Published in final edited form as:

Bioorg Med Chem. 2013 October 15; 21(20): 6101–6114. doi:10.1016/j.bmc.2013.04.023.

Programmable DNA-binding Small Molecules

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Abstract

Aberrant gene expression is responsible for a myriad of human diseases from infectious diseases to cancer. Precise regulation of these genes via specific interactions with the DNA double helix could pave the way for novel therapeutics. Pyrrole-imidazole polyamides are small molecules capable of binding to pre-determined DNA sequences up to 16 base pairs with affinity and specificity comparable to natural transcription factors. In the three decades since their development, great strides have been made relating to synthetic accessibility and improved sequence specificity and binding affinity. This perspective presents a brief history of early seminal developments in the field and highlights recent reports of the utility of polyamides as both genetic modulators and molecular probes.

1. Introduction

The ability to target DNA and precisely control transcription to elicit control of aberrant gene expression has the potential for far reaching applications in human medicine. In fact, many of the most devastating health afflictions, from cancer to infectious disease, can theoretically be conquered through targeting DNA. At the heart of this problem is the ability to design small molecules capable of interacting with the double helix with high affinity and specificity. This perspective will focus on the development of molecules that target the minor groove based upon the natural products netropsin (**1**) and distamycin A (**2**). Both these natural products are small, crescent shaped polypyrrole peptides that bind to repeating A/T tracts (where A/T is either a adenosine or thymidine) with modest affinity and specificity^{1–7} Analysis of the interactions between these molecules and DNA has led to the development of synthetic oligomers, based mostly on repeating pyrrole and imidazole units (so-called polyamides), which are capable of binding to predetermined DNA sequences with affinity and selectivity comparable to DNA-binding proteins.⁸ This perspective is not meant to be a comprehensive review of the field for which there are many excellent reviews,^{8–14} but to provide the reader with a brief history of the development of these compounds and to highlight recent advances in the field.

2. A Brief History of the Development of a Small Molecule Minor Groove Recognition Code

In 1985, Dickerson and coworkers published the X-ray crystal structure of netropsin (**1**) bound to B-DNA. The structure indicated that netropsin interacted with DNA in a 1:1

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stoichiometry by displacing the spine of hydration along the binding site in the minor groove and bound via hydrogen bonds between amide N-H protons and the N(3) of adenine and the O(2) of thymine in each A•T base pair and a van der Waals interaction between the pyrrole C-H and the C(2) of adenine (Figure 1).⁴ From this structure it was hypothesized that replacement of a pyrrole subunit with an imidazole, essentially a single atom replacement, would vastly expand the recognition code of these oligomers and allow for recognition of G•C base pairs by creating a hydrogen bond between the N(2) of imidazole and the exocyclic N-H proton of guanine.^{4, 15}

Two initial analogs of distamycin A were synthesized and tested for their ability to bind DNA. The first was a pyrrole-imidazole-pyrrole (PyImPy) oligomer by Lown and coworkers, which displayed modest efficiency and specificity. The second was an ImPyPy oligomer by Wade and Dervan, which formed the cornerstone of pyrrole-imidazole (PyIm) polyamides as synthetic DNA binding ligands.^{16, 17} Based on the 1:1 binding model, it was hypothesized that this latter ligand would bind a G(A/T)(A/T) sequence. Surprisingly, the analog bound G(A/T)C sequences with high selectivity, while an affinity cleavage reagent, in which the ImPyPy was covalently coupled to an EDTA-Fe[II] complex for sequence-directed fenton chemistry, revealed that DNA cleavage occurred on both sides of the binding site in approximately equal amounts. These results, although extremely exciting, were inconsistent with the 1:1 binding model and required re-evaluation of the binding arrangement between these ligands and the minor groove of DNA.

A key structural insight into this dilemma was provided by subsequent NMR studies from the Wemmer lab that revealed at high concentrations of distamycin, the minor groove of DNA could expand and bind distamycin in a 2:1 stoichiometry.¹⁸ A subsequent study between the Dervan and Wemmer labs delivered an NMR structure that established a 2:1 model for polyamide binding.¹⁹ The stacked ligands were oriented anti-parallel N to C-terminal along the 5' to 3' direction of the DNA helix along the binding site (5'-wGWCw-3', where w = A or T) (Figure 2). More importantly, this study provided the structural template that established that sequence specific DNA recognition could be achieved by precisely oriented pairs of aromatic amino acids, such that an Im/Py pair recognized a G•C base pair while a Py/Im pair recognized a C•G base pair. A Py/Py pairing appeared degenerate, targeting A•T as well as T•A base pairs. Subsequent studies established that covalently tethering the N-terminal of one oligomer to the C-terminal of the other could be employed to increase affinity and allow coupling of heterodimeric oligomers to dramatically increase the range of DNA sequences that could be specifically targeted. For example, an early study coupled the ImPyPy oligomer to distamycin (PyPyPy) via γ -aminobutyric acid to target 5'-GWW-3' sequences,^{20, 21} (**3**, Figure 3). This oligomer construction, more commonly referred to as a hairpin polyamide motif, showed both high affinity and specificity for its 5'-GWW-3' target sequence, returning a K_a of $7.6 \times 10^7 \text{ M}^{-1}$ for the match site and a preference of >12-fold for the match over mismatch sites.

