Heterocycl. Commun., Vol. 16(4-6), pp. 221–225, 2010 • Copyright © by Walter de Gruyter • Berlin • New York. DOI 10.1515/HC.2010.002

Synthesis and biophysical studies of hairpin polyamides targeting the Brn-3b and GATA-3 transcriptional sites

Balaji Babu, Hilary Mackay, Abby Prast, Kristin Dittenhafer, Ryan Davis, Christopher Tronrud, Toni Rice, Sameer Chavda and Moses Lee*

Department of Chemistry, Hope College, Holland, MI 49423, USA

*Corresponding author
e-mail: lee@hope.edu

Abstract

Hairpin polyamide analogs of distamycin A (**1**) were designed and synthesized to evaluate their ability to bind 5′-ATAGA-3′ and 5′-AGATA-3′ sequences which are important elements for controlling the function of the Brn-3b and GATA-3 transcriptional factors, respectively. The hairpin polyamides are composed of pyrrole and imidazole units linked together via a γ-aminobutyrate (GABA) unit. Hairpins **2b** (Py-Py-Im-γ-Py-Py-Py) and **2c** (Im-Py-Py-γ-Py-Py-Py) were synthesized to target the respective Brn-3b and GATA-3 cognate sequences. Preliminary biophysical studies including thermal denaturation and circular dichroism were performed and the results ascertained the binding of hairpins **2a** and **2b** to their respective cognate DNA sequences.

Keywords: Brn-3b; DNA; GATA-3; hairpin polyamides; minor groove; transcriptional factors.

Introduction

Recent advances in the chemical biology of the human genome have opened up new avenues for drug discovery by medicinal chemists. Because biological characteristics such as polymorphisms and gene expression profiles are closely related to drug responses, tailor-made therapy might be realized by knowledge-based design (Hurley, 2002). Many challenges, such as genome-based drug development and tailored cancer chemotherapy based on individual genomic construction or 'personalized medicine' have been the focus of considerable attention (Wittung-Stafshede, 1998). Therefore, much effort has been directed at designing and developing functional small molecules that selectively bind DNA and recognize base-pair sequences, allowing for the targeting of specific sequences (Denison and Kodadek, 1998).

The transcription factor Brn-3b is a member of the POU family of transcription factors, and they share a common bipartite DNA binding domain (Verrijzer and Van der Vliet, 1993). Brn-3b was originally identified to play a role in the nervous system where its inactivation was shown to produce defects in central nervous system development (He et al., 1989; Gerrero et al., 1993; Ninkina et al., 1993; Turner et al., 1994). In addition, Brn-3b is also expressed in reproductive tissues such as the cervix (Lillycrop et al., 1992; Ndisang et al., 1998), testis (Budhram-Mahadeo et al., 2001), and breast (Budhram-Mahadeo et al., 1998). Overexpression of Brn-3b has been detected in human neuroblastomas (Smith and Latchman, 1996; Latchman, 1997) and several human breast tumors (Budhram-Mahadeo et al., 1999; Dennis et al., 2001), suggesting a role for this transcription factor in cancer. In several breast cancers, elevated levels of Brn-3b have been determined to enhance cell proliferation and tumor growth (Budhram-Mahadeo et al., 1999; Dennis et al., 2001). A reduction in Brn-3b levels slows down such growth effects (Budhram-Mahadeo et al., 1999; Dennis et al., 2001). Additionally, elevated Brn-3b levels increase cancer cell migration (Ishrad et al., 2004) and confer resistance to growth arrest stimulus. For example, Brn-3b represses the BRCA1 tumor suppressor which normally induces cell cycle arrest (Budhram-Mahadeo et al., 1999). In addition to these direct effects on cell growth, Brn-3b can act indirectly by associating with other proteins (Budhram-Mahadeo et al., 1998). In particular, Brn-3b associates with the estrogen receptor (ER) and enhances the effect of this receptor at estrogen response elements (Budhram-Mahadeo et al., 1998). For example, the expression of HSP27, which can be directly activated by either Brn-3b or ER, is maximal when both Brn-3b and ER work cooperatively (Lee et al., 2005).

