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J. Am. Chem. Soc., **2005**, 127 (2), 742-750• DOI: 10.1021/ja044359p • Publication Date (Web): 18 December 2004

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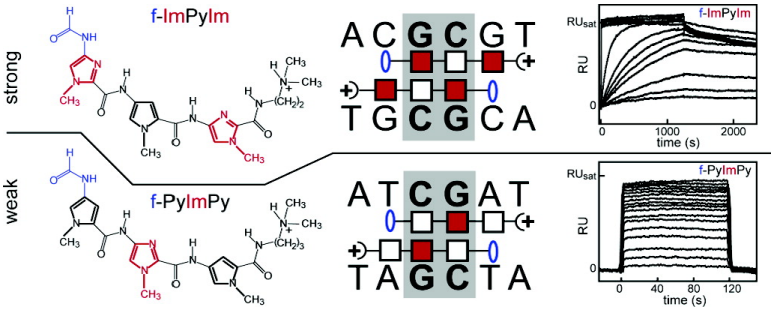

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Extending the Language of DNA Molecular Recognition by Polyamides: Unexpected Influence of Imidazole and Pyrrole Arrangement on Binding Affinity and Specificity

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Abstract: Pyrrole (Py) and imidazole (Im) polyamides can be designed to target specific DNA sequences. The effect that the pyrrole and imidazole arrangement, plus DNA sequence, have on sequence specificity and binding affinity has been investigated using DNA melting (ΔT_M), circular dichroism (CD), and surface plasmon resonance (SPR) studies. SPR results obtained from a complete set of triheterocyclic polyamides show a dramatic difference in the affinity of f-ImPyIm for its cognate DNA ($K_{eq} = 1.9 \times 10^8\ M^{-1}$) and f-PyPyIm for its cognate DNA ($K_{eq} = 5.9 \times 10^5\ M^{-1}$), which could not have been anticipated prior to characterization of these compounds. Moreover, f-ImPyIm has a 10-fold greater affinity for CGCG than distamycin A has for its cognate, AATT. To understand this difference, the triamide dimers are divided into two structural groupings: central and terminal pairings. The four possible central pairings show decreasing selectivity and affinity for their respective cognate sequences: −ImPy > −PyPy− ≫ −PyIm− ≈ −ImIm−. These results extend the language of current design motifs for polyamide sequence recognition to include the use of "words" for recognizing two adjacent base pairs, rather than "letters" for binding to single base pairs. Thus, polyamides designed to target Watson−Crick base pairs should utilize the strength of −ImPy− and −PyPy− central pairings. The f/Im and f/Py terminal groups yielded no advantage for their respective C/G or T/A base pairs. The exception is with the −ImPy− central pairing, for which f/Im has a 10-fold greater affinity for C/G than f/Py has for T/A.

Introduction

The diverse group of DNA minor groove binding imidazole and pyrrole-containing polyamides have shown considerable promise in targeting specific DNA sequences.[1−3] These molecules have medicinal potential,[4,5] in addition to providing substantial information on DNA structure and function.[6,7] Therefore, a thorough understanding of the interactions and dynamics between polyamides and DNA can have direct effects on therapeutic design and DNA molecular recognition.

Polyamides are highly versatile molecules, which can be modified to recognize specific DNA target sequences. Distamycin A (Figure 1A), a naturally occurring polyamide, with three pyrrole moieties per molecule, binds to A−T rich DNA sequences as antiparallel dimers in the minor groove.[8−10] The introduction of a single imidazole (Im) moiety in place of a pyrrole (Py) changes the sequence recognition to include a G/C base pair.[11−13] Further changes to the heterocyclic rings have been explored, such as 3-hydroxy-1*H*-pyrrole, hydroxybenzimidazole, and imidazopyridine;[14−16] however, increased specificity among the base pairs comes at the price of binding affinity.[14−17] The observations that stacked Py/Py rings (one

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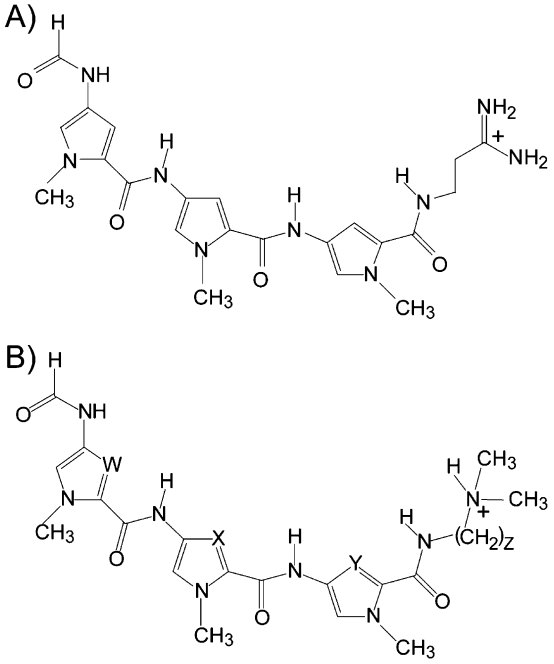

C)

GAA**CGCG**TC / CTT**GCGC**AG	**CGCG**	GGA**CTAG**TC / CCT**GATC**AG	**CTAG**
GAA**TGCA**TT / CTT**ACGT**AA	**TGCA**	GAA**TCGA**TT / CTT**AGCT**AA	**TCGA**
GAA**CCGG**TC / CTT**GGCC**AG	**CCGG**	GGA**CACG**TC / CCT**GTGC**AG	**CACG**
CG**AAATTT**C / GC**TTTAAA**G	**AATT**		

Figure 1. Polyamide and DNA sequences. (a) Distamycin A. (b) Formamido triheterocyclic polyamide (triamide) template molecule with all eight possible iterations of pyrroles and imidazoles denoted. New triamides are shown in the left column, and previously studied triamides, in the right column.[20,21] (c) Ten base pair DNA hairpin molecules, with names denoted to the right of each sequence. These DNA molecules were 5′-biotinylated for immobilization in SPR experiments.

from each molecule in the dimer) recognize A/T or T/A base pairs, stacked Im/Py rings target G/C base pairs, and stacked Py/Im rings target C/G base pairs[18−20] significantly improved the ability to design polyamides that target specific DNA sequences. In addition, Im/Im pairs bind with exceptional preference for mismatched G/T base pairs over the Watson−Crick base pairs.[7,21] Previous work has shown that the N-terminal formamido functionality imparts a 10- to 1000-fold increase in binding affinity over otherwise identical polyamides.[22] This group also promotes polyamides to preferentially

