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Comprehensive High-Resolution Analysis of Hairpin Polyamides Utilizing a Fluorescent Intercalator Displacement (FID) Assay

Winston C. Tse, Takahiro Ishii and Dale L. Boger*

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

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Abstract—Four hairpin polyamides bearing subtle N- and C-terminal substitutions were examined in a fluorescent intercalator displacement (FID) assay enlisting a library of 512 DNA hairpins that contain all possible five base pair sequences in a challenging probe of its capabilities for establishing DNA binding sequence selectivity. Not only did the assay define the global sequence selectivity expected based on known structural interactions and Dervan's pairing rules establishing the utility of the method for characterizing such polyamides, but previously unappreciated subtle substituent effects on global sequence selectivity were also revealed. Thus, we report the discovery of a novel five base pair high affinity binding site of the form 5′-WWCWW (vs 5′-WGWWW) for the polyamide ImPyPy-γ-PyPyPy-β-Dp and its structural basis.

Introduction

Hairpin polyamides composed of *N*-methylpyrrole (Py), *N*-methylimidazole (Im), and a growing set of structural analogues bind in the DNA minor groove with predictable sequence selectivity and high binding affinities[1–6] and those that can target predetermined DNA sequences have the potential to modulate gene expression.[7,8] In recent efforts to discover small molecules with novel or predefined DNA binding selectivity, we have reported the generation of combinatorial libraries based on CC-1065[9] or the distamycin A scaffolds,[10,11] novel hairpin polyamide architectures,[12,13] and explored saturated analogues of distamycin A.[14] Whereas the synthesis of such compounds has benefited from high-throughput technologies, the rapid assessment of compounds to determine DNA binding affinity and sequence selectivity remains a challenge in the discovery of new agents.

Of the techniques commonly used to establish the DNA binding properties of small molecules,[15] notably footprinting and affinity cleavage,[16–22] most are technically challenging and none are applicable to the high throughput screening required of large libraries of compounds generated by combinatorial synthesis. In addition, the techniques are capable of examining only a limited set of DNA sites at one time. Thus, the establishment of a full DNA binding profile including an assessment of modest or nonbinding sites is nearly inaccessible by these means. This inherent scope can prove to be a liability given that it is often not only a single sequence that is ideally targeted, but rather an ensemble of related sites that compose the consensus binding sequence of a nuclear hormone receptor or transcription factor. Establishing selectivity for an ensemble of sites over all other sequences requires the comprehensive assessment of binding to all possible sites.

In efforts that address some of the limitations of current techniques, we recently introduced a high throughput fluorescent intercalator displacement (FID) assay for establishing DNA binding affinity and comprehensively defining sequence selectivity.[10,11,23,24] Herein, we report the use of the FID assay in the analysis of four hairpin polyamides (**1**–**4**, Fig. 1) of complex sequence specificity, that not only serves to establish its utility for examining minor groove binding polyamides, but that also underscores the value of its comprehensive capabilities.

The polyamides **1**–**4** were chosen to examine and highlight the subtle effects of N- and C-terminal functionalization on the DNA binding selectivity of these ligands

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

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representing a challenging test of the capabilities of the FID assay. The complete binding profiles of these four molecules to all possible five base pair (bp) DNA binding sites were established using the FID assay. We show that in screening complete sequence space, information on these molecules, and the effect of terminal functional groups, may be obtained that is not readily accessible by other techniques. Not only did the assay rapidly identify the expected impact of several structural features on DNA sequence selective binding that have been reported, but a novel 5-bp DNA binding site for the polyamide ImPyPy-γ-PyPyPy-β-Dp was also identified exceeding or matching that of the expected binding site. Based on the comprehensive data set generated by the assay, a binding model for this new site was proposed that was subsequently confirmed by FID titrations.

Results

N- and C-terminal functionalization of polyamides has been shown to greatly affect their DNA binding affinity and sequence selectivity.[1–3,25,26] In some cases, the effect of polyamide substitution has been profound such that a relatively subtle structural change has a remarkable DNA binding consequence. For example, substitution of a β-alanine linker can control extended versus hairpin binding[12,13] and the substitution or deletion of a N-terminal formamido group results in altered minor groove binding and sequence selectivity.[25,26] Thus, novel derivatives may possess different and unexpected binding modes and/or sequence selectivity.

Four hairpin polyamides (**1**–**4**) with differing end group functionality were examined to establish the utility of the FID assay for examining sequence selectivity in a high throughput and comprehensive manner. Hairpin polyamide **1** contains an unsubstituted N-terminal N-methylpyrrole and C-terminal dimethylaminopropyl (Dp) tail. Hairpin **2** is structurally similar to **1** but its terminal Py contains an additional N-acetyl group. Hairpin **3** possesses an N-terminus Im versus Py and does not contain functionality appended off its N-terminal heterocycle although it retains the C-terminal Dp tail of **1** and **2**. Hairpin **4** shares the same N-terminus Im as hairpin **3**, but contains an additional β-alanine (β) spacer in its charged Dp tail.

