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Hx-amides: DNA sequence recognition by the fluorescent Hx (*p*-anisylbenzimidazole)•pyrrole and Hx•imidazole pairings

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ABSTRACT

Hx-amides are fluorescent hybrids of imidazole (I)- and pyrrole (P)-containing polyamides and Hoechst 33258, and they bind in the minor groove of specific DNA sequences. Synthesis and DNA binding studies of HxII (**5**) complete our studies on the first set of Hx-amides: Hx–I/P–I/P. HxPP (**2**), HxIP (**3**) and HxPI (**4**) were reported earlier. Results from DNase I footprinting, biosensor-SPR, CD and ΔT_M studies showed that Hx-amides interacted with DNA via the anti-parallel and stacked, side-by-side motif. Hx was found to mimic the DNA recognition properties of two consecutive pyrrole units (PP) in polyamides. Accordingly, the stacked Hx/PP pairing binds preferentially to two consecutive AT base pairs, A/T–A/T; Hx/IP prefers C–A/T; Hx/PI prefers A/T–C; and Hx/II prefers C–C. The results also showed that Hx-amides bound their cognate sequence at a higher affinity than their formamido-triamide counterparts.

Polyamides are non-fluorescent imidazole (I) and pyrrole (P)-containing analogs of distamycin A (**1**, Fig. 1). The small molecules recognize and bind specific sequences of DNA within the minor groove via a stacked, anti-parallel 2:1 ligand/DNA motif.[1–3] A set of pairing rules has been established for I and P-containing polyamide-DNA base pair recognition. A P/P pairing recognizes an A/T or T/A base pair, an I/P pairing binds to a G/C base pair, while a P/I pairing recognizes a C/G base pair. In addition, a stacked I/I pairing targets a T/G mismatch but tolerates C/G or G/C.[4–6] As a result, polyamides can be tailored to bind any DNA sequence. These molecules have been applied to target the control regions of specific genes, including those that cause cancer.[7] A number of examples have been reported,[8] including reports from our laboratories on activation of the silenced topoisomerase IIα gene in confluent cancer cells.[9,10] Specifically, we have demonstrated that low molecular weight polyamides, such as f-PIP (Fig. 1)[9] (f is the *N*-formamido group found in distamycin) and PII-γ-PPP,[10] a hairpin polyamide, were able to bind the Inverted CCAAT Box2 (or ICB2), which occurs in the promoter of the topo IIα gene and is implicated in the confluence-induced transcriptional downregulation of topoisomerase IIα through the binding of transcription factor NF-Y. Inhibition of NF-Y binding to ICB2 by these polyamides led to activation of the repressed topo IIα gene in confluent cells. Ultimately, the confluent cancer cells become sensitized to topo IIα targeted anticancer drugs, such as etoposide.[9,10] P- and I-containing polyamides are therefore potentially useful for the development of treatments for genetic or gene derived diseases, including cancer.

Despite the significant advancements that have been made in the polyamide field, further improvements in the design and development of novel polyamides are needed.[11] Specifically, there is an immediate need to develop polyamides that have good solubility in water or aqueous biological media, readily localize in the nucleus, and are fluorescent so they can be tracked in cells.[11] A number of fluorophores such as 4′,6-diamidino-2-phenylindole (DAPI),[12] thiazole orange,[13] fluorescein,[14] and bodipy[15] have been incorporated into the polyamide structure as a fluorescent tag. Although, such fluorophore-polyamide hybrids have yielded interesting results, this approach was compromised by two factors: first, the increase in molecular mass of the conjugates and second, influence of fluorescent tag on the cellular and nuclear uptake properties of the conjugates.[16] Ideally the polyamides should be inherently fluorescent yet maintaining an affinity and specificity for unique sequences. In 2007 Dervan's group made some contributions to this area by reporting hairpin polyamides that contained benzimidazole (Bz), imidazopyridine (Ip), and hydroxybenzimidazole