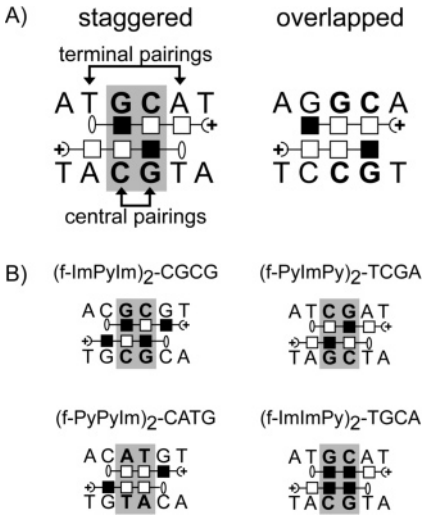

Figure 2. Staggered binding modes. (a) Staggered f-ImPyPy compared to the overlapped binding modes of nonformylated ImPyPy. The central pairings of staggered dimers (highlighted by gray boxes) and terminal pairings (not highlighted). (b) Polyamide$_2$−DNA complexes formed for the four new triamide molecules. The polyamides bind as antiparallel, staggered dimers to their respective cognate sequences. White squares, black squares, and white ovals indicate pyrrole, imidazole, and formamido groups, respectively. Boxed gray regions indicate central pairing elements.

stack in a staggered manner, which expands the sequence recognition length to six base pairs for triheterocyclic polyamides (Figure 2A).

These rules for DNA recognition by polyamides are vital in the design of novel compounds to target specific, or cognate, sequences. However, much remains unknown about how simple changes in polyamide content, stacking geometries, and arrangement of imidazole and pyrrole units alter binding affinity for DNA. For example, the binding affinities for f-ImPyPy and f-PyImIm were measured by biosensor-surface plasmon resonance (SPR) methods with their respective cognate DNA sequences, TGCA and CCGG. The affinities were determined to be 1.2×10^7 and 8.5×10^5 M^{-1}, respectively, which represented a 14-fold difference.[22] Although the rules that define the cognate sequence generally work well,[18−22] there was no way to predict that f-PyImIm would bind its cognate sequence, CCGG, weakly. Polyamides that bind DNA poorly, such as f-PyImIm, would have difficulty reaching the concentrations in the nucleus that would be required to inhibit DNA binding proteins. Therefore, polyamide design needs to address not only the synthesis of sequence specific compounds (*specificity*) but also the efficacy with which these novel polyamides will target their DNA sequences (*affinity*). Understanding the interplay between polyamide motifs and their cognate DNA sequences can only improve our ability to design compounds that bind their target DNA sequences with robust binding affinity and sequence specificity. Herein, we utilize all possible, triheterocyclic polyamides (triamides), containing only pyrroles and imidazoles, to understand binding specificity and affinity (Figure 1B). We have tested how the arrangements of pyrroles and imidazoles within the polyamide are crucial for binding affinity.

Results

Polyamide and DNA Sequence Design. The DNA binding properties of eight molecules from a subclass of synthetic pyrrole and imidazole-containing triamides and their parent amidine

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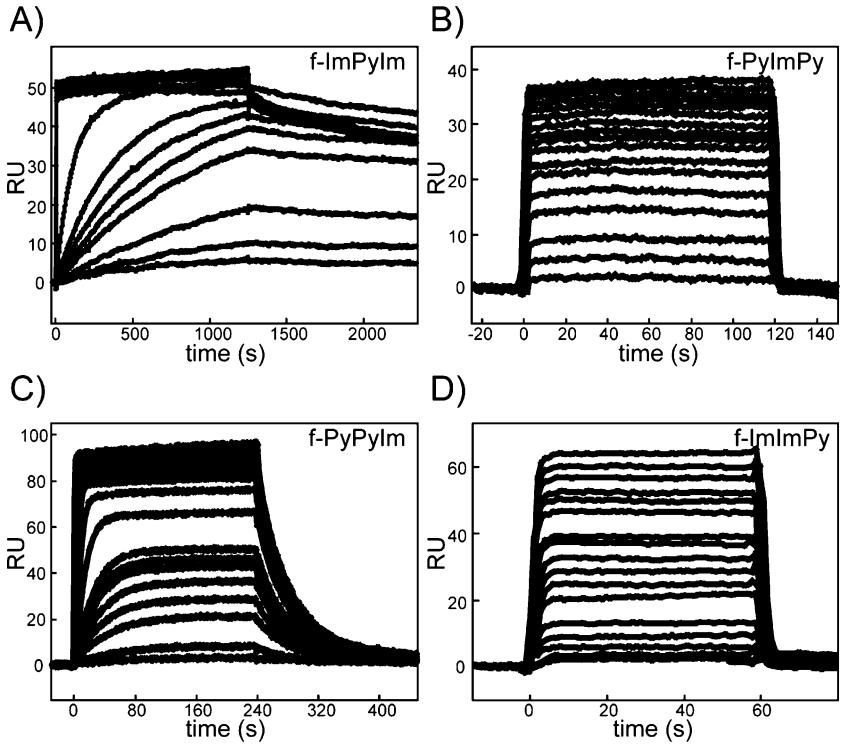

Figure 3. SPR sensorgrams of triamides with their cognate DNA. (a) f-ImPyIm with CGCG at 0.01, 0.04, 0.08, 0.14, 0.16, 0.2, 0.25, 0.5, 6, 10, 20, 30, and 40 μM triamide. (b) f-PyImPy with TCGA at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 20, 25, 30, and 40 μM triamide. (c) f-PyPyIm with CTAG at 0.1, 0.2, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.5, 2, 3, 4, 6, 8, 10, 15, 20, 30, and 40 μM triamide. (d) f-ImImPy with TGCA at 0.1, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 20, 25, and 30 μM triamide.

analogue, distamycin A, were investigated. An initial study of the DNA binding properties of five triamides (distamycin A, f-PyPyPy, f-ImPyPy, f-PyImIm, and f-ImImIm) with their cognate DNA sequences have been previously characterized (Figure 1B), and the variation in affinities indicated that the current expanded study was necessary.[21,22] All triamide molecules have been studied in detail with their respective cognate DNA sequences (Figure 1C), and the results are reported herein. These molecules were designed with an N-terminus formamido group (f), resulting in polyamides that preferentially bind in a staggered motif and exhibit increased binding affinity and binding site size over, otherwise identical, nonformylated polyamides (Figure 2A).[22] Cognate sequences of DNA hairpin molecules were designed according to known recognition elements,[18−20] and the proposed binding diagrams are shown in Figure 2. For example, f-ImPyIm should recognize the sequence 5′-(A|T)-C−G-C−G-(A|T) (Figure 2B).[1,22−24] For the remainder of this article, the 5′- and 3′-flanking A/T or T/A base pairs (written as A|T), which are the preferred base pairs for the cationic tails,[23,24] are omitted when referring to specific DNA sequences because all triamides studied contain C-terminus cationic moieties. Therefore, the DNA recognized by f-ImPyIm is termed CGCG (Figure 1C).

