequence	K ($\times 10^6$ M^{-1})	Binding stoichiometry
EthBr	5′AATT	2.7	2.92
TO	5′AATT	15	3.05

Titrant	DNA Sequence	Ethidium bromide K ($\times 10^6$ M^{-1})	Thiazole orange K ($\times 10^6$ M^{-1})
Netropsin	5′AAAT	127	113
	5′AAAA	92	71
	5′AATT	65	54
	5′AATA	64	44
	5′ATTA	45	35
	5′ATAT	41	33
	5′ATAA	34	18
	5′TAAA	26	15
	5′TTAA	11	8
	5′TATA	11	8

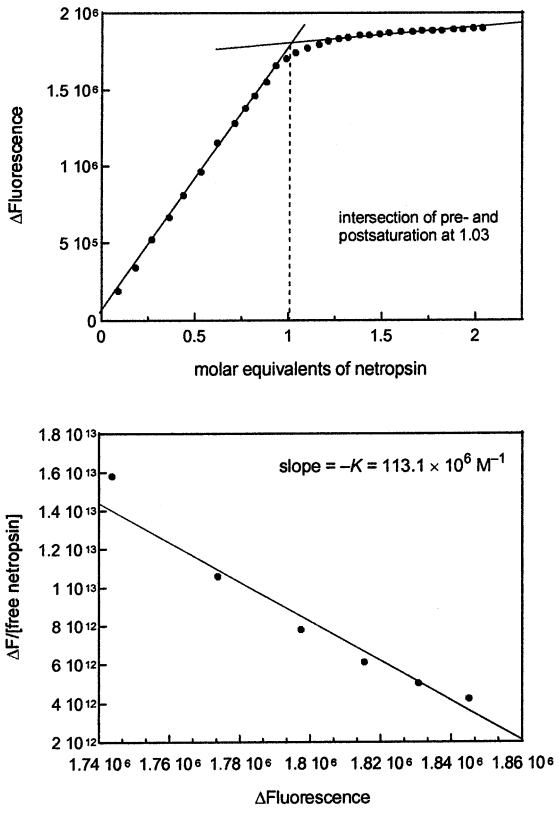
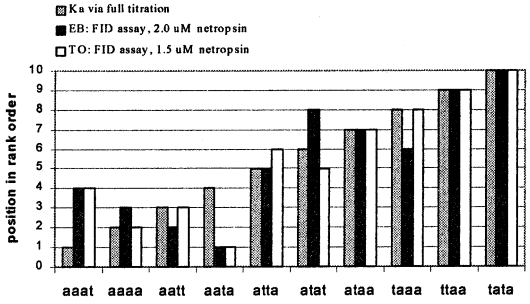


Figure 7. Titration of netropsin versus the hairpin containing 5′-AAAT at 1.5 μM with thiazole orange and the Scatchard plot for determining K_a.

With the determination of binding constants, there is now an explicit and accurate rank order of sequences preferred by netropsin. Notably, this also now permits the validation of prior footprinting rankings of all possible 4 bp AT binding sequences and defines the selectivity at a higher resolution. Importantly, the prior footprinting studies have provided trends consistent with our rank order profile, but no prior study has compared all 10 potential sequences.[14] Thus, not only are our rankings consistent with those of past studies, but also it defines the ranking at complete resolution. Comparison of this rank order with that obtained by the assay show that the assay produces a remarkably similar profile with the best results arising from the use of 2.0 μM netropsin with ethidium bromide and 1.5 μM netropsin with thiazole orange (Fig. 8). As the first eight sequences in the rank order are the best eight contiguous sequences of 136-membered library, minor reordering by these neighboring sequences was neither surprising nor unexpected. Nonetheless, we sought to ascertain the limits of fine resolution that could reasonably



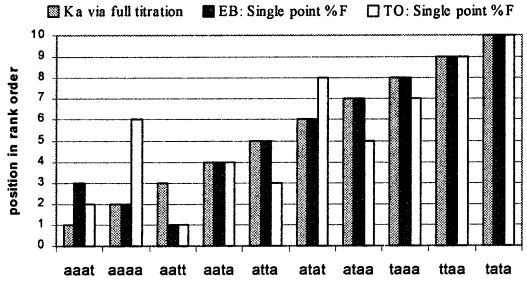
FID Assay Results

		Netropsin conc. 2.0 uM EB	1.5 uM TO
1	aaat	56.632	45.125
2	aaaa	48.908	44.804
3	aatt	48.903	44.830
4	aata	47.676	43.181
5	atta	57.582	47.056
6	atat	61.552	47.046
7	ataa	59.530	48.246
8	taaa	58.289	49.168
9	ttaa	67.619	55.414
10	tata	92.688	64.257