3. Establishing the Generality of the Pairing Rules & Exploration of Alternative Ring Systems for Minor Groove Recognition

Many of the initial studies on the hairpin motif worked to establish the generality of the pairing rules and that hairpin polyamides could be designed to recognize virtually any predetermined DNA sequence. A number of molecular recognition discoveries were made during these extensive studies and are too numerous to detail here; however, three key advances will be briefly outlined. First, it was noted that the pitch of B-DNA was less than that of the polyamide such that as the polyamide became longer it became “overwound” with respect to the duplex, thus impacting both affinity and specificity. This problem was solved by the introduction of β -alanine () as a recognition element.²² When placed within

the polyamide framework, α -alanine had the same recognition properties as a Py subunit; however, its flexible nature allowed the polyamide to essentially reset and continue to match the pitch and curvature of the duplex. As such, polyamides can be designed to recognize upwards of 16 base pairs (a unique address within the human genome) with affinities that rival that of DNA-binding proteins ($>10^{10} \text{ M}^{-22121}$). Second, it was established that diaminobutyric acid (DABA) could be employed as a γ -aminobutyric acid surrogate to incorporate an amino group into the hairpin linker²³ that could ultimately impart both additional affinity as well as provide a handle for the subsequent creation of bi-functional polyamide conjugates. Third, and arguably one of the most profound discoveries ever made in DNA minor groove recognition, was that incorporation of a *N*-methyl-3-hydroxypyrrole (Hp) subunit opposite a Py would break the A/T degeneracy recognition problem and, for the first time, provide a recognition code for all four possible base pairs (Table 1). The Hp/Py pair is able to discriminate T•A from A•T, C•G, and G•C base pairs through a combination of hydrogen bonds between the hydroxyl and O2 of thymine and steric occlusion of the asymmetric cleft between the thymine O2 and adenine C2 (Figure 4).^{24–26} The use of 3-hydroxypyrrole polyamides has been limited, however, due to their instability. Degradation occurs over weeks to months when stored in solid form, but takes mere days in solution.^{12, 14}

Having established that discrimination of A•T over T•A base pairs is possible, there has been significant effort to design alternative heterocycles that discriminate A•T from T•A base pairs that do not have the inherent instability problems of Hp. In one study, several five-membered aromatic heterocycles were synthesized and incorporated into an eight-ring hairpin polyamide. Ring pairings of Py with 3-methylthiophene (Tn) or *N*-methylpyrazole (Pz) were exceptionally selective for A•T bases and showed no binding to G•C bases. However, 4-methylthiazole (Th), furan (Fr), and 3-hydroxythiophene (Ht) abrogated binding.²⁷ Molecular modeling studies suggested that these latter ring systems induced detrimental alterations to the shape and electronic properties of the polyamide²⁷, suggesting that the available ring scaffolds for DNA minor groove binding are quite limited.

Substituted benzimidazoles have also been investigated as potential recognition units when incorporated into polyamides, as they serve as the structural template of classic Hoechst dyes that are known to bind to the minor groove of DNA.¹² Hydroxybenzimidazole (Hz) and imidazopyridine (Ip) have been fused to *N*-methylpyrrole without an intermediate amide bond and introduced to the polyamide backbone as a monomer unit to efficiently replace two subunits (Figure 5).²⁸ Ip/Py and Py/Ip pairs exhibited the expected selectivities, C•G and G•C, respectively, indicating that they are analogous to the Im/Py and Py/Im parent pairs. The Py-Hz polyamide showed 18-fold augmentation of sequence specificity for 5'-cTGTTTAt-3' vs. 5'-cTGTATAt-3' versus only 2-fold for the parent Py/Im polyamide. Although a modest 10-fold loss of affinity was observed, addition of the hydroxybenzimidazole enhanced ligand stability indicating that the Hz/Py pair represents a useful replacement for Hp/Py in biological applications. With the characterization of the benzimidazoles, a set of pairing rules for this molecule class can be extrapolated and is presented in Table 2.

4. Polyamide Synthesis

Initial syntheses of polyamides were plagued by long and difficult solution phase syntheses that could take over a month for the synthesis and purification of each polyamide. A crucial advance in the field was reported by Baird and Dervan when they reported methods for the solid phase synthesis of polyamides that included gram-scale synthesis of monomer units without chromatographic purification and shortened the synthetic timescale for a single polyamide from months to days or weeks.²⁹ Subsequent refinements of the solid-phase

approach have been reported, including the use of Fmoc-protected amino acids and an optimized synthesis of Fmoc-pyrrole monomers.³⁰

Due to initial issues of resin cleavage, both the Boc and Fmoc solid phase chemistries relied on the use of Boc- α -alanine-PAM resin and cleavage via aminolysis with *N,N*-dimethylaminopropylamine (Dp) to provide a α -Dp tail.²⁹ Sequence specificity studies revealed that the α -Dp tail and the α -turn element of hairpin polyamides both exhibit A/T specificity.³¹ To broaden available sequences for polyamide recognition, use of the Kaiser oxime resin for synthesis followed by nucleophilic cleavage with various compounds has led to polyamides with different tails that could be tested for their sequence specificity (Figure 6).³² Polyamides with methyl amide tails show comparable affinity to the α -Dp tail, but have diminished energetic preference for A•T/T•A base pairs and could allow for targeting a greater variety of DNA sequences by hairpin polyamides.

In recent years, microwave assisted solid phase synthesis has gained popularity for rapid formation of products in excellent yields and purities. The Dervan lab recently reported the first microwave assisted synthesis of a Py-Im polyamide containing the DABA hairpin linker.³³ Using the Kaiser oxime resin and Boc chemistries, the eight-ring polyamide targeting 5'-WGWWCW-3' (W = A or T) was synthesized with coupling cycles that were shortened from 190 minutes or more to 90 minutes in 56% yield after HPLC purification. Additionally, Mosher amide derivatization confirmed that the chiral α -amino acid of the DABA turn did not racemize after several coupling steps. The scope and utility of microwave assisted synthesis of polyamides remains to be rigorously established; however it is clear that the ease of the system coupled with its rapid turn around time will vastly accelerate the pace of polyamide research.