The FID assay, which relies on the measurement of a fluorescence decrease derived from the displacement of DNA-bound ethidium bromide by a DNA binding agent, was conducted as previously described enlisting a library of 512 individual hairpin deoxyoligonucleotides representing all possible 5-bp sequences.[23,24] Although there are 1024 possible sequences of a 5-bp DNA site, the position of the sequence within the hairpin was not considered making, for example, the sequence 5′-ATGCA equivalent to its complementary sequence 5′-TGCAT (Fig. 2). Hairpin sequences are referred to by the first variable region of the DNA hairpin, read 5′ to 3′. The concentration of DNA was fixed at 1.5 μM (12 μM in base pairs) and the concentration of ethidium bromide set at 6.0 μM (1:2 intercalator/base pairs). The compounds (final concentrations of 1.0, 1.5 or 2.0 μM) were added to the solutions containing the DNA hairpins saturated with ethidium bromide. The normalized percent fluorescent decrease for each DNA sequence was calculated and reported. FID sequence data was analyzed to determine affinity of the hairpin polyamides

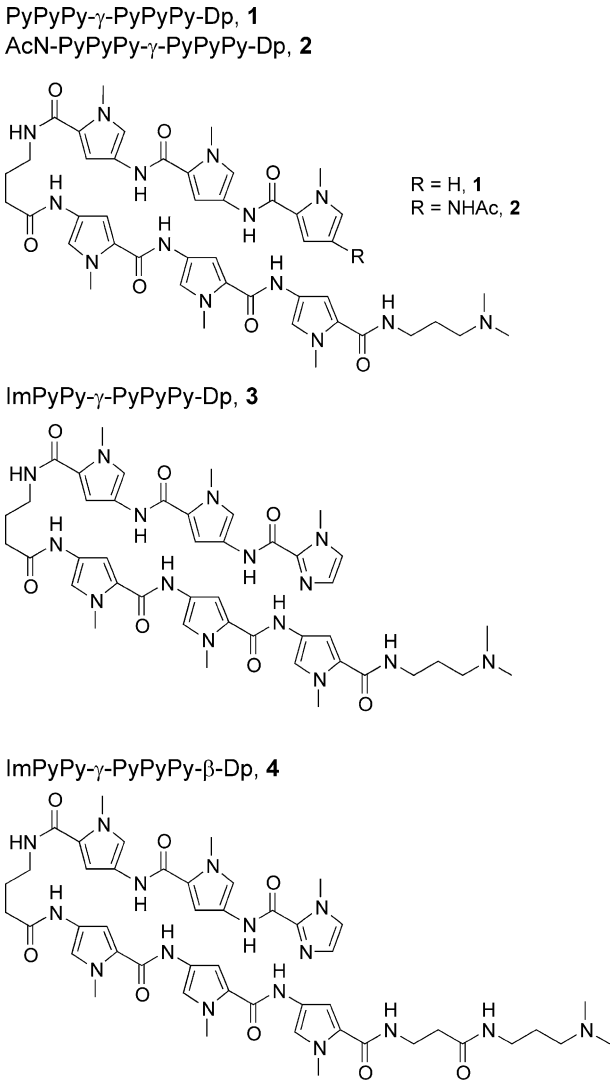

Figure 1. Hairpin pyrrole–imidazole polyamides.

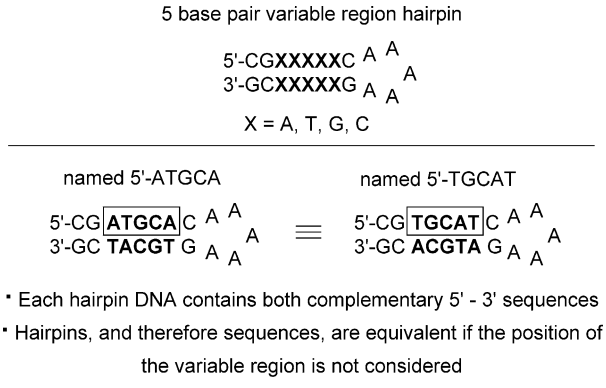

Figure 2. Structure of hairpin deoxyoligonucleotides representing all possible combinations of five base pairs. The two sequences shown are considered equivalent if the hairpin structure is not considered.

for ensembles of related sequences as well as for individual sequences.

For detailed comparisons between individual sequences, FID titration experiments were conducted based on the same principles as the assay. Compounds were titrated against hairpin deoxyoligonucleotides saturated with ethidium bromide. Scatchard analysis of the titration curves provide binding constants and a detailed means of comparing sequences.[27–29]

PyPyPy-γ-PyPyPy-Dp (1)

The FID assay was conducted with the library of 512 hairpin deoxyoligonucleotides representing all possible 5-bp sequences. A merged bar graph of the rank order binding is shown in Figure 3 and is typical of the compounds that follow. Sequence sets were rated based on the average rank position of its constituent members and by the average of a scoring system. Analysis of the sequence data generated by the FID assay (Table 1) illustrates that the highest affinity sequence set was a 5-bp site comprised entirely of A/T bps (5′-WWWWW, where W refers to either an A or T). Affinity for all other sequence sets was low to modest including the abbreviated 4-bp A/T site 5′-SWWWW (where S refers to either a C or G) for which there was a considerable distinction.

FID titration experiments of **1** to select sequences provide quantitative binding constants. Binding of **1** to the 5-bp A/T site, 5′-AAAAA, was observed with a K_a of 1.94×10^7 M^{-1} (Table 1). Binding to abbreviated 4-bp A/T sites resulted in a qualitative lower affinity, segmented titration curves, and a larger experimentally observed stoichiometry, indicating a more complex 2:1 binding mode.