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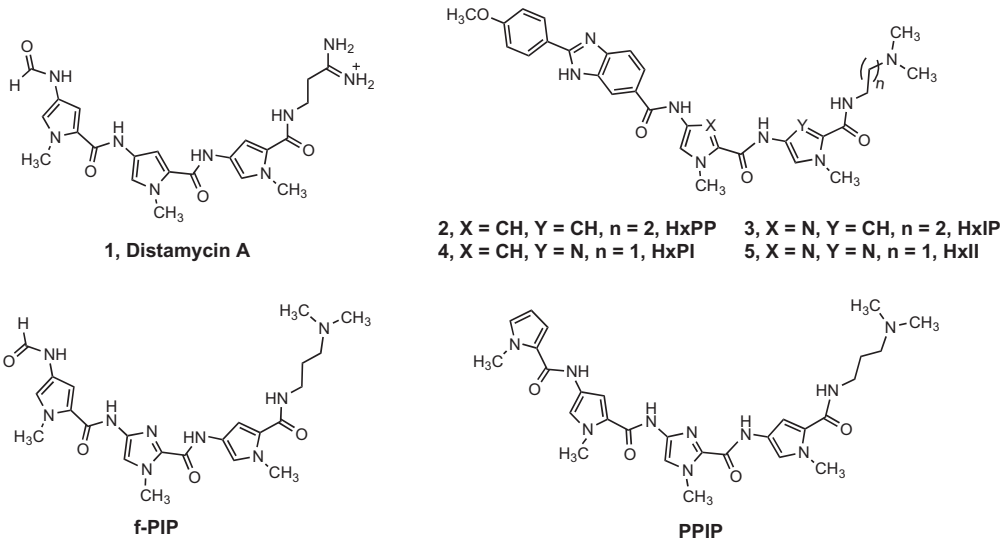


Figure 1. Structures of Distamycin A, **1**, and the complete series of Hx–I/I–I/P:HxPP (**2**), HxIP (**3**), HxPI (**4**), HxII (**5**), along with f-PIP and PPIP.

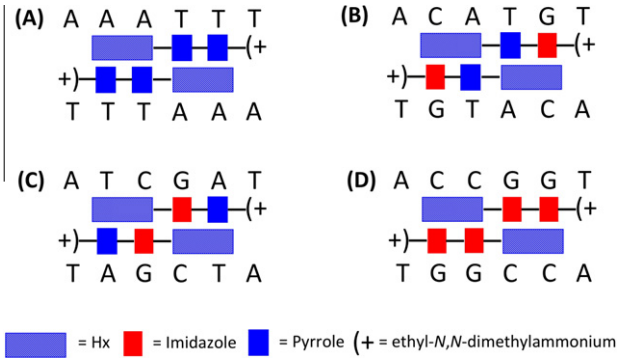
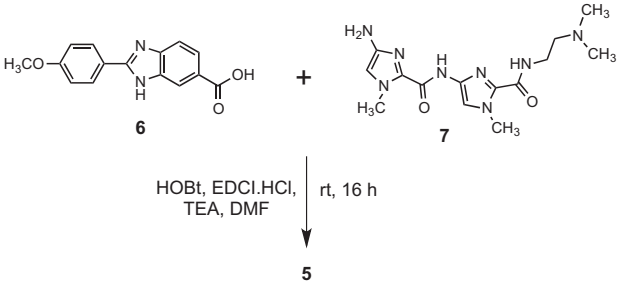


Figure 2. Binding of Hx–I/P–I/P to their respective cognate sequences through the stacked motif. (A) HxPP (**2**) and 5′-AAATTT. (B) HxIP (**3**) and 5′-ATCGAT. (C) HxPI (**4**) and 5′-ACATGT. (D) HxII (**5**) binding to its target sequence 5′-ACCGGT.



Scheme 1. Synthesis of HxII (**5**).

(Hz) units. The emission property of these structural units allowed for their detection by fluorescence studies.[17]

We have also been working on developing novel polyamides that would overcome these challenges. In 2011, we reported the design of a novel class of fluorescent and sequence specific binding polyamides. These compounds contained a fluorogenic moiety, *p*-anisylbenzimidazolecarboxamido. Hoechst 33258 inspired the design of this fluorophore and we coined it 'Hx'. Combining Hx with polyamides created a new family of DNA binders, called 'Hx-amides'. Three Hx-amides, HxPP (**2**), HxIP (**3**) and HxPI (**4**)