Figure 8. Sequence rank order comparison between orders as established by binding constants (gray bars), FID assay utilizing ethidium bromide at 2.0 μM netropsin (dark bars), and FID assay utilizing thizole orange at 1.5 μM netropsin (white bars).

be expected from the assay. From the titration curves that yielded the associative binding constants, we extracted the percent fluorescence decrease value at which 1.00 and 1.33 equivalents of netropsin has been added to the hairpin oligomer, mirroring the conditions used in the full FID assay. The rank order provided by the percent fluorescence decrease in this manner did not produce the exact rank order revealed by the binding constants obtained from the same titration curves for either ethidium bromide or thiazole orange (Fig. 9) although that of ethidium bromide is the more consistent of the two. The best three sequences, 5′-AAAT, 5′-AAAA, and 5′-AATT, are narrowly separated by a percent fluorescence decrease margin that is well within the error of the high throughput FID assay despite the fact the binding constants are spread over a 2-fold difference. The same trend is seen with the four hairpins that comprise the 5th–8th sequences in the rank order. Yet, the distinction between the two groups of sequences, #1–3 and #5–8, is clearly and easily identified by the FID assay (Fig. 8). Although the percent fluorescence decrease derived from a single point on the titration curves themselves do not precisely predict the absolute rank order as provided by the binding constants, the assay does operate at a refined resolution easily allowing rapid identification of the preferred ensemble of sequences for a given compound. However, substantive conclusions drawn about adjacent sequences should be reserved for quantitative titrations and not extrapolated from single point measurements or the 96-well format assay results.

Thiazole orange has been shown to function with comparable performance to ethidium bromide in the FID assay as well as in the quantitative titrations. Moreover, the excitation and emission maxima for thiazole orange[11] (ex. 509 nm, em. 527 nm)[21] are distinct from that of ethidium bromide (ex. 545 nm, em. 595 nm). Should the fluorescence spectrum of a DNA binding agent overlap and interfere with one intercalator, the advantages of having an alternative orthogonal fluorescence system is evident.



Single point %F from Titrations

		1.33 equiv netropsin per hairpin DNA	
		EB	TO
1	aaat	12.601	14.783
2	aaaa	12.496	20.542
3	aatt	10.953	13.090
4	aata	17.165	19.363
5	atta	20.525	17.448
6	atat	20.580	23.115
7	ataa	22.368	19.489
8	taaa	22.933	22.661
9	ttaa	50.000	38.098
10	tata	55.651	40.014

Figure 9. Sequence rank order comparison between orders as established by binding constants via full titration (gray bars), single point percent fluorescence measurement from titrations utilizing ethidium bromide (dark bars), single point percent fluorescence measurement from titrations utilizing thiazole orange (white bars).

In addition, the fluorescence enhancement upon DNA intercalation of thiazole orange has been reported to greatly exceed that of ethidium bromide.[11] We expected that this would in turn translate into a more robust and reliable measurement signal and/or the ability to conduct the assay at even lower concentrations. Small variations in readings are less significant with the larger fluorescent readings of thiazole orange and, accordingly, the consistency of our duplicate measurement readings did improve with its use. Studies directed at reducing the concentration of the assay are ongoing and preliminary results indicate that thiazole orange permits the assay concentration to be reduced at least 2–4 fold diminishing the amount and cost of the required hairpins accordingly.[22]

It also has been reported that thiazole orange displays little variation in binding affinity to double-stranded deoxyoligonucleotides, but with a typically higher binding constant.[10] In our experience, thiazole orange displayed a comparable and less sequence dependent affinity for DNA than ethidium bromide.[7] Thus, thiazole orange should behave in a manner analogous to ethidium bromide, and intrinsic errors derived from individual sequence variance could be further minimized making it a useful screening alternative to ethidium bromide. However, the absolute binding constants established through use of thiazole orange are comparable to, but typically slightly lower than, those established with ethidium bromide.

Conclusions

The fluorescence intercalator displacement (FID) assay has proven to be an inexpensive,[22] rapid, accurate, and high resolution means of identifying the sequence selectivity binding profile of netropsin to all possible four base-pair DNA sequences. In addition, the studies detailed herein have shown that thiazole orange is a viable alternative to ethidium bromide in both the 96-well format high throughput FID assay as well as in quantitative titrations for establishing binding constants. The use of thiazole orange did not compromise the quality of the information or the ultimate conclusions and may provide improvements in the sensitivity of the assay. At the very least, it provides an alternative to ethidium bromide in instances where the fluorescent excitation or emission overlaps with those of the DNA binding compounds under study.

In combination with quantitative titration experiments to establish absolute binding constants, the assay provides a high resolution technique to establish the DNA binding properties of small molecules. We anticipate that the generation of binding profiles for novel

compounds 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. Such applications are under study and will be reported in due course.