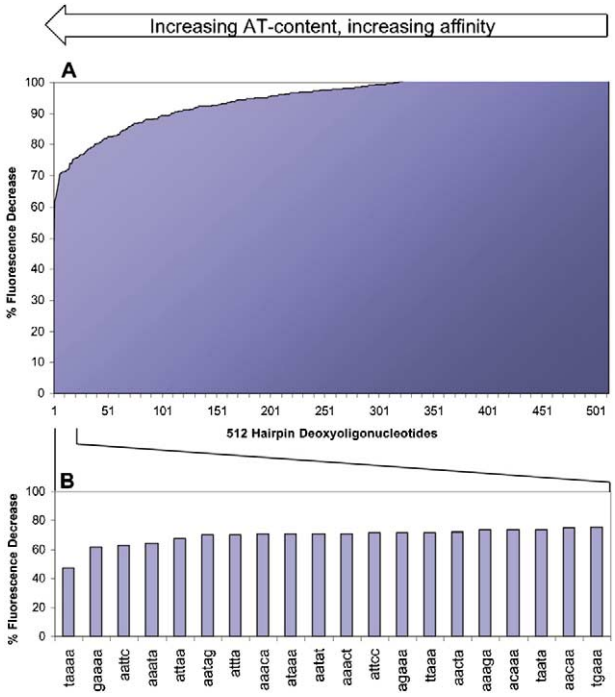

Figure 3. Screen of PyPyPy-γ-PyPyPy-Dp (2 μM concentration) against a library DNA hairpin deoxyoligonucleotides representing all possible 5-bp sequences: (A) all 512 hairpin DNAs, (B) top 20 hairpins showing highest affinity.

AcN-PyPyPy-γ-PyPyPy-Dp (2)

Analysis of the sequence rank order generated by the FID assay indicated that the expected sequence set 5′-WWWWW is the highest affinity DNA binding set (Table 2). In contrast to non-acetylated **1**, **2** also displays fairly high relative affinity to the 4-bp A/T sequence set 5′-SWWWW. All other sequence sets examined were of low affinity for this molecule including the sequence set 5′-SSWWW.

The FID assay of AcN-PyPyPy-γ-PyPyPy-Dp established that the highest affinity ensemble of sequences is the expected 5-bp site comprised entirely of A and T (5′-WWWWW), but the assay also clearly indicates that a shortened 4-bp A/T (5′-SWWWW) site is an additional relative high affinity site. Collectively, this indicates that four consecutive A/T base pairs is a minimal effective binding site. This observation is consistent with the NMR behavior revealed by Wemmer and coworkers for ligands with a N-terminal acetyl group.[25] Due to destabilizing intramolecular contacts, the C-terminal pyrrole and charged tail have been observed to flip out of the

Table 1. Sequence selectivity of PyPyPy-γ-PyPyPy-Dp[a]

FID assay sequence analysis				
Sequence	1.5 μM **1**		2.0 μM **1**	
	Avg rank	Avg score	Avg rank	Avg score
5′-WWWWW	45	0.50	29	0.49
5′-SWWWW	117	0.30	109	0.28
5′-SSWWW	215	0.12	224	0.08
5′-SSSWW	227	0.10	232	0.07

FID titration binding constants	
Sequence	K_a (M^{-1})
5′-AAAAA	1.94×10^7
5′-GAAAA	nd[b]

[a]W = A or T; S = G or C.
[b]Not determined, see text.

Table 2. Sequence selectivity of AcN-PyPyPy-γ-PyPyPy-Dp[a]

FID assay sequence analysis				
Sequence	1.5 μM **2**		2.0 μM **2**	
	Avg rank	Avg score	Avg rank	Avg score
5′-WWWWW	36	0.64	16	0.85
5′-SWWWW	78	0.46	63	0.63
5′-SSWWW	213	0.30	169	0.40
5′-SSSWW	390	0.13	372	0.22

FID titration binding constants	
Sequence	K_a (M^{-1})
5′-AAAAA	8.50×10^6
5′-GAAAA	8.23×10^6

[a]W = A or T; S = G or C.

minor groove (Fig. 4). The loss of contact to DNA results in an observable shortened minimum AT-rich binding site from 5 to 4 bp. FID titration experiments further illustrated the impact of binding by establishing associative binding constants (Table 2). Notably, both the 5- and 4-bp A/T sites have comparable affinities (8.50 and 8.23×10^6 M^{-1} respectively), but the affinities are lower than that observed for **1** (1.94×10^7 M^{-1}, see Table 1). Thus, the comparable affinities of **2** for the 5- and 4-bp A/T sites observed in the FID selectivity screen arises from a diminished affinity for 5-bp sites (relative to **1**) and not an enhancement of affinity of the 4-bp sites.