The transcriptional factor GATA-3 belongs to a family of six transcriptional factors found in the human body. Each of these GATA proteins has been linked to different types of cancer, including pancreatic, breast, ovarian, gastric, and colorectal (Guo et al., 2005). GATA-3 expression is enhanced in pancreatic cancer cells over non-cancerous cells (Gulbinas et al., 2006). GATA-3 has also been studied as a prognostic marker for breast cancer. This data shows that GATA-3 is a transcriptional activator in breast tissue and has been shown inside the nucleus of cancerous breast cells (Mehra et al., 2005). However, there is still uncertainty on the exact role of GATA-3 in the pathogenesis of cancer.

Owing to the connection of GATA-3 and Brn-3b in affecting cancer growth, they represent excellent targets for the design of novel gene-focused cancer control agents. The use of polyamide analogs of distamycin **1** (Figure 1) to interrupt the binding of transcriptional factors and to inhibit gene function is under intense investigation by the authors' laboratory and others (Mackay et al., 2008). Pyrrole (Py) and imidazole (Im) containing polyamides can be synthesized to bind with high affinity and selectivity in the minor groove of the

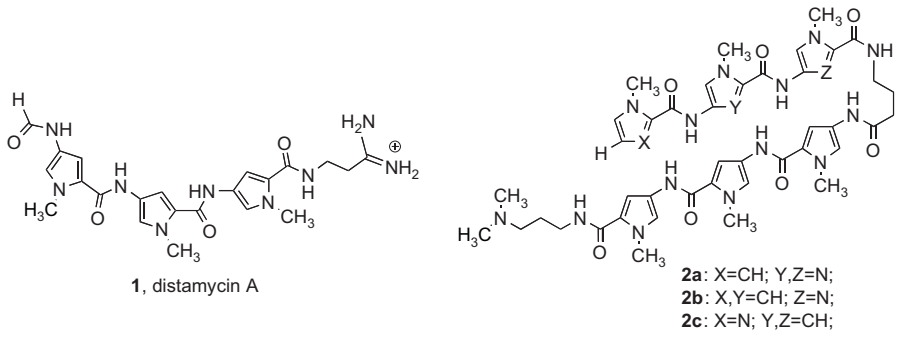


Figure 1 Structures of distamycin **1** and hairpin polyamides **2a**, **2b**, **2c**.

desired DNA sequence by following the established 'pairing rules': Im/Py binds G·C and Py/Im binds C·G, Py/Py binds either to A/T or T/A, and Im/Im binds T/G mismatched base pairs (Kopka et al., 1997; Yang et al., 1999; Dervan and Edelson, 2003; Lacy et al., 2004). Previous research by the authors' laboratory demonstrated that hairpin polyamide **2a** (Figure 2) was capable of entering the cell nucleus and inhibiting the action of nuclear factor Y (NF-Y) at the inverted CCAAT box 2 (ICB2) site on the topoisomerase IIα (topo IIα) promoter (Henry et al., 2004). Anticancer agents such as etoposide and doxorubicin, which target topo IIα, become ineffective in confluent cancer cells where the expression of this enzyme is downregulated. NF-Y affects this downregulation by binding to the ICB2 site (5′-A*TTGGT*-3′). As depicted in Figure 2, hairpin **2a**, which has a polyamide sequence Py-Im-Im-γ-Py-Py-Py was designed to bind to the 3′-flank of this sequence and it was found to interfere with NF-Y binding and its downstream events. DNaseI footprinting studies showed binding to the desired site and electrophoretic mobility shift assay studies demonstrated inhibition of NF-Y binding to ICB-2 (Henry et al., 2004). Incubation of confluent NIH 3T3 cells with **2a** demonstrated increased expression of topo IIα (Hochhauser et al., 2007). Following exposure to etoposide, increased DNA double-stand breaks were observed by the comet assay.