Triamides: f-ImImPy, f-PyImPy, and f-PyPyIm. The triamides, f-ImImPy, f-PyImPy, and f-PyPyIm, were studied by SPR with their respective cognate DNA sequences and AATT (previously referred to as A_3T_3 in refs 21 and 22), a noncognate DNA for these triamides. Example SPR sensorgrams are shown in Figure 3. The data for f-ImImPy, f-PyImPy, and f-PyPyIm binding their respective cognate DNA hairpins are best fit using a steady-state binding model that accounts for two molecules of polyamide bound to a single DNA hairpin (Figure 4). Two equilibrium constants (K_1 and K_2) were determined for each polyamide−cognate DNA complex,[22] and to compare monomer and dimer complex formation the macroscopic binding constants (K_{eq}) are reported as $(K_1K_2)^{1/2}$. The 2:1 complex (triamide/cognate DNA) is substantiated by the observation that experimental RU values, at polyamide concentrations approaching saturation binding (RU_{sat}), were approximately twice the calculated RU_{max}. f-ImImPy, f-PyImPy, and f-PyPyIm were additionally tested for their binding to the noncognate AATT

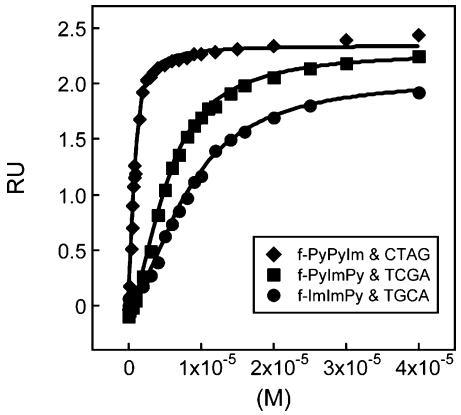

Figure 4. Steady-state analysis of $(f\text{-}PyPyIm)_2$·CTAG (diamonds), $(f\text{-}PyImPy)_2$·TCGA (squares), and $(f\text{-}ImImPy)_2$·TGCA (circles) from SPR experiments. Data were fit by $((K_1[\text{triamide}]) + (2K_1K_2[\text{triamide}]^2))/(1 + (K_1[\text{triamide}]) + (K_1K_2[\text{triamide}]^2))$, where triamide concentrations are reported in molarity and represent the free (unbound) concentration.

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Table 1. Binding Constants, K_{eq} (M^{-1}), for All Eight Triamide Molecules and Distamycin A

		CGCG	TGCA	CCGG	AATT	CTAG	TCGA	CACG
f-lmPylm[a]	M^{-1}[b]	**1.9×10^8**[d]		2.2×10^5	5.3×10^4[e]			5.4×10^5
	°C	**7.8**		1.1	0.9			−0.5
f-lmPyPy	M^{-1}	8.8×10^4	**1.2×10^7**[f]				9.4×10^4	
	°C	2.0	**11.0**		3.5		2.5	
distamycin	M^{-1}				**1.7×10^7**[g]			
	°C	0.0		2.0	**13.8**			−1.0
f-PyPyPy	M^{-1}				**3.2×10^6**[f]			
	°C	0.9		1.6	**9.3**			−1.0
f-PyPylm	M^{-1}				4.2×10^5	**1.1×10^6**		
	°C				5.8	1.0		
f-Pylmlm	M^{-1}	3.2×10^3		**8.5×10^5**[g]	2.5×10^4[g]			
	°C	0[g]		**3.4**[g]				
f-PylmPy	M^{-1}				1.6×10^5[e]		**1.8×10^5**	
	°C				2.5		**0.0**	
f-lmlmlm	M^{-1}	**8.4×10^4**[f]		**2.1×10^5**[g]	5.9×10^3[g]			
	°C			1[h]	0.1			
f-lmlmPy	M^{-1}		**1.1×10^5**		5.8×10^4[e]			
	°C		**0.5**		−0.5			

[a] Experiments performed at 25 °C in MES20 at pH 6.2, and unless otherwise noted all data exhibited 2:1 triamide/DNA complex formation. [b] Data in rows designated M^{-1} were generated from SPR experiments; DNA sequences are those shown in Figure 1. [c] Data in rows designated °C were from ΔT_M experiments; DNA sequences are shown in Materials and Methods. [d] Fit by kinetic isotherms. [e] 1:1 binding. [f] Previously reported in ref 21. [g] Previously reported in ref 20. [h] Reported in ref 40.

DNA hairpin, which is the recognition sequence for distamycin A. Binding of f-ImImPy and f-PyImPy to AATT were each fit best by steady-state isotherms using a 1:1 binding model (K_{eq}), and the calculated RU_{max} values were similar to the RU_{sat}, indicating that f-ImImPy and f-PyImPy bind AATT as a monomer (data not shown). For the f-PyPyIm·AATT complex, the RU_{max} value was approximately one-half the RU_{sat}, and the data were best fit using the 2:1 steady-state model, indicating that f-PyPyIm binds AATT as a dimer.

f-ImImPy binds to TGCA (cognate DNA) and AATT (a noncognate DNA) with affinities of $K_{eq} = 1.1 \times 10^5\ M^{-1}$ and $K_{eq} = 5.8 \times 10^4\ M^{-1}$, respectively (Table 1). Interestingly, binding of the first molecule of f-ImImPy to TGCA is weaker ($K_1 = 2 \times 10^4\ M^{-1}$) than binding by the second molecule ($K_2 = 6 \times 10^5\ M^{-1}$), thus this triamide exhibits positive cooperativity with a factor (K_2/K_1) of 30. Thermal melting experiments show only a 0.5 °C increase in DNA denaturation midpoint upon binding by f-ImImPy to TGCA (Table 1). This value is significantly lower than the 14.7 °C measured for the strong binder distamycin A with its cognate DNA, AATT. Even though f-ImImPy binds to its cognate DNA as a dimer and AATT as a monomer, the K_{eq} values show that f-ImImPy has less than 2-fold specificity for TGCA over AATT. In summary, the SPR and ΔT_M data show that f-ImImPy exhibits relatively weak association to its cognate DNA (Figures 3D and 4).