Although the development of solid phase synthesis methods allowed for the rapid synthesis of polyamides for *in vitro* studies as well as pilot animal studies, it does not easily provide the gram quantities of compounds necessary for animal studies. To overcome this limitation, novel solution methods were developed to synthesize an eight-ring hairpin polyamide, ImPyPyPy-(*R*)-ImPyPyPy-(+)-IPA (**9**, Figure 7), to target and antagonize the androgen receptor.³⁴ Using a convergent synthesis, the Dervan lab exploited solubility differences between reactants and products to isolate pure material via precipitation. The core polyamide was synthesized in multi-gram scale without chromatographic purification and featured a C-terminus ready for derivatization with a number of different tail moieties.

5. Genetic Modulation by Pylm Polyamides

Each cell in the human body contains approximately 20,000 genes whose transcription is tightly regulated by transcription factors, proteins that act to either aid or inhibit transcription of the gene of interest. Many disease states, including some hereditary conditions and cancer, are the result of aberrant gene expression. Given their sequence specificity and superior DNA binding affinity, polyamides have been extensively investigated as synthetic transcription factors to either inhibit or promote gene transcription with the goal of ultimately correcting aberrant gene expression and providing therapeutic options for many human diseases.

5.1 Polyamide-mediated Transcriptional Inhibition

An initial collaboration between the Gottesfeld lab and Dervan lab established that polyamides could inhibit transcription. Using an eight-ring polyamide targeted to the recognition site of transcription factor TFIIIA, transcription of the downstream 5S RNA genes was suppressed both *in vitro* and in *Xenopus* kidney cells.³⁵ Subsequent studies, investigated regulation of genes transcribed by RNA polymerase II and found that

polyamides designed to bind to the RNA polymerase II binding site were capable of inhibiting transcription factor binding, while the utility of this approach was established by demonstration that these polyamides prevented HIV-1 transcription in a cell-free assay.^{36–39}

Transcription inhibition has been further demonstrated with an eight-ring hairpin polyamide targeted to the hypoxia response element (HRE) sequence 5'-WTWCGW-3'.⁴⁰ Under hypoxic conditions, hypoxia-inducible factor 1 (HIF-1) forms a heterodimer with aryl hydrocarbon receptor nuclear translocator (ARNT), subsequently binds to a cognate HRE, and recruits additional transcriptional co-activators to induce transcription of hypoxia-inducible genes, including vascular endothelial growth factor (VEGF). VEGF is a mitogenic factor involved in angiogenesis that has been implicated in cancer metastasis. The designed polyamide featured a 3-chlorothiophene ring in the N-terminal position to provide T•A base pair specificity⁴¹ and was conjugated at the C-terminus to a fluorescein dye conjugate for imaging studies (Figure 8). DNase I footprint titrations revealed a K_a value of $6.3 \times 10^9 \text{ M}^{-1}$ for the designed polyamide at the HRE binding site. Furthermore, the polyamide ligand was able to inhibit binding of the HIF-1 /ARNT heterodimer at concentrations as low as 0.25 nM *in vitro*. Cell culture studies revealed that treatment with the hairpin polyamide effectively inhibited luciferase activity when under the control of the VEGF promoter. Gene expression analysis supported this result, revealing that VEGF mRNA expression in hypoxic cells was reduced by approximately 60% in addition to several other hypoxia-inducible genes.

Expanding on these promising results, Viger and Dervan synthesized a series of hairpin polyamides featuring six-five fused rings based on the benzimidazole pairing rules (Figure 9).⁴² A novel benzimidazole-chlorothiophene (BiCt) dimer was introduced to mimic the chlorothiophene-pyrrole pair used in the initial HRE studies. With the exception of an all-fused ring hairpin **16**, binding affinities were found to be comparable to the parent five-ring polyamides with K_a values ranging from $5.9 \times 10^8 \text{ M}^{-1}$ to $2.3 \times 10^{10} \text{ M}^{-1}$. As expected, the HzPy pair in polyamide **12** showed a 10-fold increase in sequence specificity for the 5'-ATACGT-3' match site versus a 5'-ATTCGT-3' mismatch site. Interestingly, when hydroxybenzimidazole is paired against benzimidazole, a pyrrole mimic, specificity is a modest 3- to 5-fold higher than the parent Py/Py pair. These studies highlight the utility of fused benzimidazole rings for increasing sequence specificity while maintaining suitable DNA binding affinity.

Fukuda and colleagues recently reported another example of transcription inhibition by targeting transforming growth factor- β 1 (TGF- β 1). TGF- β 1 is a multifunctional regulatory cytokine involved in cell growth, proliferation, differentiation, and apoptosis with implications in renal diseases including glomerulosclerosis and nephritis.⁴³ In this study, a ten-ring hairpin polyamide was designed and synthesized to target an AP-1 transcription factor binding site in the TGF- β 1 promoter region that showed homology between rats and humans.⁴⁴ Using gel mobility shift and surface plasmon resonance (SPR) assays, polyamide binding was shown to have a dissociation constant (K_d) of $1.43 \times 10^{-9} \text{ M}$. Treatment of HEK-293 cells with the ligand showed a marked and concentration-dependent decrease in TGF- β 1 promoter driven luciferase activity. *In vivo* experiments revealed that the polyamide was a potent inhibitor of TGF- β 1 and related processes. Fluorescein labeled polyamide was shown to localize to the nuclei of nephrotubuli and glomeruli within 24 hours after administration. Rats fed a high salt diet exhibited increased expression of TGF- β 1, CTGF, collagen type 1 α 1, and fibronectin, leading to increased urinary protein and albumin levels. Treatment with the polyamide was shown to reduce urinary protein and albumin levels as well as mRNA levels of the associated proteins, suggesting that polyamide treatment could modulate renal damage by inhibiting TGF- β 1 and its downstream functions. Later studies showed that 3% of transcripts were affected by polyamide treatment and that most of these

transcripts were renal-injury associated factors, indicating that polyamides may present attractive gene-silencing therapeutics for advanced kidney disease.⁴⁵