ImPyPy-γ-PyPyPy-Dp (3)

Sequence analysis of data generated by the FID assay for polyamide **3** indicated that the highest affinity site was the expected 5′-WGWWW sequence set (Table 3).[30,31] All other sequence sets examined were of modest to low affinity for this molecule. FID titration of **3** to the sequence 5′-TGTTA generated a binding constant (3.08×10^7 M^{-1}) consistent with that ascertained by footprinting experiments (7.6×10^7 M^{-1}).[30]

ImPyPy-γ-PyPyPy-β-Dp (4)[32]

Analysis of the sequence data provided by the FID assay of **4** identified two high-affinity sites: the expected sequence set 5′-WGWWW[30] and an unanticipated site 5′-WWGWW (Table 4A). All other sequence sets examined were of modest to low affinity including sequence sets differing at the second and fourth positions, which have been previously examined in quantitative footprinting studies.[30]

In investigating this unexpected sequence selectivity observed in the FID assay, we analyzed the data in alternative fashions. As part of this analysis, the position of the variable region within the DNA hairpin was also considered such that the two hairpins in Figure 2 are recognized to be unique rather than equivalent entities. Therefore, complementary sequences formerly considered redundant were considered unique. It should be noted that in prior validation studies with distamycin A and netropsin, no such positional difference was observed.[23,24] In the present case, we do see a minor orientational binding preference resulting from the unsymmetrical nature of the DNA hairpins and we will return to a discussion of this issue below. Utilizing this data analysis (Table 4B), it was confirmed that polyamide **4** does have affinity to 5′-WWCWW greater or equal to that of the expected high affinity sites 5′-WWWCW and 5′-WGWWW. Interestingly, the complementary site (5′-WWGWW) to the highest affinity sequence set displayed a more modest binding affinity.

While high-binding affinity to 5′-WGWWW and 5′-WWWCW was expected, the identification of 5′-WWCWW that is of equal or greater affinity was unanticipated. Footprinting studies have investigated the sequence specificity of **4** and the independent selectivity of the γ-turn and the β/Dp tail.[31,33] NMR studies have probed in detail the binding interactions of this hairpin polyamide to DNA.[25] Yet the combined information in these detailed studies failed to predict the highest binding affinity site for this molecule in a 5-bp context (5′-WWCWW). This was only realized through the ability of the FID assay to sample the full panel of possible sequences.

The comprehensive data set provided by the FID assay also allowed formulation of a binding model that accounts for the observed selectivity. In this model, Dervan's pairing rules[4–6] are maintained due to the strong hydrogen bond interaction between the *N*-methylimidazole and the exocyclic amine of the guanosine. In the expected high affinity binding to the sequence 5′-WGWWW, the γ-turn of the polyamide binds to a single degenerate A/T bp, and the charged tail likewise binds to a single degenerate A/T bp. Sequence sets examined with G/C bps at the end positions show a greatly reduced binding affinity demonstrating the preference for both the γ-turn and the β/Dp to degenerate A/T bps.

Table 3. Sequence selectivity of ImPyPy-γ-PyPyPy-Dp[a]

FID assay sequence analysis				
Sequence	1.5 μM **3**		2.0 μM **3**	
	Avg rank	Avg score	Avg rank	Avg score
5′-WGWWW	54	0.43	60	0.47
5′-WWWWW	122	0.32	124	0.32
5′-WCWWW	164	0.20	126	0.25
5′-WGWCW	241	0.08	191	0.13

FID titration binding constants	
Sequence	K_a (M^{-1})
5′-TGTTA[b]	3.08×10^7

[a]W = A or T.
[b]Lit. $K = 7.6\times10^7$ M^{-1}, footprinting.[30]

Table 4. Sequence selectivity of ImPyPy-γ-PyPyPy-β-Dp[a]

A. Analysis utilizing complementary sequences				
Sequence	1.5 μM **4**		2.0 μM **4**	
	Avg rank	Avg score	Avg rank	Avg score
5′-WGWWW	55	0.47	54	0.47
5′-WWGWW	82	0.44	36	0.55
5′-WCWWW	97	0.32	105	0.28
5′-WWWWW	131	0.27	111	0.27

B. Secondary analysis				
Sequence	1.5 μM **4**		2.0 μM **4**	
	Avg rank	Avg score	Avg rank	Avg score
5′-WWCWW	55	0.54	20	0.67
5′-WWWCW	59	0.50	51	0.50
5′-WGWWW	55	0.45	58	0.45
5′-WWGWW	125	0.28	64	0.35

[a]W = A or T.

The binding mode to the novel sequence set (5′-WWCWW) does not differ greatly from that of the expected high-affinity sequence set (Fig. 5). The key to its behavior rests with the realization that **4** (with the β/Dp tail) really requires 6 bp (not an expected 5) for full binding. Forced binding into a 5-bp context results in non-optimal binding contacts. Although the γ-turn and the β/Dp tail both have a sequence preference for binding degenerate A/T base pairs, the affinity of the tail for a 2 (not 1)-bp A/T site out ranks the affinity of the γ-turn for a single A/T bp. The consequence is that the while pairing rules are still operating, the A/T specificity of the γ-turn is sacrificed in deference for the tail binding to two A/T base pairs.

This model was confirmed by obtaining quantitative binding constants to selected sequences (Table 5) utilizing a DNA hairpin containing the 7-bp sequence used in prior footprinting studies of **4**.[30] The binding constant obtained for the full sequence (5′-TAACAAT) of 2.39×10^8 M^{-1} was comparable to the footprinting binding constant of 2.9×10^8 M^{-1} previously reported.[30] By systematically shortening the 7-bp binding site from both ends, our studies clearly establish that a 6-bp binding site is required for full binding affinity (Table 5, 1 and 2). Reduction to any 5-bp site results in a near 10-fold reduction in K_a (Table 2 vs 3 or 5). Furthermore, removal of an A/T bp from the turn (Table 5, 1 vs 4) is less detrimental than removal of the first of two A/T bp from the tail (Table 5, 1 vs 3). Finally, direct comparison of a member from each 5-bp sequence set identified by the FID assay reveals that the hairpin polyamide does exhibit higher affinity to 5′-AACAA than to 5′-TGTTA (Table 5, 10 vs 11).