Experimental

Fluorescent intercalator displacement assay

Concentrations of hairpin deoxyoligonucleotides were determined at 90 °C to ensure accurate concentration determination with single-strand millimolar extinction coefficients and diluted to a uniform volume and concentration. Each well of a Costar black 96-well assay plate was loaded with Tris buffer containing the fluorescent intercalator (70 μL of 6.43 μM EthBr or TO in 0.1 M NaCl, 0.1 M Tris, pH 8.0). To each well was added one hairpin deoxyoligonucleotide of the library (10 μL, 15 μM, 105 μM in base-pairs in H_2O) followed by netropsin (20 μL, 5, 7.5, or 10 μM in H_2O). Final concentrations were 1.5 μM DNA hairpin, 4.5 μM intercalator, and 1.0, 1.5, or 2.0 μM netropsin. After incubation at 25 °C for 30 min, each well was read (average of eight readings) on a Molecular Devices Spectra Max Gemini fluorescent plate reader (TO: ex. 504 nm,[21] em. 527 nm, cutoff 515 nm; EthBr: ex. 545 nm, em. 595 nm, cutoff 590 nm) in duplicate with two control wells (no agent = 100% fluorescence, no DNA = 0% fluorescence). Fluorescence readings are reported as percent fluorescence relative to the control wells. In our experience, fluorescence plate readers show a variability of ±10% depending on the fluorescent intercalator, but surface effects (e.g., bubbles and dust) and pipetting errors may contribute to larger variations requiring a second set of measurements.

Titration utilizing the fluorescent intercalator displacement

A 3 mL quartz cuvette was loaded with Tris buffer (0.1 M NaCl, 0.1 M Tris, pH 8.0) and fluorescent intercalator (4.5 μM final concentration). The fluorescence was measured on a JYHoriba Spex FluoroMax-3 spectrofluorometer at the respective excitation and emission maxima and normalized to 0% fluorescence. The hairpin deoxyoligonucleotide of interest was added (1.5 μM, 10.5 μM in base-pairs final concentration), and the resulting fluorescence was normalized to 100%. Titrations were conducted by adding aliquots of netropsin (2 μL, 0.2 mM or 2 μL, 0.1 mM in H_2O) and measuring the resultant fluorescence decrease after a 5 min equilibration time. Additions were continued until the system reached saturation and the fluorescence remained constant with subsequent compound additions.

Direct fluorescence titration with ethidium bromide and thiazole orange

A 3 mL quartz cuvette was loaded with Tris buffer (0.1 M NaCl, 0.1 M Tris, pH 8.0) and hairpin deoxyoligonucleotide (1.1 μM, 7.7 μM in base-pairs final concentration). The fluorescence was measured at the respective excitation and emission maxima and normalized to 0% fluorescence. Titrations were conducted by adding aliquots of ethidium bromide or thiazole orange (2 μL, 0.5 mM) and measuring the resultant fluorescence increase after a 5 min equilibration time. Additions were continued until the system reached saturation and the fluorescence remained constant with subsequent compound additions.

Determination of binding constants by Scatchard analysis

The ΔF was plotted versus molar equivalents of agent and the ΔF_{sat} was determined mathematically by simultaneous solving the equations representing the pre and post saturation regions of the titration curve. Utilizing eqs (1)–(3), a Scatchard plot was generated where ΔF/[Free Agent] was plotted versus ΔF. The slope of the region immediately preceding complete saturation of the system provided $-K$. In these equations, [free agent] = concentration of free agent, $[DNA]_T$ = total concentration of DNA, X = molar equiv of agent versus DNA, ΔF_x = change in fluorescence, and ΔF_{sat} = change in fluorescence at the point where DNA is saturated with ligand.

$$\left(\frac{\Delta F_x}{\Delta F_{sat}}\right)\frac{1}{X} = \text{Fraction of DNA–Agent Complex} \quad (1)$$

$$\left[1 - \left(\frac{\Delta F_x}{\Delta F_{sat}}\right)\frac{1}{X}\right] = \text{Fraction of Free Agent} \quad (2)$$

$$[DNA]_T\left[X - \frac{\Delta F_x}{\Delta F_{sat}}\right] = [\text{Free Agent}] \quad (3)$$

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21. Due to the proximity of the emission wavelength to the excitation wavelength, use of an excitation wavelength of 509 nm with a top reading fluorescence plate reader may led to inconsistent results. We have found that use of 504 nm as the excitation wavelength produces more consistent and reliable results.
22. The quantities of the 136-membered hairpin library required for a single assay conducted in duplicate may be purchased at a current cost of approximately $25–30.