This group applied the same polyamide and polyamide-fluorescein conjugate to evaluate the broad utility of polyamides for treatment of TGF- β 1-controlled injury response. In 2009, the role of TGF- β 1 in restenosis of intracoronary stents was investigated.⁴⁶ Restenosis is caused most frequently by TGF- β 1 mediated intimal vascular smooth muscle cell (VSMC) hyperplasia and deposition of extracellular matrix at the stent site. To mimic the effects of stent placement, male rats were treated to create a distending and de-endothelializing injury of the carotid artery. Fluorescence imaging revealed that the polyamide localized to the injured arterial wall within 30 minutes post-injury and that strong nuclear localization occurred within 24 hours after administration. mRNA levels of connective tissue growth factor (CTGF), fibronectin, collagen type 1, and LOX-1, which were significantly increased in injured arteries, were reduced by a mere 10 minute incubation with the polyamide. Inspection of arterial cross-sections 21 days post-injury showed significantly reduced restenosis in polyamide-treated arteries in a concentration-dependent manner. Similar results were seen when the polyamide was applied to a model of corneal alkali burns.⁴⁷ After ocular chemical burns, TGF- β 1 promotes migration of corneal epithelial cells and keratocytes. Overexpression of TGF- β 1 post-injury exacerbates damage to the injured cornea and leads to scarring, hazy corneas, and impaired vision. Corneas treated with polyamide post-injury healed within seven days and showed stratified epithelium and undetectable or minimal scarring, and clear corneas. Taken together, these studies indicate that polyamides may become a useful tool to prevent TGF- β 1-associated complications after injury or treatment.

Recently, polyamides have entered preclinical investigation as antiviral compounds. Sugiyama and coworkers designed Py-Im hairpin polyamides to target Epstein-Barr virus (EBV) via the highly conserved dyad symmetry (DS) element of the viral genome, which, upon binding with viral protein EBNA1, initiates recruitment of viral DNA replication components.⁴⁸ DSE-3 (Im ImPy-(R) -ImPyPyPy -Dp) showed binding to all four DS match sites, despite one or two base-mismatch sequences in sites 1, 2, and 4. Treatment of latently EBV-infected cells with DSE-3 showed an anti-proliferative effect in EBV episome-positive cell lines, indicating selective targeting to the EBNA1 binding site. Furthermore, five-day treatment with DSE-3 reduced EBV genome copy numbers whereas similar treatment with hydroxyurea, a ribonucleotide reductase inhibitor used to suppress DNA replication, did not. Chromatin immunoprecipitation (ChIP) assays revealed that DSE-3 was indeed acting by inhibiting EBNA1 binding to DNA, thereby destabilizing the EBV episome.

Similar episome reduction levels were observed for a hairpin polyamide designed to target AT-rich regions of the human papillomavirus (HPV) genome.⁴⁹ The designed polyamide (17, Figure 10), showed an apparent IC₅₀ of 0.036 μ M against HPV16, a common strain of HPV responsible for most cervical cancers, whereas distamycin A had an apparent IC₅₀ of 33 μ M. Although distamycin A reduced HPV viral DNA copies, its antiviral activity largely tracked with cellular toxicity while the polyamide showed no appreciable cellular toxicity up to 200 μ M, giving it a selectivity index greater than 1000-fold superior to distamycin. Histological examination of epithelial tissues infected with HPV and tested with the designed polyamide confirmed that polyamide treatment caused a dose-dependent loss of HPV episomal DNA via suppressed viral DNA transcription. Taken together, these latter antiviral studies build upon the seminal anti-HIV work of the Dervan lab^{36-38, 50-52} and indicate the emerging role of polyamides as methods for studying the role of particular DNA binding elements in viral replication and highlight a novel method for targeted antiviral therapeutics.

5.2 Gene Silencing via Site-Specific DNA Alkylation

One of the initial observations made by Gottesfeld and Dervan was that hairpin polyamides were unable to inhibit gene expression when bound in the coding regions of DNA.⁵³ Once RNA polymerase started transcription, it was essentially a “freight train” that could not be de-railed by a non-covalently bound low molecular weight ligand in its way. In an effort to utilize the more specific coding sequences for precise transcriptional inhibition, the Dervan lab sought to use the sequence specificity of the polyamides to deliver molecules capable of covalently modifying DNA. Creation of bifunctional ligands via conjugation of Py-Im hairpin polyamides to DNA alkylating agents has paved the way for development of site-specific alkylating agents capable of selectively silencing genes. Using either the nitrogen mustard chlorambucil (CHL)⁵³ or the natural product CC-1065,⁵⁴ these alkylating agents were originally tethered to polyamides via coupling to the α -amine of the α -aminobutyric acid turn unit (Figure 11). Both agents showed sequence-specific alkylation of the N3 of adenine in the minor groove when conjugated to polyamides (Figure 12). Once alkylated, DNA repair machinery leads to base excision and strand breakage, thereby terminating gene elongation and effectively performing genetic surgery.¹³ Sugiyama later developed a polyamide-clopropylpyrroloindole (CPI) conjugate based on the alkylating moiety of duocarmycin A, which also targets minor groove adenines adjacent to the polyamide binding site.⁵⁵ Subsequent studies have investigated linker length, composition, and location to determine optimal conjugate composition for alkylation. These discoveries have paved the way for a host of polyamide-alkylator conjugates targeting numerous medically interesting genes.