Binding affinities of f-PyImPy to its cognate, TCGA, and noncognate, AATT, DNA hairpins were determined to be 1.8×10^5 and $1.6 \times 10^5\ M^{-1}$, respectively (Table 1). Binding of the second molecule of f-PyImPy to TCGA was 20-fold stronger than binding by the first, indicating a positively cooperative association of the polyamide dimer to the DNA. In addition, the ΔT_M between the bound $(f\text{-PyImPy})_2$·TGCA complex and free TGCA was only 0.5 °C. These results show that f-PyImPy is not a strong binder to its cognate DNA, TGCA (Figures 3B and 4). Ultimately, f-PyImPy exhibits surprisingly low affinity for TGCA and binds AATT with essentially identical affinity and, thus, has no specificity for its cognate DNA, TGCA.

f-PyPyIm binds to CTAG and AATT with $K_{eq} = 1.1 \times 10^6$ and $4.2 \times 10^5\ M^{-1}$, respectively (Table 1). The first molecule of f-PyPyIm binds CTAG more weakly than does the second molecule ($K_2/K_1 = 3$), indicating slight positive cooperativity in forming the complex. In contrast, binding of the first molecule of f-PyPyIm to AATT is stronger than binding of the second molecule ($K_2/K_1 = 0.04$) and, therefore, binding is negatively cooperative. These findings are consistent with our previous studies, where binding of imidazole-containing polyamides to GC rich sequences have different characteristics than those of polyamides binding within the narrow minor groove of A_nT_n rich sequences.[21−26]

The association and dissociation rates for the $(f\text{-PyPyIm})_2$·CTAG complex were sufficiently slow for quantification of kinetic constants by SPR (see Table 2). The combination of slow association rates (k_a) and even slower dissociation rates (k_d) correlate with good DNA binding,[21,22] as anticipated by the relationship $K_{eq} = (k_{a1}/k_{d1} \times k_{a2}/k_{d2})^{1/2}$ for the 2:1 triamide-to-DNA complex, where K_{eq} increases as k_{d1} and k_{d2} decrease. The equilibrium constant, $5.9 \times 10^5\ M^{-1}$, as determined from the rate constants, is marginally lower than that determined by steady-state analysis ($1.1 \times 10^6\ M^{-1}$) but is macroscopically equivalent. f-PyPyIm is a more promising compound than f-ImImPy or f-PyImPy, because it has a strong binding affinity for its cognate DNA sequence, CTAG (Figures 3C and 4). The degeneracy of Py/Py and Im/Im pairings results in multiple cognate recognition sites for each of these triamides. For example, f-PyPyIm recognizes its three cognate sequences, CTAG, CATG, and CAAG, within 5-fold affinity of one another (SPR data not shown). In addition, all three of these polyamides exhibit low binding specificity between their respective cognate sequences and their noncognate AATT DNA, which is due to relatively weak binding to their respective cognate DNA sequences and not an increase in binding to AATT. Therefore,

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Table 2. Kinetic Rate Constants Derived Directly from SPR

compound	DNA sequence	k_{a1}[a] (M^{-1} s^{-1})	k_{d1} (s^{-1})	k_{a2} (M^{-1} s^{-1})	k_{d2} (s^{-1})	K_{eq}[b] (M^{-1})	K_{eq}[c] (M^{-1})
f-ImPyIm	CGCG	5.9×10^4	0.017	1.1×10^6	1.1×10^{-4}	1.9×10^8	ND[g]
f-ImPyPy[d]	TGCA	8.2×10^4	0.54	7.7×10^5	1.4×10^{-3}	9.1×10^6	1.2×10^7
f-PyPyIm	CTAG	6.6×10^4	0.41	4.6×10^6	0.022	5.9×10^5	1.2×10^6
f-PyImIm[e]	CCGG	7.0×10^4	0.58	2.3×10^5	0.030	9.9×10^5	8.3×10^5
f-PyImPy[f]	TCGA	$>10^6$	>1	$>10^6$	>1	ND	1.8×10^5
f-ImImPy[f]	TGCA	$>10^6$	>1	$>10^6$	>1	ND	1.1×10^5

[a] Data were fit as previously described in ref 21. [b] K_{eq} is calculated directly from the kinetic analysis. $K_{eq} = (K_1K_2)^{1/2} = [(k_{a1}/k_{d1})*(k_{a2}/k_{d2})]^{1/2}$. [c] K_{eq} is calculated from steady-state measurements; see Table 1. [d] Data previously reported in ref 21. [e] Data previously reported in ref 20. [f] Association and dissociation rates were too fast for the detection limits of BIACORE. [g] Not determined.

the existence of multiple recognition sites and low specificity for these sites over noncognate sequences limits the potential effectiveness of these compounds.

Triamide: f-ImPyIm. Binding affinity of f-ImPyIm to its cognate DNA hairpin, CGCG, was tested by SPR. Within reasonable experimental time, the steady state response could not be obtained at concentrations well below the saturation limit because the association rate was very slow (Figure 3A); therefore, the binding affinity of f-ImPyIm to CGCG could not be reliably calculated from the steady state. Data for f-ImPyIm binding to CGCG could be reproducibly fit by kinetic isotherms,[22] and the equilibrium constant (1.9×10^8 M^{-1}) was calculated from the association and dissociation rate constants. The data were best fit according to a 2:1 binding model, and all four kinetic rate constants are shown in Table 2. A K_2/K_1 of ~3000 shows that f-ImPyIm binds with strong positive cooperativity. In addition, the experimental RU_{sat} values were consistent with 2:1 binding. Circular dichroism studies show that f-ImPyIm binds as a dimer to CGCG and will be discussed in more detail below. Thermal melting studies confirmed that f-ImPyIm binds to CGCG with high affinity (ΔT_M of 7.3 °C). Thus, f-ImPyIm follows the polyamide recognition rules that have been previously stated[18−20,22] and clearly binds to its predicted cognate sequence with exceptional affinity for a nonhairpin polyamide. f-ImPyIm binds to CGCG ($K_{eq} = 1.9 \times 10^8$ M^{-1}) with greater affinity than binding of distamycin A, a natural product, binds its cognate recognition sequence, AATT (1.7×10^7 M^{-1}), as measured by SPR under the same buffer and temperature conditions.[22]