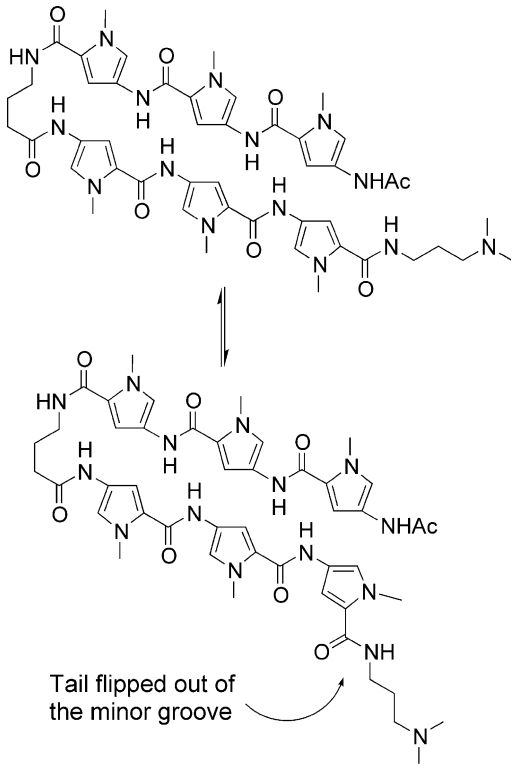

Figure 4. Proposed orientation of AcN-PyPyPy-γ-PyPyPy-Dp when binding to the minor groove of DNA. Due to destabilizing intramolecular interactions, the C-terminal pyrrole and charged tail may rotate out of the minor groove.

In conducting these studies, we also examined the polyamide binding orientation within the hairpin deoxyoligonucleotide. Although we observed very minor differences in binding affinity for hairpins containing the 7-bp variable region (Table 5, 1–5 vs 6–9) and observed a slightly greater difference with the 5-bp hairpin DNA (Table 5, 10 vs 12), it is important to note the magnitude of the orientation preference as suggested by FID assay data but quantitated by binding constants. Although the differences in rank order binding for complimentary sequence sets seem to differ significantly in the alternative data analysis (e.g., 5′-WWCWW and 5′-WWGWW differ by 50 in average rank for its constituent members), binding constants provided by FID titrations reveal that the actual difference in K_a is less than 2-fold for the complimentary sequences 5′-AACAA and 5′-TTGTT. Thus, these differences are minor and unlikely to alter global selectivity conclusions that can be drawn from a FID analysis. This is especially true of ensembles of sequences that are statistically represented on both the leading and complementary strand effectively canceling any such orientational effects. However, they are sufficient to distinguish subsets of otherwise identical sequences and quantitative comparisons are best made with identical placements within such hairpins. The basis of this orientation preference is not yet clear though it is possible that the hairpin DNA possesses a dipole due to the negative charge of the hairpin 5A loop. This in turn may bias polyamide binding such that the positively charged Dp tail resides closest to the 5A loop to complement that dipole.

Table 5. Binding constants for ImPyPy-γ-PyPyPy-β-Dp obtained by titration experiments

		Sequence	K_a (M^{-1})
5′-CG T A A C A X X C A A 3′-GC A T T G T X X G A A	1.	5′-TAACAAT[a]	2.39×10^8
	2.	5′-TAACAAC	1.73×10^8
	3.	5′-TAACACC	1.33×10^7
5′-CG X A A C A A T C A A 3′-GC X T T G T T A G A A	4.	5′-GAACAAT	3.31×10^7
	5.	5′-GAACAAC	2.84×10^7
5′-CG X X T G T T A C A A 3′-GC X X A C A A T G A A	6.	5′-ATTGTTA	9.87×10^7
	7.	5′-GTTGTTA	9.89×10^7
	8.	5′-GGTGTTA	2.57×10^7
	9.	5′-GTTGTTC	3.30×10^7
	10.	5′-AACAA	2.62×10^7
	11.	5′-TGTTA	1.69×10^7
	12.	5′-TTGTT	1.46×10^7

[a]Lit. $K=2.9\times10^8$ M^{-1}, footprinting.[15]