In another example of sequence specific mediated gene control, Sugiyama and coworkers hypothesized that bifunctional polyamides could be used to alkylate the double stranded region of human telomere repeats and abrogate telomerase activity. Human chromosomes terminate in telomeres, long sequences of double stranded tandem repeats of 5'-TTAGGG-3', which are essential for chromosomal stability.⁵⁶ Telomeres typically lose approximately 100 bp with each cell division. However, in immortal cell lines and up to 85% of cancers, telomerase activity is upregulated to prevent telomere shortening and ultimately cell death; therefore, targeting aberrant telomerase activity is an attractive chemotherapeutic option for cancer treatment.^{57, 58} The first generation telomere-targeting polyamide, PyImImIm- α -PyPyPyLDu86, bound and alkylated the sequence 5'-CCCTAA-3' (A represents the alkylated adenine), while⁵⁹ control experiments using single stranded DNA showed no alkylation, indicating that the designed polyamide is, as expected, specific for the double stranded telomere repeats. In tests against human cancer cell lines the conjugate showed differential growth inhibition profiles having the greatest activity against LOX-IMVI (melanoma), DMS114 (lung cancer), HCT-116, and HCC2998 (both colon cancer).

To overcome difficulties associated with the synthesis and alkylating efficiencies of earlier conjugates using CPI and a vinyl linker, use of *seco*-1-chloromethyl-5-hydroxy-1,2-dihydro-3H-benz[e]indole (CBI) and an indole linker were investigated (Figure 13).⁶⁰ When tested against the same cancer cell lines, the *seco*-CBI conjugates displayed 50–100-fold increased antitumor activity than for the previously described CPI conjugates. Although these derivatives showed improved alkylating efficiency, they did show some propensity for binding and alkylation of mismatch sequences. Sequence specificity was improved by use of a heterotrimeric mixture of Py-Im polyamides or polyamides and distamycin A (Figure 14).⁶¹ A linear conjugate, specifically bound and alkylated sequence 5'-GGTTAGGGTTA-3' when combined with distamycin A. Although the linear conjugate showed nanomolar toxicity against Jurkat cells, the proof of concept for using heterotrimeric systems to increase sequence recognition length and specificity in the context of DNA

alkylation remains intriguing and underexplored. An extension of this system was recently reported by tethering chlorambucil to either the C- or N-terminus of a linear polyamide conjugate. Using distamycin A as the small binding partner,⁶² it was found that while sequence specificity was better using *seco*-CBI, chlorambucil provided synthetic ease and the ability to tether alkylating agent to either the C- or the N-terminus, thereby targeting both strands of the DNA for alkylation; C-terminal linked CHL alkylated the polyamide bound strand, while N-terminal linked CHL alkylated the opposite strand.

Another of the earliest conceived applications of a polyamide-alkylator conjugate was the silencing of oncogenes to treat cancer. A collaboration between the Gottesfeld and Dervan labs led to the generation of a small library of pyrrole-imidazole polyamide-CHL conjugates that were screened for activity against a human colon carcinoma cell line. One conjugate, 1R-Chl was found to alter cell morphology and cause cell cycle arrest at the G2/M stage. 1R-Chl was also active in a murine model of cancer. Microarray analysis revealed that the histone *H4c* gene was down-regulated two-fold, leading to a 50–70% reduction in histone H4 protein, but few other genes were affected.

Subsequent studies revealed that 1R-Chl binds and alkylates several members of the H4 gene family leading to depletion of histone protein and loss of nucleosomes.^{63–65} Nucleosome depletion causes global alkylation within the genomic DNA, leading to cell cycle arrest. In cell culture studies, 1R-Chl inhibited proliferation of chronic myelogenous leukemia (CML) cell line K562 at nanomolar concentrations. The therapeutic utility of 1R-Chl was explored more fully by testing its efficacy, both alone and in combination with a common Abl kinase inhibitor, imatinib, against BCR-ABL positive bone marrow cells.⁶⁶ 1R-Chl proved effective at blocking proliferation of BCR-ABL positive cells and displayed no toxicity toward non-cycling cells. It worked additively with imatinib, indicating that a combination therapy could one day prove effective. Most interestingly, 1R-Chl displayed excellent dose tolerance in a murine model, displaying liver toxicity only at levels near ten times the therapeutic dosage.

A recent study by Sugiyama and coworkers described a photocontrollable polyamide-*seco*-CBI conjugate targeted to the sequence 5'-WGWCCA-3' (W = A or T).⁶⁷ The photocaged alkylating agent was designed to prevent the alkylating activity of *seco*-CBI via protection of the naphthalene hydroxyl group, which could be removed upon exposure to UV irradiation. In a cell-free assay, the photocaged polyamide conjugate was able to selectively alkylate DNA in the presence of UV irradiation (365 nm) for 5 minutes. Cellular studies with human lung cancer cell line A549 revealed that treatment with the photocaged conjugate showed no cytotoxicity or morphological changes until photocleavage by UV irradiation, which proved toxic. These early findings could pave the way for increased spatiotemporal control of gene regulation via polyamide-alkylator conjugates.

5.3 Polyamide-mediated Transcriptional Activation

Polyamides have also been investigated for their potential to serve as scaffolds for the construction of artificial transcription factors (TFs). TFs are typically modular proteins that contain a DNA binding region to direct binding to specific regions of the genome, as well as dimerization and activation regions to drive transcription. Therefore, one of the first questions addressed in this field was whether the DNA binding region of a TF could be replaced by a hairpin polyamide. To answer this question, Mapp *et al.* designed and characterized the first of these synthetic activators by tethering a hairpin polyamide to a peptide dimerization element and an acidic helical peptide activation domain (Figure 15).⁶⁸ A glutamate-rich acidic helical (AH) activation domain and coiled-coil dimerization element from yeast factor GCN4 were coupled to a Py-Im hairpin polyamide targeting the sequence 5'-TGTTAT-3' with low nanomolar affinity ($K_D = 1.1$ nM). Binding of the designed

activator to template DNA with three palindromic binding sites led to a 13-fold increase in transcription in yeast nuclear extracts. To test the necessity for each of the three components (polyamide, dimerization domain, and AH domain), conjugates bearing a non-functional element of each component were prepared and evaluated for transcription activation. Constructs without the AH domain or bearing a mismatch polyamide showed no enhanced transcriptional activation above basal levels. However, replacement of the dimerization element with a flexible straight-chain linker projecting the AH away from DNA was still able to activate transcription at approximately 50% efficacy as compared to the parent synthetic activator.