A gain in DNA affinity is often accompanied by a loss of specificity.[14−17] Thus, the binding affinities of f-ImPyIm for other DNA hairpins, CCGG, AATT, and CACG, were measured to determine whether the improved binding affinity for CGCG correlated with a loss of sequence specificity. The CCGG DNA hairpin contains the same nucleotide content and contains only a reversal of the central GC step to CG, resulting in a change in the context of two base pairs (Figure 2B). The AATT DNA hairpin is AT rich and includes CG base pairs only at the ends of the double stranded DNA. The CACG sequence has a single base pair change within the cognate sequence. The binding isotherms and the calculated RU_{max} values both showed that f-ImPyIm binds CCGG and CACG as a dimer and AATT as a monomer. The steady-state derived equilibrium binding constants of f-ImPyIm with CCGG and AATT were 4 orders of magnitude weaker than the kinetically derived binding constant of f-ImPyIm binding to CGCG. The change of a single base pair within the recognition sequences (C*G*CG to C*A*CG) lowers the binding affinity by at least 2 orders of magnitude. The specificity of f-ImPyIm for CGCG is confirmed by thermal melting studies. ΔT_M values of 0.9, −0.5, and 1.1 °C were observed for the noncognate DNAs, AATT, CACG, and CCGG, respectively, and 7.8 °C was observed for the cognate DNA, CGCG (Table 1). The SPR and ΔT_M data show that f-ImPyIm has impressive specificity for its cognate DNA, CGCG, compared to the similar DNA sequences, CCGG and CACG, and an AT rich DNA, AATT. Thus, the high binding affinity of f-ImPyIm for CGCG does not correlate with a loss in specificity. It is also important to note that a f-ImPyIm dimer, stacked in a staggered fashion, contains no degenerate recognition elements (i.e., Py/Py pairing), and therefore, there is only the single cognate sequence available to f-ImPyIm.

The good specificity of f-ImPyIm correlates with the strong binding affinity for its cognate sequence, rather than weak binding to both the cognate and noncognate sequences as is observed for the triamides with low specificity, f-ImImPy, f-PyImPy, and f-PyPyIm. It is important to note that noncognate sequences generally have low binding constants near 10^5 M^{-1} (Table 1), which is probably a general, nonspecific DNA binding value for triamides under these conditions.

Circular Dichroism Studies. Circular dichroism (CD) experiments were utilized to monitor the mode of binding and saturation limit for the four new triamide molecules. Molecules that bind in the minor groove of DNA typically exhibit the induction of a positive CD band.[21−23,27] f-ImPyIm, f-PyPyIm, f-PyImPy, and f-ImImPy each exhibit positive CD inductions at ~320 nm that indicate binding in the DNA minor groove of their respective cognate sequences, CGCG, CATG, TCGA, and TGCA (Figure 5A). At 3:1 ratios of triamide/DNA the induced signal for $(\text{f-ImPyIm})_2$·CGCG is considerably stronger than those for the other three triamides with their cognate sequences (Figure 5A). This is in good agreement with the observation that f-ImPyIm is a stronger cognate DNA binder than f-PyPyIm, f-PyImPy, and f-ImImPy. Visual perusal of the CD data allows for two important observations. First, the DNA bands remain essentially in the B-form upon addition of triamide, indicating that binding does not significantly alter the global DNA conformation. Second, the CD spectra show clearly defined isodichroic points indicating that these triamides bind via a single mechanism to the DNA minor groove (Figure 5B and C).

Stoichiometry of the polyamide/DNA complex can be directly determined for molecules that demonstrate strong binding. f-ImPyIm clearly shows 2:1 binding to its cognate, CGCG (Figure 5B and D), and its noncognate, CACG (data not shown). At similar mole ratios of ligand-to-DNA, f-ImPyIm shows a stronger DNA-induced ligand peak at ~325 nm when bound to CGCG rather than to AATT (Figure 5C and D), indicating that f-ImPyIm recognizes CGCG with much better affinity than

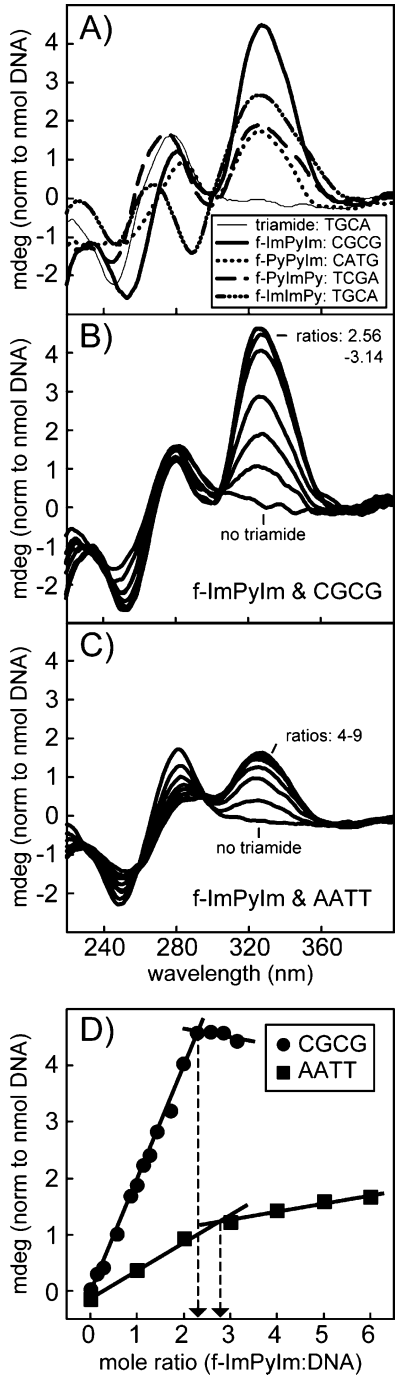

Figure 5. Circular dichroism studies of the four novel triamide molecules. (a) Typical B-form hairpin DNA (TGCA) and f-ImPyIm, f-PyPyIm, f-PyImPy, and f-ImImPy with their respective cognate DNA sequences at saturating polyamide concentrations (3:1, 4:1, 4:1, and 4:1 triamide-to-DNA, respectively). f-ImPyIm titrated to (b) its cognate DNA, CGCG (0, 0.57, 1.00, 1.42, 2.00, 2.56, 2.85, and 3.14 mole ratios), and (c) the noncognate DNA, AATT (0, 1, 2, 3, 4, 5, 7, and 9 mole ratios). (d) The induced peak maximums at 328 and 325 nm for f-ImPyIm binding to CGCG and AATT, respectively, are plotted against the mole ratio. The intersection of linear curve fits (solid lines) indicates the saturation limit of DNA binding sites. All data were normalized = mdeg response/nmol DNA.