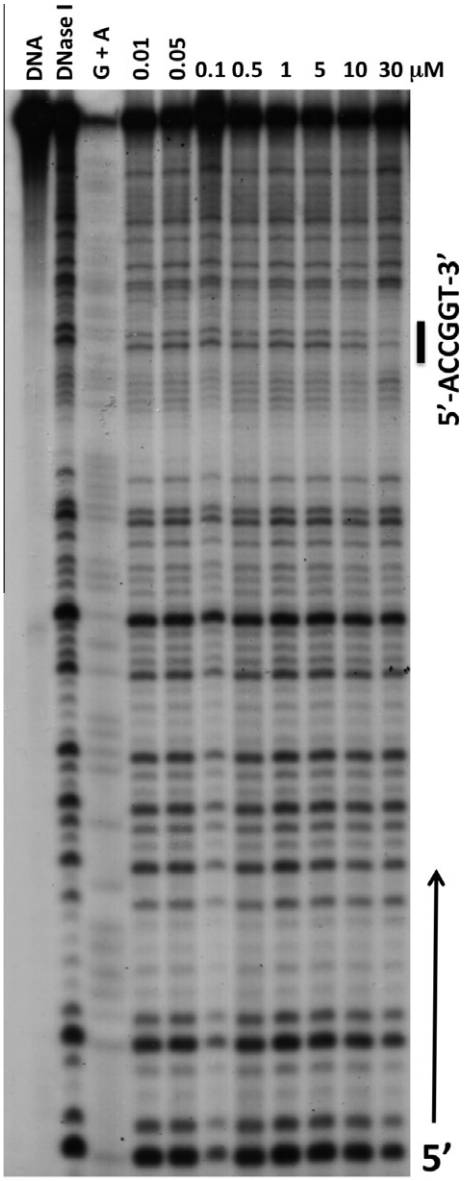


Figure 3. Autoradiogram of a DNase I footprinting experiment of HxII (**5**). Concentrations are expressed in μM.

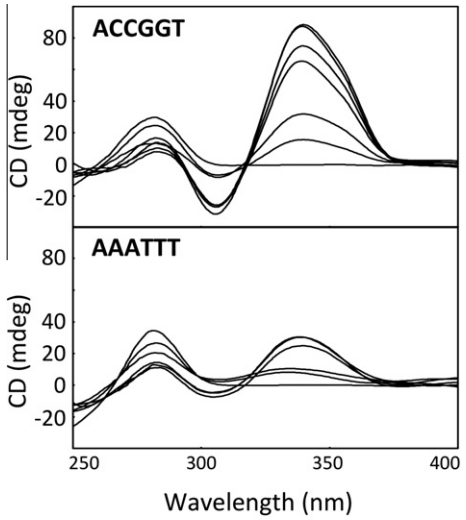


Figure 4. CD titration studies of HxII (**5**) to its cognate sequence 5′-ACCGGT and to a non-cognate sequence 5′-AAATTT. CD experiments were carried out using 160 μL of a 9 μM DNA solution, which was titrated with 1 mol equiv of **5**, past the point of saturation.

(Fig. 1) were recently reported.[18] These compounds bound in the minor groove and preferentially at specific sequences via the stacked dimer motif. Specifically, HxPP preferred 5′-AAATTT and HxPI preferred 5′-ACATGT. HxIP bound selectively to 5′-ATCGAT and 5′-TACGAT.[18] The latter sequence is located at the 5′-flank of the ICB2 site of the topoisomerase IIα promoter. Results from Hx-amides **2**–**4** suggest that the Hx-moiety behaved comparably to two consecutive pyrroles or PP in polyamides. In this communication, we complete the studies on the first set of Hx-amides Hx–P/I–P/I by synthesizing the fourth molecule, HxII (**5**) (Fig. 1). According to the model in which Hx-amides bind their cognate sequences given in Figure 2, HxII is expected to target 5′-ACCGGT-3′ DNA sequence.

HxII polyamide (**5**) was synthesized according to the reaction depicted in Scheme 1. 2-(4-Methoxyphenyl)benzimidazole-6-carboxylic acid (**6**)[19] was coupled with amino-ImIm-*N*,*N*-dimethyl-2-aminoethylamine (**7**)[20] in anhydrous dimethylformamide in the presence of EDCI, hydroxybenzotriazole (HOBt), and triethylamine at room temperature. Purification of the product by column chromatography using silica gel afforded the desired HxII as a yellow solid. The compound was characterized by TLC, infrared, 400 MHz ^{1}H NMR, and mass spectrometry.[21]

The sequence binding preference of HxII (**5**) was established through a DNase I footprinting experiment using a 5′-^{32}P labeled 131 base pair DNA fragment that contain the cognate sequence (5′-ACCGGT). As shown in Figure 3, cleavage inhibition specifically at the cognate site is observed at 10 μM and 30 μM. This result demonstrates the selectivity of HxII for the target 5′-ACCGGT sequence. The results provide direct evidence of the binding of HxII to the target 5′-ACCGGT site.