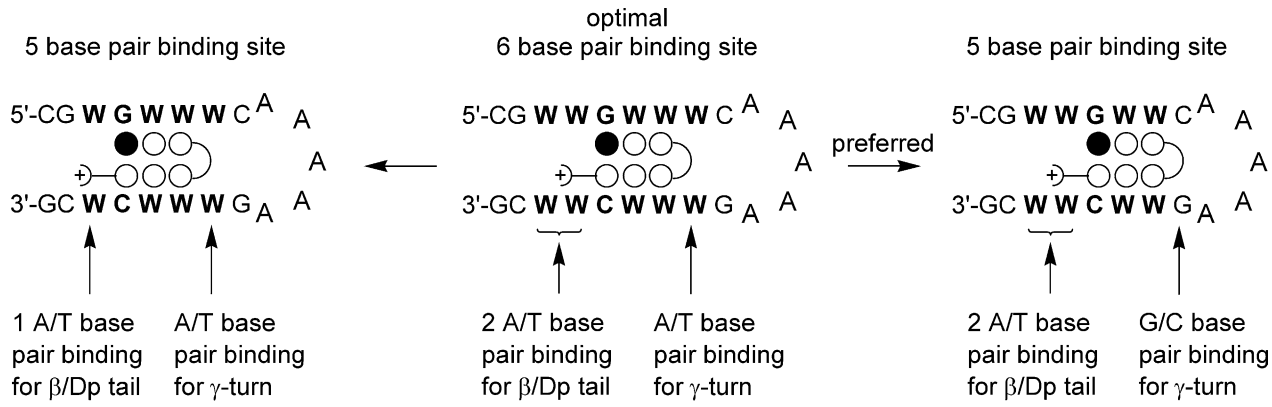

Figure 5. Binding modes for ImPyPy-γ-PyPyPy-β-Dp to the expected highest affinity 5-bp site 5′-WGWWW, the observed highest affinity 5-bp site 5′-WWGWW, and the proposed optimal 6-bp site 5′-WWGWWW. Filled circles represent imidazoles, and open circles, pyrroles.

Discussion

Relatively minor structural changes in hairpin polyamide design may lead to relatively large changes in DNA binding mode or sequence selectivity. While the most common techniques of analysis such as footprinting or NMR spectroscopy provide detailed information, the ability to detect global changes in sequence selectivity is relativity limited due to their inherent scope. In the current study, we have illustrated that the FID assay, with the capability to rapidly screen all possible DNA binding sites at high resolution, is in a unique position to comprehensively define the sequence selectivity.

The hairpin polyamides PyPyPy-γ-PyPyPy-Dp (**1**) and AcN-PyPyPy-γ-PyPyPy-Dp (**2**) have long been recognized to have high affinity to sequences comprised entirely of A/T base pairs. Only more recently has the effect of a N-terminal acetyl group on hairpin polyamides come to be appreciated.[25,26] NMR studies of such molecules bound to deoxyoligonucleotides have illustrated that an intramolecular steric clash with the *N*-acetyl group is responsible for loss of contacts between the small molecule tail and the DNA minor groove.[25] These results suggest reduced binding affinities and an abbreviated binding site for such *N*-acetylated hairpin polyamides. Demonstration of such, however, was beyond the scope of the studies. FID assay of **1** has shown that the minimal binding site for the non-acetylated polyamide **1** is a full 5-bp A/T site. In contrast, FID assay of **2** demonstrated that this *N*-acetylated hairpin polyamide binds with comparable affinity to 5- and abbreviated 4-bp A/T sites and that **2** exhibits a reduced affinity relative to **1**. Thus, while NMR spectroscopy studies have indicated the molecular interactions that may be responsible for altered *N*-acetylated hairpin polyamide binding, only upon full sequence screening is this translated into a defined sequence selectivity.

The hairpin polyamide ImPyPy-γ-PyPyPy-Dp (**3**), like **1**, displayed the most straightfoward correlation with the expected sequence specificity of the four molecules assayed. Selectivity was clearly displayed for the expected sequence set 5′-WGWWW. Previously determined preferences for the heteroaromatic pairings, for the Dp tail, and for the γ-hairpin turn were all observed to give a well defined 5-bp binding site.

For the hairpin polyamide ImPyPy-γ-PyPyPy-β-Dp (**4**), the ability of the assay to screen complete sequence space resulted in the discovery of an unexpected 5′-WWCWW sequence specificity. Although detailed studies have probed the ligand–DNA interactions of this molecule and its sequence selectivity has been established, its effective binding to such sequences had not been observed. Use of the FID assay to comprehensively screen DNA binding sites has provided a more complete picture of its DNA binding sequence selectivity. Notably, the quantity and quality of the data set generated by the FID assay also provided the basis for a binding model. This binding model established that the polyamide **4** really has an optimal binding site of 6 bp (not 5) of the form 5′-WWGWWW, and that the β/Dp tail combination requires two degenerate A/T base pairs. FID titration experiments confirmed the model by providing quantitative binding constants. While it was previously shown that the interactions of the alkyl tail with DNA are the dominant source of an orientational preference for these hairpins (5′-3′ *N* to *C* binding), we now show that the tail can also dominate the sequence specificity in a binding context that is less than its optimal 6-bp binding site.

Conclusions

The fluorescent intercalator displacement (FID) assay has been shown to be a reliable, technically non-demanding, high-throughput, high-resolution means of establishing the DNA binding affinity and sequence selectivity of small molecules. Herein, the assay has shown special utility in examining Py/Im polyamides with its quantitative characteristics sufficiently accurate to detect and measure subtle distinctions only previously recognized by detailed structural (NMR spectroscopy) studies. Additionally, a novel high affinity 5-bp binding site for ImPyPy-γ-PyPyPy-β-Dp was revealed along with the molecular basis for this specificity. More fundamentally, this illustrates that the comprehensive nature of the assay provides access to

information on small molecule DNA binders that is not readily accessible by other means. For small molecule programs where small changes in structure might affect global DNA binding selectivity, current techniques may not provide the full scope necessary to monitor the consequences of such structural alterations. The FID assay provides an alternative and complementary technique that can fully define the DNA binding selectivity of small molecule DNA binders.