AATT. Binding saturates at an ~3-fold excess of f-ImPyIm to its noncognate, CCGG (data not shown). This CD data are in good agreement with the SPR and ΔT_M that f-ImPyIm is specific for CGCG over AATT, CACG, and CCGG. f-PyPyIm, f-PyImPy, and f-ImImPy each required at least a 4-fold excess of triamide to their cognate DNA sequences and AATT to reach saturation (data not shown). Thus, weak binders require higher mole ratios (>3:1) of triamides than those expected by SPR to reach saturated spectral signals.[23]

Discussion

Previous limited studies suggest large variations in polyamide−DNA binding constants. To investigate this finding in detail, we have conducted systematic structural and thermodynamic investigations on the binding of imidazole and pyrrole-containing polyamides to their cognate sequences. Using a combination of SPR, thermal DNA melts (ΔT_M), and CD titration studies, the DNA binding results for eight polyamides and distamycin A with seven different oligonucleotide hairpins were examined. Our results provide the first systematic set of evidence that the arrangements of imidazole and pyrrole units within the polyamides, as well as the DNA recognition sequence, have significant and direct effects on binding affinity and specificity.

Cooperativity. We have observed that positively versus negatively cooperative binding is dependent on the DNA sequence, which is consistent with prior work.[21−25] All of the eight synthetic triamides, with the exception of f-PyPyPy, bind to their respective cognate sequences with positive cooperativity.[21,22] However, distamycin A and f-PyPyPy bind AATT with negative cooperativity.[21,22] The cognate sequences for all of the triamides except distamycin A and f-PyPyPy are mixed, GC rich sequences; whereas, AATT is an AT rich DNA. The minor groove is narrow, 0.3−0.4 nm, in some AT rich DNAs, such as AATT,[28] but mixed sequences have wider DNA minor grooves, 0.5−0.6 nm.[29] The thickness of one polyamide ring is about 0.34 nm;[28] therefore, a single distamycin A molecule can fit snugly and make favorable van der Waals contacts with the walls of the minor groove in AATT. Binding of the second molecule, however, requires the minor groove to significantly widen to accommodate the two stacked distamycin A molecules, resulting in a lower affinity for the second molecule.[29] The wider groove in GC rich DNA promotes positive cooperativity with optimum contacts to the groove walls, even at low ratios (less than 1:1) of compound-to-DNA. Thus, GC sequences can more readily accommodate stacked dimers than AT counterparts. In addition, some AT rich sequences, such as T_nA_n, have wider minor grooves and can bind stacked minor groove binding benzimidazole dication dimers with positive cooperativity.[26]

f-PyPyIm binds the noncognate DNA, AATT, with negative cooperativity ($K_2/K_1 = 0.04$), but it binds its cognate DNA, CATG, with slight positive cooperativity ($K_2/K_1 = 3$), further evidence that DNA sequence influences cooperativity. Interestingly, f-ImPyIm binds its cognate, CGCG, with a large positive cooperativity (K_2/K_1 ~3000). The results suggest that the f-ImPyIm dimer and CGCG must have complementary structural and conformational features that provide optimum interactions. These results support our previous findings that imidazole-containing polyamides, which bind to GC-sequences, normally exhibit positive cooperativity.[22]

Pyrrole and Imidazole Arrangement: Central Pairings. The over 300-fold difference in kinetic rate derived binding

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affinity, between f-ImPyIm with its cognate, CGCG ($K_{eq} = 1.9 \times 10^8$ M^{-1}), and f-PyPyIm with its cognate, CTAG ($K_{eq} = 5.9 \times 10^5$ M^{-1}), clearly indicates the need for the conventions of polyamide design to be expanded beyond those for base-pair definition. Several observations from this research provide important contextual information for understanding polyamide−DNA recognition. First, any triamide homodimer can be divided into two distinct categories: the central pairings (highlighted in gray in Figure 2) and the terminal pairings (remainder of triamide dimer not highlighted in Figure 2). These complexes involve symmetric homodimers, and each dimer contains identical f/Im or f/Py pairings but not both; therefore, the terminal pairings are treated as one group. The strength of binding affinity is dictated by the content of the central pairings:

$$\begin{matrix}\text{–ImPy–}\\ \text{–PyIm–}\end{matrix} > \begin{matrix}\text{–PyPy–}\\ \text{–PyPy–}\end{matrix} > \begin{matrix}\text{–PyIm–}\\ \text{–ImPy–}\end{matrix} \approx \begin{matrix}\text{–ImIm–}\\ \text{–ImIm–}\end{matrix}$$

For simplicity, the central pairings trend in binding affinity can be written as the top line (−ImPy− > −PyPy− ≫ −PyIm− ≈ −ImIm−), with the complementary portion implied. For the subclass of triamides described in this report, the trend in binding affinity is reflected in Table 1, where the triamide−cognate complexes are ranked from strongest to weakest (top to bottom). The binding affinities for three nonformylated tetraheterocyclic polyamides (tetramides) with identical Im/Py terminal pairings and different central pairings (underlined) have been studied previously by quantitative DNAse I footprinting.[30,31] Im*ImPy*Py, containing the central −ImPy− pairing, binds to its cognate, GGCC, as an overlapped dimer with a binding affinity of 2×10^7 M^{-1}.[30] However, Im*PyIm*Py binds its cognate sequence, GCGC, with 100-fold lower binding affinity than Im*ImPy*Py binds to GGCC.[30] In addition, Im*PyPy*Py binds GATC with an affinity of 9×10^6 M^{-1}.[31] Interestingly, these tetramides have decreasing binding affinities for their respective cognate sequences that follow the same central pairing trend observed for the triamides: −ImPy− > −PyPy− ≫ −PyIm−. The behavior of these tetramides, in conjunction with the triamides, provides conclusive evidence that the arrangement of heterocyclic moieties within a polyamide can have significant impact on the binding affinity for DNA. Therefore, the language of polyamide sequence recognition and binding affinity must be extended to include "words", such as −ImPy−, which recognize two base pairs, rather than the use of "letters" that recognize only a single base pair.