Binding of HxII to its preferred sequence 5′-ACCGGT was further corroborated by DNA thermal denaturation studies. Using 3 mol equiv of the HxII-polyamide to DNA (bp), ΔT_M values of 5 °C, 0 °C, and 5 °C were recorded for 5′-AAATTT, 5′-ACGCGT, and its cognate sequence 5′-ACCGGT, respectively. It is worth noting that a switch of CG in the core site of ACCGGT to GC reduced the ΔT_M value to 0 °C. Binding of HxII to AAATTT is presumably due to electrostatic stabilization of the positively charged polyamide and the polyanionic backbone of the DNA. According to CD titrations studies (Fig. 4), emergence of a strong positive DNA induced ligand band at about 330 nm inferred the binding of HxII in the minor groove.[22] The clear isodichroic point at about 310 nm for ACCGGT also suggested that HxII bound through a single mechanism, presumably in the minor groove and as a stacked dimer. In contrast, an isodichroic point was not apparent for AAATTT indicating the binding was not by a single mechanism.

The biosensor-SPR method was employed to ascertain the binding affinity of HxII to its cognate and non-cognate sequences. The studies were conducted using methodologies similar to those reported for HxPP (**2**), HxIP (**3**), and HxPI (**4**).[18] Thus the results are directly comparable. The SPR results given in Figure 5 allowed the determination of the binding constant of HxII for its cognate 5′-ACCGGT to be $2 \times 10^6\ M^{-1}$. This result was calculated using a model of 2:1 Hx-amides:DNA stoichiometry. The binding constants for the non-cognate sequences are as follows: 5′-ATGCAT, $1 \times 10^6\ M^{-1}$, and 5′-AGCGCT, $5 \times 10^5\ M^{-1}$. Interestingly, these results led to the following deductions: first, consistent with Hx-amides **2**–**4**, HxII (**5**) binds its cognate sequence with a higher affinity than its non-cognate sequences. Second, its preference for

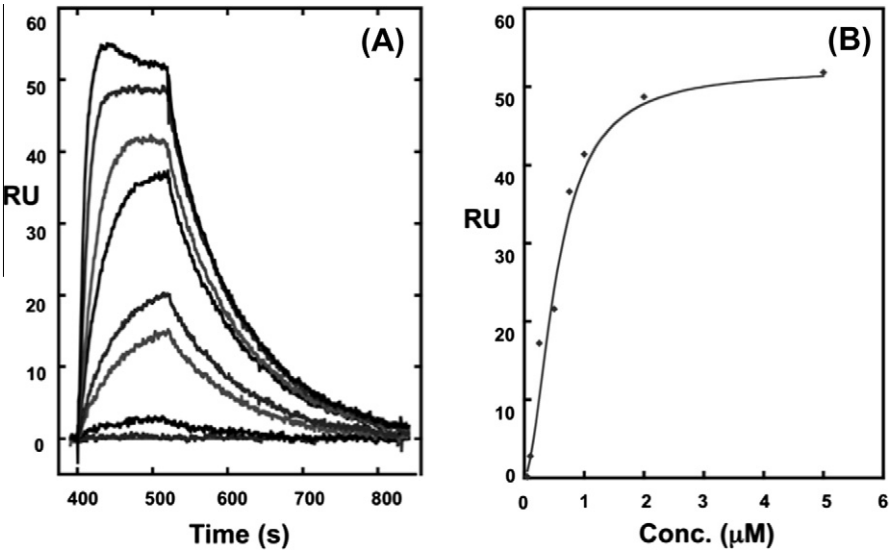


Figure 5. (A) SPR sensograms of HxII upon binding to its cognate sequence 5′-ACCGGT. (B) Fitting of steady-state RU versus concentration using a two-site model. The steady-state values at the lowest concentrations were determined by prediction from kinetics fits to the full curves.