Experimental

Synthesis of polyamides

PyPyPy-γ-PyPyPy-Dp (**1**), AcN-PyPyPy-γ-PyPyPy-Dp (**2**), and ImPyPy-γ-PyPyPy-Dp (**3**) were synthesized according to procedures previously developed.[34] ImPyPy-γ-PyPyPy-β-Dp (**4**) was provided by Chamberlin and Krutzik and prepared according to their solid-phase methodology.[32]

Fluorescent intercalator displacement assay

Concentrations of hairpin deoxyoligonucleotides were determined by UV at 90 °C to ensure accurate concentration determination with single-strand extinction coefficients. Each well of a TC-treated Costar black 96-well assay plate was loaded with Tris buffer containing ethidium bromide (70 μL of 8.57 μM EtBr solution 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 of 15 μM hairpin solution, 120 μM in bp, in H_2O). To this, compound stock solutions were added (20 μL of 5, 7.5, or 10 μM agent in H_2O). Final concentrations were 1.5 μM DNA hairpin, 6 μM EtBr, and 1.0, 1.5, or 2.0 μM agent. After incubation at 25 °C for 30 min, each well was read (average of six readings) on a Molecular Devices Spectra Max Gemini fluorescent plate reader (ex. 545 nm, em. 595 nm, cutoff 590 nm). Compound assessments were conducted in duplicate or triplicate with two control wells (no agent = 100% fluorescence, no DNA = 0% fluorescence). Fluorescence readings are reported as percent fluorescence decrease relative to the control wells.

FID data analysis

Sequences were sorted according to percent fluorescent decrease. Sequence sets were evaluated based on two criteria: (1) The average rank was calculated for the constituent members of a sequence set and used for evaluation (ranking 1–512). (2) All sequences were assigned a score reflective of its percent fluorescent decrease relative to the percent fluorescent decrease of the best sequence. Thus, if a sequence had a %F of 55% and the best sequence had a %F of 40%, then its score would be (100−55)/(100−40) = 0.75 and the best sequence would carry a score of 1. Thus, the calculated score is a better representation of the actual binding profile than the rank position. The average score was then calculated for all the members of the sequence set and used for evaluation.

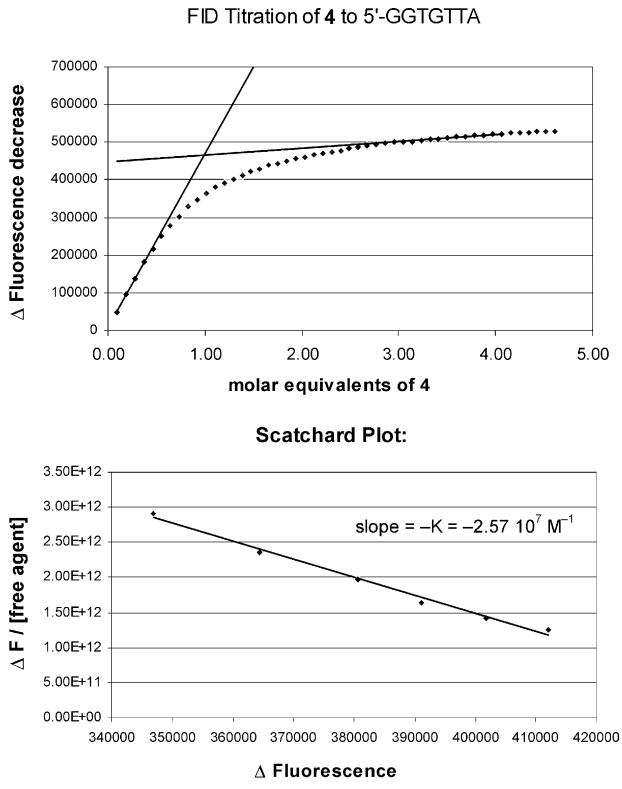

Figure 6. (a) Titration of ImPyPy-γ-PyPyPy-β-Dp versus the hairpin containing 5′-GGTGTTA. Intersection of the pre- and post-saturation linear portions of the curve indicates the stoichiometry of binding; (b) Scatchard analysis of the titration curve provides an associative binding constant (*K*).

Titrations utilizing fluorescent intercalator displacement

A 3-mL quartz cuvette was loaded with Tris buffer (2.5 mL, 0.1 M NaCl, 0.1 M Tris, pH 8.0) and ethidium bromide (6 μM final concentration) The fluorescence was measured on a JY Horiba Spex FluoroMax-3 spectrofluorometer and normalized to 0% fluorescence. The hairpin deoxyoligonucleotide of interest was added (1 μM hairpin final concentration), and the resulting fluorescence was normalized to 100%. Titrations were conducted by adding aliquots of compound (3 μL, 0.05 mM in DMSO) and measuring the resultant fluorescence decrease after a 5-min equilibration time (Fig. 6). Additions were continued until the system reached saturation and the fluorescence remained constant with subsequent compound additions.