The weakness of −ImIm− for C/G and G/C Watson−Crick base pairs is consistent with the preference of Im/Im pairings for G/T or T/G mismatched base pairs and has been well documented by kinetic, thermodynamic, and structural means.[7,21,25] The Im/Im·G/T complex is stabilized by two hydrogen bonds; whereas, the Im/Im·GC complex is stabilized only by a single bifurcated hydrogen bond,[32] which results in more than 1 order of magnitude decrease in binding affinity.[21] The strength of −PyPy− for A/T rich DNA, with favorable van der Waals interactions and optimal steric orientation, has also been well documented, for naturally occurring distamycin A and many synthetic polyamides.[8−10]

The unexpected strength of −ImPy− was not previously recognized, and now the repertoire for constructing novel polyamide molecules has been extended. Depending on the terminal pairings, the affinity of −ImPy− for its cognate −GC− sequence is at least 10^7 M^{-1}. If the central pairings are switched to −PyIm−, the cognate sequence is changed to −CG−, and the binding affinity drops to 10^5 M^{-1} (Table 2). The content of the central pairing remains the same in both situations, the terminal pairings for both compounds are f/Im, and both triamides recognize pure GC cognate DNA sequences; however, there is a significant, intrinsic difference between −ImPy− and −PyIm−. This difference is probably due to a number of factors, which may include DNA structure, polyamide structure, DNA dynamics, and final complex conformation. Changes in base pair sequence can appreciably affect DNA structure; for example, 5′-CpG-3′ and 5′-GpC-3′ steps are structurally dissimilar[32,33] and their conformations are highly dependent on the neighboring base pairs in the DNA.[34] The unique structure of the 5′-CpG-3′ site has been identified as a prevalent protein binding site and a site of cytosine methylation and is easily mutated.[31,32] Interestingly, NMR and X-ray crystal studies conducted on CGCG and CCGG containing DNA, respectively, show that the conformations of both of these DNA sequences are essentially identical.[37,38] Thus, if DNA is part of the answer to the uniqueness of (f-ImPyIm)$_2$·CGCG, then differences must arise from subtle features, such as flexibility or hydration, between these DNA molecules that are not apparent in these structural studies.

Triamide binding affinity is independent of the type of neighboring terminal pairing of −ImIm−, −PyIm−, and −PyPy− central pairings. For example, f-PyPyPy and f-PyPyIm bind their cognate DNA sequences (AATT and CTAG, respectively) within 3-fold affinity of each other. f-PyImIm and f-PyImPy bind CCGG and TCGA within 5-fold affinity (Table 1). This terminal pairing is necessary as previously reported;[22] however, f/Im and f/Py have similar effects on binding affinity. The one exception in this subclass is with the central pairing, −ImPy−. f-ImPyIm binds to CGCG with approximately 1 order of magnitude higher affinity to CGCG than f-ImPyPy to TGCA; thereby, providing additional evidence for the unique behavior of f-ImPyIm and its cognate DNA, CGCG.

Systematic studies, such as the one described herein, are vital to the elucidation of contextual trends and, therefore, can result in the rational design of polyamides with strong DNA binding affinity and with high sequence discrimination, especially in the creation of novel gene control agents. For example, binding of a nuclear transcriptional factor NF-Y to the inverted CCAAT box-2 (or ICB2) sequence (binding site given in bold, 5′-CAAGCT*ACG***ATTGG**TT-3′) of the topoisomerase II α gene down regulates gene expression.[39] Molecular biology experiments from our laboratories have demonstrated that f-PyImPy,

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which contains a central −PyIm− pairing, binds to the underlined ACGA sequence and was capable of inhibiting NF-Y binding to ICB2 (data not shown). According to the newly discovered central pairing trend, it would be imperative to design agents that contain an −ImPy− core, such as f-ImPyPy, which should bind to the underlined AGCT sequence adjacent to the ICB2 site. f-ImPyPy binds 66 times more strongly than f-PyImPy to their respective cognate sequences (Table 1); therefore, f-ImPyPy is a logical choice for future NF-Y inhibition experiments. Even though the f-ImPyPy binding site does not overlap ICB2, precedence exists for significant protein binding inhibition by polyamides that bind adjacent to the protein binding site,[3] and therefore, f-ImPyPy is a good candidate for inhibiting the activity of NF-Y. Biological experiments designed to test this idea are underway in our laboratories. Thus, more investigations should be made toward other polyamide systems and, ultimately, to understand the fundamental structural and thermodynamic properties that result in these trends.

It is generally believed that short linear stacked polyamides do not bind to their cognate DNA sequences with the same affinity as their hairpin counterparts, which have consequently been widely investigated as potential gene control agents.[3] In hairpin polyamides, the heterocyclic units are linked together by a γ-aminobutyrate group and they fold into each other like a stacked dimer. However, recent data showed that hairpin compounds have difficulty in penetrating cellular and nuclear membranes, thereby compromising their potential biological applications.[3] This limitation has created a need for alternative mechanisms that will target polyamides-to-DNA sequences in cells. The results from this study provide a potential solution because linear polyamides are approximately half the molar mass of the corresponding hairpins, and they have been shown to readily enter cells.[41] These findings provide unequivocal evidence that appropriately designed stacked polyamides can achieve similar binding affinity and selectivity to DNA as their hairpin counterparts. For example, f-ImPyIm and f-ImPyPy have comparable binding affinities for their cognate sequences as the hairpin polyamide **JH-37**, which was recently developed in our laboratory.[42] We found from SPR studies that **JH-37** (Py-Im-Im-γ-Py-Py-Py-Dp; γ and Dp represent the linker and the C-terminus dimethylaminopropyl group, respectively) bound to its six-base pair cognate sequence 5′-ATTGGT-3′, found in the ICB2 site of the topoisomerase IIα promoter, with a binding constant of 2.8×10^7 M^{-1}.

Materials and Methods

General. A 10 mM MES (2-(*N*-morpholino)ethanesulfonic acid), pH 6.2, buffer with 200 mM NaCl, 1 mM EDTA (disodium ethylenediamine tetraacetic acid), and 0.001% P20 surfactant was utilized for the SPR experiments.[22]

DNAs. Three DNA hairpins, named CATG, TCGA, and CACG (Figure 1C), were chemically synthesized with a 5′-biotin group and AE-HPLC purified by Midland Certified Reagent Company, Inc. The other four DNA hairpins, CGCG, TGCA, CCGG, and AATT, denoted in Figure 1C were reported previously.[22] The DNAs were resuspended in MES20 (pH 6.2) and used without further purification.