Building on the success of the above strategy, the current report describes the synthesis and evaluation of hairpin polyamides **2b** and **2c** (Figure 1), designed specifically to bind the respective promoters and hopefully inhibit Brn-3b and GATA-3. Altering the structure of **2a** by replacing the second Im group with Py (Py-Py-Im-γ-Py-Py-Py) produces hairpin **2b** that should recognize the sequence 5′-ATAGA located on the 3′-flank of the Brn-3b site (Figure 3). Replacing the top piece with Im-Py-Py produces hairpin **2c** that should recognize the sequence 5′-AGATA-3′ (Figure 3). The binding characteristics of **2b** and **2c** were investigated preliminarily using thermal denaturation (ΔT_m) and circular dichroism (CD) (Lacy et al., 2002; Westrate et al., 2009).

Results and discussion

Synthesis of 2b and 2c

The synthetic approach towards obtaining polyamides **2b** and **2c** is outlined in Scheme 1. The desired hairpin polyamides were synthesized using standard Schotten-Baumann coupling of amines and acid chlorides. The bottom piece *N*-(*N*′,*N*′-dimethylaminopropyl)-4-[4-(1-methyl-4-nitropyrole-2-carboxamido)-1-methylpyrrole-2-carboxamido]-1-methylpyrrole-2-carboxamide **3** was synthesized using the previously described procedure in 95% yield (Matsumoto et al., 1990).

The intermediate **11** was synthesized using the readily available 1-methyl-4-nitro-2-trichloroacetylimidazole **4** (Jaramillo et al., 2004). Reaction of **4** with ethyl γ-aminobutyrate hydrochloride yielded the previously reported compound **5**. Reduction of **5** using 5% Pd/C in cold ethanol, followed by coupling with acid chloride (prepared from **6** using $SOCl_2$), yielded compound **7**, which in turn was reduced using the same protocol as discussed above and coupled to acid **8** using PYBOP, diisopropylethylamine (DIPEA) in dry DCM yielding **10** in 25% yield. Hydrolysis of **10** followed by coupling of the carboxylic acid **11** with the amine derived from hydrogenation of **3** using PyBOP and DIPEA in dry DCM gave the desired hairpin polyamide **2b**.

The intermediate **16** desired for the synthesis of **2c** was synthesized starting from 1-methyl-4-nitro-2-trichloroacetyl-

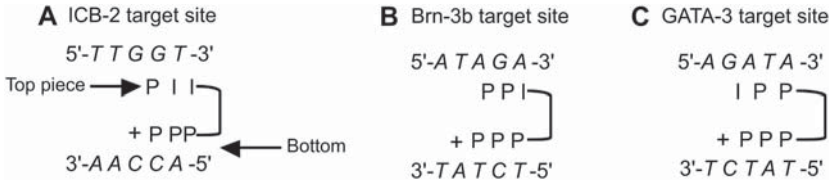


Figure 2 Target sequences for **2a**, **2b** and **2c**.

5'-ACGCGT-3': 5'-GA ACGCGT CG CTCT CGACGCGTTC-3'
5'-A3T3-3': 5'-CG AAATTT CC CTCT GG AAATTT CG-3'
5'-ATAGA-3': 5'-GCAATAGA GCCTCTGCTCTATTGC-3'
5'-AGATA-3': 5'-GCAAGATA GCCTCTGCTATCTTGC-3'

Figure 3 Cognate and non-cognate DNA sequences for **2b** and **2c**.

pyrrole **12** (Xiao et al., 2000). Compounds **13** and **14** were synthesized as described previously in the literature (Jaramillo et al., 2004). Reduction of **14** using 5% Pd/C in cold ethanol, followed by coupling with acid chloride (prepared from **9** using $SOCl_2$), yielded the unknown compound **15** in 95% yield. Hydrolysis of **15** followed by coupling of acid **16** with the amine derived from hydrogenation of **3** using PyBOP and DIPEA in dry DCM gave the desired hairpin polyamide **2c**. Both the new hairpin polyamides **2b** and **2c** were characterized using IR, NMR and HRMS spectral data.