Determination of binding constants[23]

The ΔF was plotted versus the molar equivalents of compound and the ΔF_{sat} was determined mathematically by simultaneously solving the 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 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 compound versus DNA, ΔF_x = change in fluorescence, and ΔF_{sat} = change in fluorescence at the point

where DNA is saturated with ligand.

$$(\Delta F_x/\Delta F_{sat})(1/X) = \text{Fraction of DNA} - \text{Agent Complex} \quad (1)$$

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

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

Supplementary Material

Full experimental details of the synthesis of PyPyPy-γ-PyPyPy-Dp (**1**), AcN-PyPyPy-γ-PyPyPy-Dp (**2**) and ImPyPy-γ-PyPyPy-Dp (**3**) are available upon request from the authors or can be found in the online version of the paper.

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References and Notes

1. Dervan, P. B. *Bioorg. Med. Chem.* **2001**, *9*, 2215.
2. Neidle, S. *Nat. Prod. Rep.* **2001**, *18*, 291.
3. Bailly, C.; Chaires, J. B. *Bioconjugate Chem.* **1998**, *9*, 513.
4. Pelton, J. G.; Wemmer, D. E. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 5723.
5. Geierstanger, B. H.; Mrksich, M.; Dervan, P. B.; Wemmer, D. E. *Science* **1994**, *266*, 646.
6. Mrksich, M.; Dervan, P. B. *J. Am. Chem. Soc.* **1995**, *117*, 3325.
7. Gottesfeld, J. M.; Neely, L.; Trauger, J. W.; Baird, E. E.; Dervan, P. B. *Nature* **1997**, *387*, 202.
8. Dickinson, L. A.; Gulizia, R. J.; Trauger, J. W.; Baird, E. E.; Mosier, D. E.; Gottesfeld, J. M.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 12890.
9. Boger, D. L.; Schmitt, H. W.; Fink, B. E.; Hedrick, M. P. *J. Org. Chem.* **2001**, *66*, 6654.
10. Boger, D. L.; Fink, B. E.; Hedrick, M. P. *J. Am. Chem. Soc.* **2000**, *122*, 6382.
11. Boger, D. L.; Dechantsreiter, M. A.; Ishii, T.; Fink, B. E.; Hedrick, M. P. *Bioorg. Med. Chem.* **2000**, *8*, 2049.
12. Woods, C. R.; Ishii, T.; Boger, D. L. *J. Am. Chem. Soc.* **2002**, *124*, 10676.
13. Woods, C. R.; Ishii, T.; Wu, B.; Bair, K. W.; Boger, D. L. *J. Am. Chem. Soc.* **2002**, *124*, 2148.
14. Woods, C. R.; Faucher, N.; Eschgfaller, B.; Bair, K. W.; Boger, D. L. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2647.
15. Fox, K. R. *Drug–DNA Interactions Protocols; Methods in Molecular Biology*; Humana: Totowa, NJ, USA, 1997; Vol. 90.
16. Galas, D. J.; Schmitz, A. *Nucleic Acids Res.* **1978**, *5*, 3157.
17. Royer-Pokora, B.; Gordon, L. K.; Haseltine, W. A. *Nucleic Acids Res.* **1981**, *9*, 4595.
18. Van Dyke, M. W.; Hertzberg, R. P.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 5470.
19. Tullius, T. D.; Dombroski, B. A.; Churchill, M. E.; Kam, L. *Methods Enzymol.* **1987**, *155*, 537.
20. Kuwabara, M. D.; Sigman, D. S. *Biochemistry* **1987**, *26*, 7234.
21. Taylor, J. S.; Schultz, P. G.; Dervan, P. B. *Tetrahedron* **1984**, *40*, 457.
22. Hardenbol, P.; Wang, J. C.; Van Dyke, M. W. *Bioconjugate Chem.* **1997**, *8*, 617.
23. Boger, D. L.; Fink, B. E.; Brunette, S. R.; Tse, W. C.; Hedrick, M. P. *J. Am. Chem. Soc.* **2001**, *123*, 5878.
24. Boger, D. L.; Tse, W. C. *Bioorg. Med. Chem.* **2001**, *9*, 2511.
25. Hawkins, C. A.; Pelaez de Clairac, R.; Dominey, R. N.; Baird, E. E.; White, S.; Dervan, P. B.; Wemmer, D. E. *J. Am. Chem. Soc.* **2000**, *122*, 5235.
26. Lacy, E. R.; Le, N. M.; Price, C. A.; Lee, M.; Wilson, W. D. *J. Am. Chem. Soc.* **2002**, *124*, 2153.
27. Scatchard, G. *Ann. N. Y. Acad. Sci.* **1949**, *51*, 660.
28. Browne, K. A.; He, G. X.; Bruice, T. C. *J. Am. Chem. Soc.* **1993**, *115*, 7072.
29. Satz, A. L.; Bruice, T. C. *Bioorg. Med. Chem.* **2000**, *8*, 1871.
30. Parks, M. E.; Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1996**, *118*, 6147.
31. deClairac, R. P. L.; Geierstanger, B. H.; Mrksich, M.; Dervan, P. B.; Wemmer, D. E. *J. Am. Chem. Soc.* **1997**, *119*, 7909.
32. Krutzik, P. O.; Chamberlin, A. R. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 2129.
33. Swalley, S. E.; Baird, E. E.; Dervan, P. B. *J. Am. Chem. Soc.* **1999**, *121*, 1113.
34. Full experimental details are provided in supplementary material that can be requested from the authors.