The use of tethered polyamide-peptide conjugates as synthetic transcriptional activators was further evaluated using a minimal activation sequence derived from VP16, a potent viral activator.⁶⁹ To determine the minimal activation sequence, the 20-residue AH activation domain was replaced with derivatives of the VP16 domain made of either eight (VP1) or 16 (VP2) residues. A flexible polyether linker was employed to link the activation domain to the polyamide. This linker was varied both in length and placement to create an optimal conjugate with minimal molecular weight and potent activity. Relative activation activity of the conjugates was determined based on *in vitro* transcription from a DNA template with three palindromic binding sites upstream from the adenovirus major late TATA box. The polyamide-VP2 conjugate showed a 20-fold increase in transcription levels over basal levels. Conjugation of the activation domain via a 36-atom linker tethered to the *N*-methyl position of an internal pyrrole showed a 60% increase in activation over the C-terminal linked isomer. Further insight into the optimal linker spacing between the polyamide and peptide activator was achieved by replacing the polyether linker with a rigid oligoproline linker and studying the effect on transcription activation as a function of linker length.⁷⁰ Conjugates featuring linkers made of 6, 9, 12, and 15 poly-L-proline residues separated the polyamide from the activation domain by 18, 27, 36, and 45 Å, respectively, were examined. Optimal activation was achieved by conjugates with linker lengths between 36 and 45 Å.

The cellular activity of these early synthetic transcriptional activators was limited by their permeability in living cells.⁷¹ The Kodadek lab successfully developed a cell-permeable synthetic transcriptional activator linking a hairpin polyamide to a hexameric peptoid shown to bind to KIX, a mammalian transcriptional coactivator, via a C-terminal poly-ether linker. To measure transcriptional activation in living cells, HeLa cells were transiently transfected with a plasmid-borne luciferase reporter gene under the control of a promoter with six polyamide binding sites. Extracellular administration of the polyamide-peptoid conjugate to the HeLa cells showed a dose-dependent increase in luciferase expression with a maximum five-fold increase over basal levels observed. Control experiments indicated that compounds lacking the DNA-binding hairpin polyamide or the peptoid AD surrogate were incapable of activating luciferase expression. Additionally, reporter plasmids with only two polyamide binding sites showed no gene activation, indicating that multiple peptoid-polyamide conjugates are required for gene activation. Whole genome analysis identified 45 genes upregulated at least three-fold by treatment with the polyamide conjugate. Each gene contained multiple polyamide binding sites in the promoter region, indicating that the designed transcriptional activator was potentially capable of regulating endogenous gene function in living cells.

Recently, Sugiyama and coworkers took a novel approach to transcriptional activation by Py-Im polyamide hairpin conjugates.⁷² Rather than tether the DNA-binding polyamide to a known activator domain, a suberoylanilide hydroxamic acid (SAHA) moiety, known to act as a histone deacetylase (HDAC) inhibitor with broad epigenetic activity was used. The goal of this study was to induce transcription of pluripotency genes required to convert somatic fibroblasts to stem cells. Sixteen hairpin polyamides targeted to specific DNA sequences

were synthesized and coupled to the SAHA moiety via a double α -alanine linker on the N-terminus. Screening for the ability to induce endogenous expression of known reprogramming factors (*Oct-3/4*, *Nanog*, *Sox2*, *Klf4*, and *c-Myc*) without inducing overt cellular toxicity led to identification of one polyamide, **20** (Figure 16), which showed no cytotoxicity even at concentrations where free SAHA reduced cellular viability. ChIP assays against the *Oct-3/4* promoter revealed that cells treated with the polyamide-SAHA conjugate showed a five- to six-fold increase in acetylation of H4K and H3K9 and a six- to seven-fold enrichment of acetylation at H3K14. Analysis of trimethylation levels at histone H3 Lys4 (H3K4), a marker associated with active chromatin, showed a four- to five-fold increase. Similar results were seen for the *Nanog* promoter. In a subsequent study, the group showed that a second-generation polyamide-SAHA conjugate at nanomolar concentrations could induce multiple pluripotency genes, including *Rex1* and *Cdh1*, by tenfold in only 24 hours.⁷³ Furthermore, cells treated with the polyamide-SAHA conjugate were able to overcome epithelial transition, a rate-limiting step in transition to pluripotency, by inducing a complex transcriptional network.

6. Polyamides as Molecular Probes

Since their discovery, polyamides have been identified as potentially useful molecular probes. A large number of polyamide-fluorophore conjugates have been characterized for studies ranging from verification of polyamide binding models to determining the cellular localization of specific DNA sequences.⁷⁴ The Dervan lab first published results showing that conjugation of Py-Im polyamides to tetramethylrhodamine (TMR) (**21**) via an internal pyrrole ring could produce a specific fluorescence signal in the presence of bound dsDNA (Figure 17). Py-Im-TMR conjugates were largely quenched in solution; however, upon binding to designed match sites the conjugates gave a greater than 10-fold increase in fluorescence, whereas titration of the conjugate with a mismatch DNA template showed attenuated signal.⁷⁵ This type of fluorescence detection of specific dsDNA sequences was subsequently expanded to include fluorescent intercalators.⁷⁶ Utilizing thiazole orange (TO) cyanine dye conjugated to the C-terminus of a hairpin polyamide (**22**), greater than 1000-fold enhancement of fluorescence was observed upon binding to the cognate DNA sequence with only a small enhancement of fluorescence in the presence of mismatch DNA. Furthermore, the TO-polyamide conjugates were able to disrupt the DNA duplex with an unwinding angle, θ , of approximately 8° . Use of 6-5 fused dimer modules (**23**) generated sequence-specific dsDNA binding molecules showing a marked inherent fluorescence excitation at 340 nm upon binding to cognate DNA sequences.⁷⁷ Lee and coworkers expanded the repertoire of inherently fluorescent polyamides via incorporation of a *p*-anisylbenzimidazolecarboxamido (Hx) derivative of Hoechst 33258.⁷⁸ Targeting A/T base pairs, the Hx moiety strongly emits blue light after excitation at 322 nm and allows the direct measurement of polyamide binding both *in vitro* and *in vivo*.