Thermal denaturation for compounds 2b and 2c

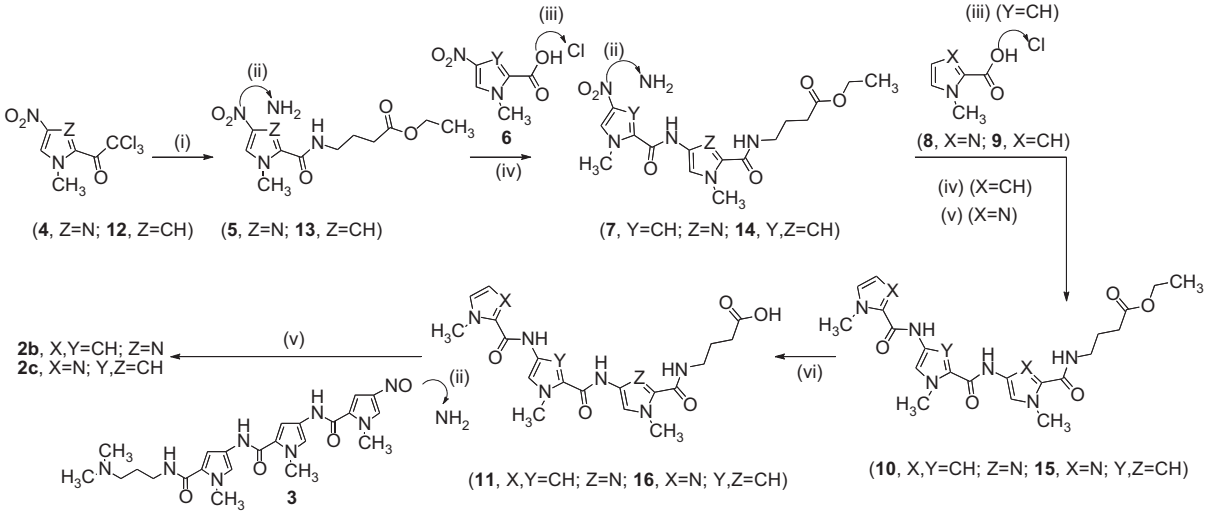
Thermal denaturation (ΔT_m) studies with hairpin **2b** were carried out using three DNA hairpins that contain a single binding site representing the cognate, Brn-3b site (5′-ATAGA-3′), an A/T rich non-cognate site (5′-AAATTT-3′), and a C/G rich non-cognate site (5′-ACGCGT-3′). No change in the melting temperature (ΔT_m) between DNA alone and DNA in the presence of compound **2b** was observed for any of the sequences. This was unexpected in the case of the cognate site, as a melt had been observed with hairpin **2a** and the ICB2 sequence in previous studies (8°C) (Henry et al., 2004). However, a similar result was observed with other polyamides that have been shown to bind to its cognate sequence by other biophysical methods but have shown ΔT_m values of 0°C. This is probably owing to low binding affinity and the polyamide/DNA complex was not stable at higher temperatures; hence, a ΔT_m was not observed.

Thermal denaturation (ΔT_m) studies with hairpin **2c** were carried out using three DNA hairpins with a single binding site representing the cognate, GATA site (5′-AGATA-3′), an A/T rich non-cognate site (5′-AAATTT-3′), and a C/G rich non-cognate site (5′-ACGCGT-3′). However, there was a small change in thermal melt with the cognate sequence (ΔT_m=2°C), and interestingly it gave a higher melting temperature with A/T rich non-cognate sequence (ΔT_m=3°C). This is not unusual especially considering that the cognate and A/T rich non-cognate sequences differ by only one G/C base pair. Imidazole-containing polyamides have been shown to tolerate and bind AT-rich sequences and the affinity is attributed mainly to electrostatic attraction (Lown et al., 1986). AT-rich sequences have the most negative molecular electrostatic potential and the polyamides are cationic at physiological pH (Lown et al., 1986).