5′-ACCGGT as determined by SPR and other studies provided further evidence that Hx behaves similarly to two consecutive pyrroles, PP. Third, Hx-amides produced enhanced binding affinity over their formamido counterparts. For example, the binding constants of HxIP (**3**), f-PIP, and PPIP (Fig. 1) for 5′-ATCGAT were $2 \times 10^6\ M^{-1}$, $2 \times 10^5\ M^{-1}$, and $2 \times 10^4\ M^{-1}$, respectively.[18] In this study, HxII (**5**) also bound two-times more strongly to its cognate sequence 5′-ACCGGT than f-PII: $2 \times 10^6\ M^{-1}$ versus $1 \times 10^6\ M^{-1}$,[23] respectively. Fourth, given the inherent fluorescence property of Hx-polyamides, including HxII (excitation at 320 nm and emission at 360 nm), Hx-amides are readily traceable in cells (data will be reported elsewhere). Fifth, as hydrochloride salts Hx-amides readily dissolved in water at 1 mM. This is a significant improvement over the formamido triamides and the non-formamido compounds.

Finally, the results provide evidence that Hx functions as a PP doublet and a set of pairing rules for DNA sequence recognition is summarized in Figure 2. Specifically, Hx/PP pairing preferentially binds in the minor groove of two A/T base pairs, or A/T–A/T (Fig. 2A). The Hx/IP pairing binds a C–A/T doublet (Fig. 2B), Hx/PI recognizes A/T–C, and Hx/II binds C–C.

In conclusion, we have completed the synthesis and DNA binding studies of all four members of Hx–I/P–I/P set of Hx-amides. The results provide evidence that Hx is a versatile and useful DNA sequence recognition entity. Our group is actively engaged in optimizing and further enhancing its sequence recognition properties. Biological studies of Hx-amides are underway and the results will be reported in due course.

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21. *p*-Anisylbenzimidazole-imidazole-imidazole-dimethylaminoethylamine (HxII) (**5**): 2-(4-methoxyphenyl)benzimidazole-6-carboxylic acid (**6**)[18,19] (0.051 g, 0.19 mmol) was dissolved in anhydrous DMF (1.5 mL), and EDCI·HCl (0.11 g, 0.6 mmol) and dry triethylamine (0.11 mL, 0.8 mmol) were added under nitrogen atmosphere at room temperature. The reaction mixture was stirred for 5 min at room temperature and HOBt (0.087 g, 0.6 mmol) was added and the mixture was stirred for another 5 min. A solution of amino-ImIm-*N*,*N*-dimethyl-2-aminoethylamine (**7**)[20] (0.053 g, 0.16 mmol) dissolved in anhydrous DMF (2.0 mL) was added at room temperature. After the mixture was stirred at room temperature for 16 h the reaction was complete, as monitored by TLC [CH_3OH:NH_4OH:$CHCl_3$ (1:0.15:5.5)], the solvent was removed under vacuum (45–50 °C). The residue was purified by silica gel column chromatography using a gradient $CHCl_3$/MeOH (0:100–100:0%, v/v) solvent system. The product **5** was isolated as a yellow solid (8.0 mg, 8.3%); mp: 135–138 °C; R_f: 0.59 (1:0.15:5.5 v/v, CH_3OH:NH_4OH:$CHCl_3$); FT-IR: 3256, 2960, 1630, 1572, 1527, 1467, 1431, 1259, 1152, 1098, 987, 880, 807, 740, 696 cm^{-1}; ^1H NMR (CD_3OD): δ 8.20 (s, 1H), 8.07 (d, J = 8.0 Hz, 2H), 7.83 (d, J = 8.0 Hz, 1H), 7.66 (d, J = 8.0 Hz, 1H), 7.59 (s, 1H), 7.45 (s, 1H), 7.09 (d, J = 8.0 Hz, 2H), 4.08 (s, 3H), 4.01 (s, 3H), 3.88 (s, 3H), 3.49 (t, J = 7.0 Hz, 2H), 2.56 (t, J = 7.0 Hz, 2H), 2.31 (s, 6H); LRMS (ES^+) m/z 585 ($M+H^+$, 100%); HRMS $[M+H]^+$ calcd for m/z $C_{29}H_{32}N_{10}O_4$: 585.2686, obsd. 585.2706.
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