Several studies have used polyamide-dye conjugates to study specific chromosomal loci and events in both *Drosophila* and mammalian cells.^{79–82} Laemmli and colleagues designed polyamides targeted to AT-rich satellites I, III, and scaffold associated regions (SARs) and the purine-rich GAGAA satellite V in *Drosophila melanogaster* polytene chromosomes.⁸⁰ Fluorescein-conjugated polyamides were used to confirm nuclear and chromosomal uptake of the polyamides. Poly-pyrroles designed to bind the extended AT-rich satellite sequences stained both the AT tracts in SARs and the AT-rich satellites I and III. Im ImPy Im Im Dp was synthesized to bind the purine rich satellite V, representing approximately 6% of the *Drosophila* genome. In *Drosophila* Kc cell nuclei, both polyamides were able to stain their appropriate cognate sequences without overlapping. Additionally, the oligopyrrole compounds were found to induce opening of chromatin domains, as seen by an enhanced sensitivity to restriction enzymes and DNA topoisomerase II.⁷⁹

Trask and coworkers have used polyamide-fluorescein conjugates to differentiate chromosomes via microscopy and flow cytometry that were traditionally difficult to resolve using standard Hoechst and chromomycin dyes.⁸¹ Polyamide-dye conjugates targeted to the sequence 5'-TCCA-3', which is highly redundant in heterochromatic regions of chromosomes 1, 9, and Y, stained the same chromosomal regions as a fluorescein-tagged oligonucleotide ((TTCCA)₄). One major advantage of the polyamides was the ability to stain the chromosomes without denaturing the double helix. These studies highlight the potential for polyamide-dye conjugates as diagnostic tools in pre-natal screenings and cancer diagnosis.

Polyamide-fluorophore conjugates have also been reported as a potential diagnostic tool for triplet repeat-based diseases.^{83, 84} Generational expansion of trinucleotide CAG repeats past threshold levels results in the development of hereditary disorders including Huntington's disease, spinobulbar muscular atrophy, and various ataxias.⁸⁵ Conjugation of pyrene moieties to two pyrrole residues via a butyl spacer created a conjugate capable of recognizing a CAG repeat sequence with an emission profile (475 nm) that directly related to the concentration of cognate sequence. In an effort to design a probe with a longer excitation wavelength and more stable emission profile, the group modified the linker from the flexible butyl chain to a more rigid alkyne. The excitation wavelength of these molecules shifted to the red with the lead conjugate having an excitation wavelength centered around 495 nm and an emission intensity that remained at over 85% after four hours.

7. Cellular and Nuclear accessibility

In solution, Py-Im polyamides display robust DNA binding activities. However, their effectiveness in cell culture and animal studies has been hampered by variable cellular and nuclear uptake. Several studies have determined that efficient nuclear localization is dependent on cell type, polyamide structure, identity of conjugated fluorophores, and imidazole content.⁸⁶⁻⁹¹ Polyamide-BODIPY conjugates were shown to efficiently localize to the nuclei of live cell types that had shown robust responses to polyamides, including primary human T-cells.⁹¹ However, in other cell lines the conjugates were excluded from the nucleus and sequestered in lysosomes and other cytoplasmic vesicles. The extent of nuclear localization varied with polyamide structure, suggesting that redesign and use of different dyes may affect cellular distribution.

To address this issue, Dervan and colleagues synthesized a series of fluorescein-labeled polyamides and tested their nuclear localization against a panel of live mammalian cells, including 11 human cancers, human transformed kidney cells, and a murine leukemia cell line.⁹⁰ Based on these studies, some basic localization determinants were derived. Generally, nuclear localizing polyamides are composed of an eight-ring DNA binding domain with one or more positive charges on either the linker or the turn residue when conjugated to fluorescein. An expansion of this initial study was reported a year later,⁸⁸ in which over 100 polyamide-fluorophore conjugates were assayed for nuclear uptake. The conjugates were chosen to systematically deduce the localization effects of several structural variables, including molecular weight, imidazole content, ordering of rings and aliphatic building blocks, charge, and dye composition and placement. While molecular weight and imidazole content do not appear to affect nuclear uptake, the positions of imidazole residues have significant effects on nuclear access, though no general trends for imidazole placement was deduced. Generally, a β -alanine within the tail and lack of a cationic alkyl amine negatively affect nuclear uptake, while an acetylated 2,4-diaminobutyric acid turn unit enhanced nuclear uptake.

Kodadek and coworkers employed a cell based reporter assay to determine the effects of linker size and composition on cellular permeability of polyamide-dexamethasone conjugates acting as transcriptional activators.⁸⁷ The reporter assay utilizes dexamethasone (dex) conjugates of polyamides incubated with cells expressing a fusion protein containing yeast Gal4 DNA-binding domain (Gal4DBD), human glucocorticoid receptor ligand-binding domain (GRLBD), and VP16, the C-terminal activation domain of herpes simplex virus (HSV) transcriptional activator VP16. In its apo form, the fusion protein remains sequestered in the cytoplasm via interaction with heat shock protein 90 (Hsp90). Upon interaction with the dex conjugate, a GRLBD-dex interaction causes release of the fusion from Hsp90 and concomitant translocation of the entire complex to the nucleus where it activates transcription of a Gal4-controlled luciferase (Luc) reporter gene (Figure 18). The luciferase activity readouts are dose dependent and can indicate the relative permeability of the test polyamide in relation to a standard polyamide. These studies confirmed earlier findings by Dervan that the α -alanine-3,3'-diamino-*N*-methyldipropylamine linker negatively affected cellular permeability and that removal of the α -alanine improved uptake and the corresponding luciferase signal. Furthermore, use of a shorter ethylenediamine (Et) linker gave increased permeability profiles, even among large (10-ring) polyamides with poor uptake.