CD studies for compounds 2b and 2c

CD studies were carried out using the same DNA hairpins described above. Minor groove binding of **2b** to the cognate sequence (5′-ATAGA-3′) (Figure 4A) is demonstrated by the appearance of an induced CD band at 330 nm, which is typical of polyamides that bind in this manner (Lacy et al., 2002; Hochhauser et al., 2007; Westrate et al., 2009). Interestingly, binding was observed at the A/T rich non-cognate sequence (Figure 4B). This is consistent with the ΔT_m studies as discussed above. It appears that **2b** can tolerate this one base mismatch, although binding to this sequence gave weaker band than that of the cognate (Figure 4A), as suggested by the reduced CD response at 330 nm (8 vs. 13 mdeg, respectively). By comparison, no binding was observed to the C/G rich non-cognate sequence (Figure 4C), demonstrating that **2b** has some degree of selectivity.

CD studies were carried out using cognate DNA sequence for compound **2c**. Minor groove binding of **2c** to (5′-



Scheme 1 (i) Ethyl γ-aminobutyrate, dry Et_3N, dry DCM, reflux, 18 h; (ii) 5% Pd/C, cold EtOH, rt; (iii) $SOCl_2$, 15 min, reflux; (iv) dry Et_3N, dry DCM, 0°C-rt; (v) PyBOP, DIPEA, dry DCM, 2 days, rt; (vi) a: NaOH, H_2O, MeOH, reflux, 30 min.

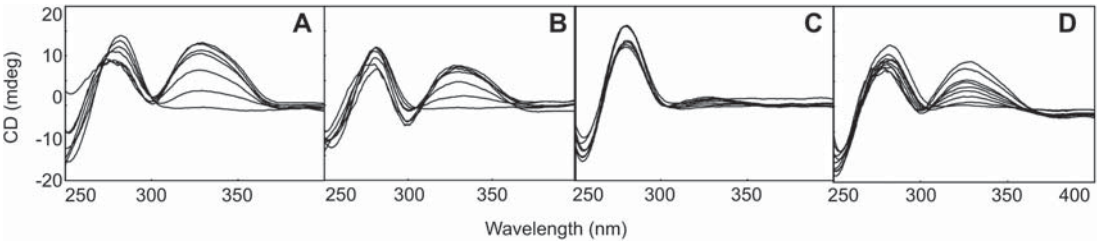


Figure 4 CD spectra of hairpin **2b** with (A) the cognate sequence 5′-ATAGA-3′ and two non-cognate sequences, (B) 5′-A_3T_3-3′ and (C) 5′-ACGCGT-3′. (D) CD spectra of hairpin **2c** with cognate sequence 5′-AGATA-3′. Note that the *x*-axis represents wavelength (nm), whereas the CD in mdeg is represented on the *y*-axis.

AGATA-3′) (Figure 4D) was demonstrated by the induced CD band at 330 nm.

Conclusion

Small molecules that specifically bind to any sequence in the human genome would be useful tools in molecular biology and potentially in human medicine. Thus, two new hairpin polyamides having some sequence specificity and binding affinity towards their cognate DNA sequences, as indicated by biophysical studies, have been synthesized and characterized. The goal in disclosing compounds **2b** and **2c** is to offer the scientific community working in this area, an opportunity, to utilize these compounds in biological studies.