Eleven base pair DNA hairpins were used in the ΔT_M and CD studies. These DNAs were chemically synthesized and desalted by Qiagen, Inc. The DNAs are as follows: *CGCG*, **CG**G AAC GCG TC CTCT GA CGC GTT C**CG**; *TGCA*, **CG**G AAT GCA TT CTCT AA TGC ATT C**CG**; *CCGG*, **CG**G AAC CGG TC CTCT GA CCG GTT C**CG**; *AATT*, **GG**C GAA ATT TC CTCT GA AAT TTC G**CC**; *CTAG*, **GC**G GAC TAG TC CTCT GA CTA GTC C**GC**; *TCGA*, **CG**G AAT CGA TT CTCT AA TCG ATT C**CG**; *CACG*, **CG**G GAC ACG TC CTCT GA CGT GTC C**CG**. The sequences were identical to those reported in Figure 1C, except that two base pairs were added to the open end (bolded letters shown above). Therefore, the sequences were named the same as those reported in Figure 1C. The general agreement between ΔT_M and SPR experiments (see Table 1) indicates that the lengthened DNAs used for ΔT_M and CD experiments did not affect the binding properties of the polyamides because the target sequences and immediately flanking base pairs were not altered.

Compounds. Except for f-PyPyIm, syntheses of all other triamides have been previously reported.[21−25] ***N*-[-2-(Dimethylamino)ethyl]-1-methyl-4-{1-methyl-4-[4-formamido-1-methyl-2-pyrrolecarboxamido]pyrrole-2-carboxamido}imidazole-2-carboxamide, f-PyPyIm.** *N*-[2-(Dimethylamino)ethyl]-1-methyl-4-{1-methyl-4-[4-nitro-1-methylpyrrole-2-carboxamido]pyrrole-2-carboxamido}imidazole-2-carboxamide (40.0 mg, 0.083 mmol) and 5%Pd/C (20 mg) were suspended in chilled MeOH (10 mL). The mixture was degassed and stirred under hydrogen at atmospheric pressure and room temperature. After TLC analysis showed that all the starting material had been reduced, the catalyst was removed by filtration. The filtrate was concentrated under reduced pressure. The amine was coevaporated with CH_2Cl_2 (4 mL, twice), then dissolved in dry CH_2Cl_2 (3 mL), and kept at 0 °C. A flask containing dry acetic acetic anhydride [prepared from 2.0 mL of acetic anhydride and 1.0 mL of formic acid at 55 °C 15 min, then kept at 0 °C until it was used] was slowly dripped into the amine solution. The solution was allowed to stir overnight under a nitrogen atmosphere and at room temperature. The reaction was quenched by addition of MeOH (20 mL), and the mixture was concentrated under reduced pressure. The residue was taken up in $CHCl_3$ (50 mL) and washed with a NaOH solution (20 mL) at pH 10. The aqueous layer was back extracted with $CHCl_3$ (50 mL) and ethyl acetate (50 mL). The organic layers were collected, dried with Na_2SO_4, and concentrated under reduced pressure. The residue was purified on a silica gel column using a $CHCl_3$/MeOH solvent system (from 0% MeOH to 30% MeOH, with 2.5% increments every 50 mL of solvent) to give the desired product as a white powder (23.0 mg, 0.047 mmol, 57%). Mp 138−146 °C. TLC (30% MeOH/$CHCl_3$) R_f 0.32. IR (Neat) 3290, 3070, 1652, 1538, 1470, 1404, 1249, 754. ^{1}H NMR (500 MHz, DMSO-d_6): 10.38 (s, 1H), 10.03 (s, 1H), 9.91 (s, 1H), 8.12 (s, 1H), 7.72 (t br, 1H), 7.50 (s, 1H), 7.31 (d, 1.2, 1H), 7.16 (d, 1.2, 1H), 7.03 (d, 1.2, 1H), 6.86 (d, 1.2, 1H), 3.94 (s, 3H), 3.84 (s, 3H), 3.83 (s, 3H), 3.31 (q, 6.0, 2H), 2.38 (t, 6.0, 2H), 2.17 (s, 6H). TOF-MS-ES *m*/*z* (relative intensity): 484 ($M + H^+$, 100). HRMS (TOF-MS-ES): calcd for $C_{22}H_{30}N_9O_4$, 484.2421; obsd, 484.2421.

Surface Plasmon Resonance (SPR). Kinetic and steady state experiments were performed by SPR, using either a BIACORE 2000 or 3000 instrument (Biacore AB). Approximately 300 response units (~0.3 ng DNA per mm^2) of 5′-biotinylated DNA hairpins were immobilized via streptavidin-dextran matrix to the gold chip (sensor chip SA, purchased from Biacore AB). Polyamide compounds were dissolved in MES20 to $\sim 5 \times 10^{-4}$ M, and the concentration was determined by UV−vis spectroscopy (ϵ_{305} for f-ImImPy = 2.4×10^4 M^{-1} cm^{-1}; ϵ_{308} for f-PyImPy = 2.5×10^4 M^{-1} cm^{-1}; ϵ_{307} for f-ImPyIm = 2.4×10^4 M^{-1} cm^{-1}; ϵ_{304} for f-PyPyIm = 3.5×10^4 M^{-1} cm^{-1}). Stock polyamide solutions were stored at 4 °C and were stable within the time frame in which they were used (2 weeks). Dilutions ranging from 0.01 to 40 μM of each polyamide were flowed across the immobilized DNA at 25 μL/min, and the change in relative response units (RU) was analyzed using biaevaluation software (Biacore AB).[22] Stoichiometric, steady-state, and kinetic data were analyzed as described

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previously.[22] The RU_{max} values were calculated from the product of the response units of DNA immobilized on the chip, the molecular weight of the polyamide, the refractive index of the polyamide (1.4), and the inverse of the molecular weight of the DNA.[22] Fitting errors, due to random point scatter, are less than ±5% for K_{eq} and are approximately 25% for the individual K_1 and K_2 values due to correlation of variables. Experimental errors estimated from reproducibility of results are ±10% for K_{eq} or k_d values between 5×10^5 and 5×10^7 M^{-1} or 0.1 and 0.001 s^{-1}, respectively. Errors increase to ±20% for K_{eq} values between 5×10^7 and 5×10^8 M^{-1} and for k_d between 0.1 and 1 s^{-1}. It is difficult to accurately determine k_d values that are greater than 1 s^{-1} by biosensor-SPR methods. k_a values that could be determined for the compounds in these experiments have ±10% errors.

Acknowledgment. Support from the Camille and Henry Dreyfus Foundation Scholar/Fellow program, the National Science Foundation-REU program, Research Corporation (M.L.), NIH GM61587, and the Georgia Research Alliance (W.D.W.) is gratefully acknowledged.

JA044359P