In the same issue, Jacobs and Dervan reported synthesis of a focused polyamide library using four core polyamide sequences and fifteen C-terminal tail modifications to evaluate effects on cellular permeability using quantitative real-time PCR (RT-PCR).⁹² Use of a C3 aliphatic linker tethered to an aromatic group through an oxime linkage gave enhanced polyamide potency. After exploring the structure of the C-terminal tail, focus turned toward derivatization of the α -turn unit as a method for increasing cellular and nuclear uptake (Figure 19).⁹³ Addition of aryl groups at the α -aminobutyric acid turn unit improved the biological effects of a hairpin polyamide targeting 5'-WGWWCW-3' (W = A or T) by up to two orders of magnitude. Analysis by confocal microscopy and quantitative flow cytometry showed increased nuclear uptake may cause the enhanced potency. Incorporation of the aryl-turn modification into several other hairpin polyamides revealed globally enhanced uptake, though the upper limit of nuclear accumulation remained variable.

More recent studies have focused on improving polyamide solubility in aqueous solution, which can be a major barrier to animal studies. The Lee and Wilson laboratories have approached this problem via addition of amine or ammonium groups to pyrrole residues within the polyamide structure.^{94, 95} Addition of an orthogonal alkylammonium side chain in the backbone was found to maintain DNA sequence selectivity while exhibiting enhanced water solubility.⁹⁴ Sugiyama and coworkers PEGylated the C-terminus of hairpin polyamide-*seco*-CBI conjugates targeted to an eight-bp DNA sequence, 5'-WGGCCA-3'.⁹⁶ They found that the conjugates exhibited high cytotoxicity against A549 and DU145, two human cancer cell lines, while also improving water solubility over the parent polyamide. Rather than alter the structure of the polyamides, the Dervan lab increased aqueous solubility via addition of 2-hydroxypropyl- α -cyclodextrin (Hp CD), a carbohydrate solubilizing agent.⁹⁷ Use of Hp CD-solubilized polyamides in mice allowed for improved injection conditions and improved cellular uptake of polyamides, likely due to decreased aggregate formation in plasma.

Conclusion

Pyrrole-imidazole polyamides represent a powerful class of programmable small molecules that can be designed to target specific predetermined DNA sequences. In the nearly three decades since their discovery, the utility and versatility of polyamides have been extensively demonstrated by their ability to act as transcriptional activators or repressors, to modulate

protein-DNA interactions, to act as sequence-specific gene silencers, and to function as molecular probes. Future studies will undoubtedly focus on the application of polyamides to potentially solve many of the unmet needs in human medicine.

Acknowledgments

The authors would like to acknowledge the NIH (RO1GM055769) and NCTracs for their generous support.

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Figure 1. DNA minor-groove binding small molecules. Netropsin (**1**) and distamycin A (**2**) are shown with diagrammatic representations of molecular interactions involved in DNA binding.

Figure 2. Pairing rules derived from 2:1 polyamide:DNA complexes. Potential hydrogen bonds between the polyamides and the minor groove of DNA are represented with dotted lines.

Figure 3.
Hairpin polyamide

Figure 4. Molecular Basis for A/T Discrimination by Hp. Steric occlusion prevents interaction of the Hp hydroxyl with the asymmetric cleft between thymine O2 and adenine C2 (left), whereas an additional hydrogen bond with the O2 of thymine stabilizes the interaction (right).

Figure 5. Comparison of pyrrole-imidazole dimers to their corresponding six-five fused benzimidazole derivatives.

Figure 6. Polyamide Tail Derivatives. a) polyamide with the -Dp tail afforded by aminolytic cleavage from -alanine-PAM resin; b) polyamide with various tails accessed via nucleophilic cleavage from the Kaiser oxime resin.

Bioorg Med Chem. Author manuscript; available in PMC 2014 October 15.

Figure 8.
Fluorescein-polyamide conjugate targeting VEGF

Figure 9.
Six-five Fused Ring Polyamides to target VEGF.

Figure 10.
Polyamide designed to target HPV.

Figure 11.
Representative Polyamide-alkylator Conjugates. The alkylating moiety is boxed.

Figure 12.
DNA-alkylating mechanism of Polyamide-chlorambucil conjugates.

Figure 13.
Variations in alkylator and linker groups to target telomere repeats

Figure 14.
Heterotrimeric polyamides to target telomere repeats.

Figure 15.
Polyamide-based synthetic transcriptional activators

Figure 16.
Polyamide-SAHA conjugate 18.

Figure 17.
Polyamide-based fluorophores.

Figure 18.
Cell-based assay to probe cellular and nuclear uptake of polyamides.

Figure 19.

Aryl turns imparting improved biological function to hairpin polyamides.

Table 1

Pairing Rules for Pyrrole-Imidazole Polyamides

Pair	G•C	C•G	T•A	A•T
Im/Py	+	–	–	–
Py/Im	–	+	–	–
Hp/Py	–	–	+	–
Py/Hp	–	–	–	+

Table 2

Pairing rules for benzimidazole ring derivatives

Pair	G•C	C•G	T•A	A•T
Ip/Py	+	–	–	–
Py/Ip	–	+	–	–
Hz/Py	–	–	+	–
Py/Hz	–	–	–	+
Bi/Py	–	–	+	+
Py/Bi	–	–	+	+