Experimental

General procedure for the synthesis of 2b and 2c

Compound **3** (1 eq.) was reduced in the presence of hydrogen over 5% Pd/C (50% wt/wt) in cold MeOH (100 ml) at RT for ~18 h. The reaction mixture was filtered over celite and the catalyst was washed thoroughly with MeOH. The solvent was removed by evaporation and residual MeOH removed by co-evaporation with dry DCM (3×2 ml). The resulting product was dried under high vacuum and protected from light. Anhydrous **11** or **16** (1.2 eq.) and PyBOP (2.1 eq.) were added to the dry amine. The atmosphere was evacuated out of the reaction mixture by purging with argon. This process is repeated for two or three times to ensure the atmosphere is dry and inert. The mixture was dissolved in dry DCM (~10 ml) and diisopropylethylamine (3.1 eq.) was added. The reaction was allowed to stir for 2 days in the dark with an argon balloon. A basic aqueous work-up was performed by using 2 M NaOH and the products were extracted with DCM (3×20 ml). The combined organic layers were washed with brine followed by water and dried over anhydrous Na_2SO_4. The dried solution was evaporated under reduced pressure to obtain the residue, which was purified by flash column chromatography (silica, gradient 0:100–100:0 with ammonium hydroxide % v/v, MeOH/$CHCl_3$) to yield the desired compounds **2b** or **2c** as light yellow orange/orange solids.

Compound 2b

Yellow solid; yield=9%; mp: 277–279°C; IR (neat) 3200, 1659, 1650, 1538, 1260 cm^{-1}; ^1H NMR: (400 MHz, DMSO-d_6) δ 10.29 (s, 1H), 9.91 (s, 1H), 9.89 (s, 1H), 9.84 (s, 1H), 8.08 (br t, 1H), 8.04 (br t, 1H), 7.50 (s, 1H), 7.33 (br d, 1H), 7.24 (d, 1H), 7.18 (d, *J*=1.2 Hz, 1H), 7.17 (d, *J*=2 Hz, 1H), 7.06 (d, *J*=1.6, 1H), 7.03 (d, *J*=1.6 Hz, 1H), 6.94 (m, 2H), 6.871 (d, *J*=1.6 Hz, 1H), 6.84 (s, 1H), 6.05 (br t, *J*=3.2 Hz, 1H), 3.93 (s, 3H), 3.87 (s, 3H), 3.85 (s, 3H), 3.84 (s, 3H), 3.83 (s, 3H), 3.80 (s, 3H), 3.43 (quart, *J*=6 Hz, 2H), 3.19 (m, 2H), 2.54 (t, *J*=6.5 Hz, 2H), 2.28 (t, *J*=6 Hz, 2H), 2.21 (s, 6H), 1.81 (quin, 2H), 1.63 (quin, 2H); ES^+: 906 (M^+); HRMS: Calcd., 906.4487; Obsd.: 906.4490.

Compound 2c

Yellow orange solid; yield=3%; mp: 230–237°C; IR (neat) 3733, 3584, 2921, 2850, 1715, 1556, 1540, 1506, 1471, 1456, 1435, 1404, 1260 cm^{-1}; ^1H NMR: (400 MHz, DMSO-d_6) δ 10.46 (s, 1H); 10.29 (s, 1H); 9.93 (s, 1H); 9.92 (s, 1H); 9.89 (s, 1H); 9.85 (s, 1H); 8.55 (s, 1H); 8.32 (s, 1H); 8.07 (s, 1H); 8.07 (s, 1H); 7.59 (s, 1H); 7.39 (s, 1H); 7.29 (s, 1H); 7.24 (s, 1H); 7.18 (s, 1H); 7.16 (s, 1H); 7.04 (s, 1H); 7.03 (s, 1H); 6.91 (s, 1H); 6.88 (s, 1H); 6.82 (s, 1H); 3.99 (s, 3H); 3.84 (s, 6H); 3.83 (s, 3H); 3.80 (s, 3H); 3.79 (s, 3H); 3.24 (m, 2H), 3.19 (m, 2H); 2.33 (m, 2H); 2.23 (t, *J*=6.8 Hz, 2H), 2.13 (s, 6H); 1.79 (quin, *J*=6.4 Hz, 2H) 1.60, (quin, *J*=6.8 Hz, 2H); ES^+: 906 (M^+); HRMS: Calcd., 906.4487; Obsd.: 906.4469.

Acknowledgements

The authors are thankful to the support from the National Science Foundation (CHE 0809162).

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