

Genomic Effects of Polyamide/DNA Interactions on mRNA Expression

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Summary

Here we characterize the biological activity of a hairpin polyamide **1** that inhibits binding of the minor-groove transcription factor LEF-1, constitutively expressed in colon cancers. Genome-wide analysis of mRNA expression in DLD1 colon cancer cells treated with **1** reveals that a limited number of genes are affected; the most significant changes correspond to genes related to cell cycle, signaling, and proteolysis rather than the anticipated WNT signaling pathway. Treated cells display increased doubling time and hypersensitivity to DNA damage that most likely results from downregulation of DNA-damage checkpoint genes, including YWAE (14-3-3 ϵ protein) and DDIT3. Promoter analyses on a genomic level revealed numerous potential polyamide binding sites and multiple possible mechanisms for transcriptional antagonism, underscoring the utility of gene expression profiling in understanding the effects of polyamides on transcription at the cellular level.

Introduction

Chemical approaches for selectively regulating protein expression have emerged as powerful methods for elucidating gene function [1–7]. One approach involves the use of polyamides that selectively bind the minor-groove of dsDNA to downregulate gene expression by blocking transcription factor binding [7]. Examples of chemical approaches for modulating gene expression include antisense oligonucleotides [1] and peptide nucleic acids [2, 3] as well as dsRNA interference and siRNA approaches [4–6]. The ability to directly target transcription requires molecules that either bind and block a transcription factor or selectively compete for their DNA targets in the genome. Minor-groove binding pyrrole-imidazole polyamides, derived from the natural products

distamycin and netropsin [7, 9], are small molecules that can bind DNA in a sequence-selective manner [7]. Polyamide hairpins are designed based on pairing rules; pyrroles opposite pyrroles in the hairpin bind to either AT or TA base pairs, whereas imidazole opposite pyrrole binds specifically to GC base pairs [7]. Such compounds have been shown to effectively compete with transcription factors for binding to specific DNA sites in a number of studies. Examples include interfering with transcription factor TFIIIA binding and altering expression of 5S RNA in *Xenopus* kidney cells [10]; targeting the Ets binding site of the HER2/*neu* promoter in vitro [11]; interfering with the transcription of genes controlled by RNA polymerase II; and antagonizing enhancer/promoter elements of HIV-1 that include Ets-1, LEF-1, and TBP binding sites in vitro and in vivo [12]. However, the selectivity of these DNA ligands on a genomic level has not been established, and this is a key issue when one considers them as tools for cell biology and ultimately as therapeutics.

Results and Discussion

To begin to explore the specificity of polyamides on a cellular level, we designed compound **1** to target the lymphoid enhancer factor (LEF)/T cell factor (TCF) binding sites of the human cyclin D1 (CD1) promoter ([13]; Figure 1). LEF-1 makes substantial contacts in the minor groove of its DNA binding sites [14] and has been successfully targeted by polyamides in previous studies [12]. In complex with β -catenin, LEF-1 participates in the WNT signaling pathway, whose aberrant activation is thought to be a cause of colorectal cancer [8]. The ability of **1** to target the LEF-1 site was evaluated by surface plasmon resonance, electrophoretic gel mobility shift assays, and luciferase reporter assays. Biotinylated hairpin DNA sequences were linked to streptavidin-coated SPR chips [15] corresponding to the binding sequence for polyamide **1**, and a dissociation constant (K_d) of 150 nM was determined from the response curves. The reported K_d for LEF-1 is in the range of 1–5 nM [13], indicating that **1** should be able to competitively bind common target sequences if it is in molar excess. To test whether **1** can effectively inhibit LEF-1 binding to its target DNA sequence, we performed electrophoretic mobility shift assays (EMSA) (Figure 2). Consistent with the relative magnitudes of the K_d values, we found that a ratio of polyamide **1**:LEF-1 of roughly 500:1 is required for greater than 90% inhibition of LEF-1 binding. We tested the effects of polyamide **1** on transcription in cell culture by transfecting Hela cells with a luciferase reporter plasmid driven by 961 bp of the CD1 promoter [16]. Compound **1** decreased the activation of a CD1 promoter-luciferase construct induced by β -catenin in Hela cells [16] by as much as 80% at concentrations ≥ 50 μ M. Another hairpin polyamide (ImPyPyPyPy- γ -PyPylmPyPy- β -Dp) with the same composition of pyrroles and imidazoles as **1** but different sequence selectivity did not show activity in the reporter assay.

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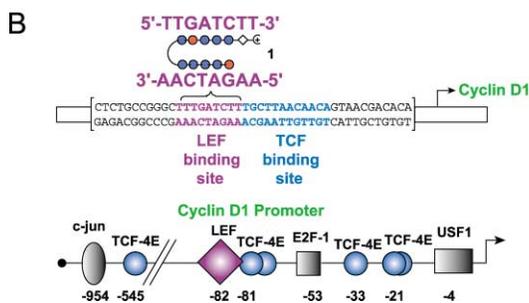
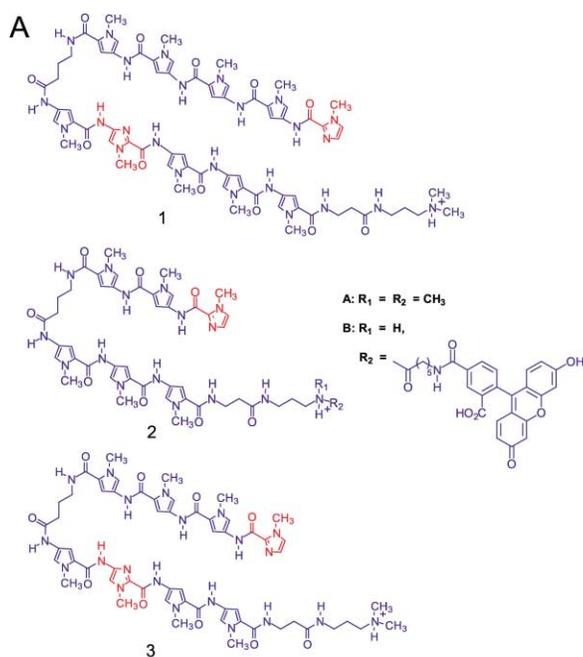


Figure 1. Structures of Polyamides Studied and Interactions with an LEF-1 Binding Site within the Promoter of the Cyclin D1 Gene (A) Structures of polyamides ImPyPyPyPy- γ -PylmPyPyPy- β -Dp (1), ImPyPy- γ -PyPyPy- β -Dp (2A) and the corresponding fluorescein conjugate (2B), and ImPyPyPy- γ -PylmPyPy- β -Dp (3). (B) Expanded cyclin D1 promoter region showing the target sequences and binding models for polyamides, with pyrrole rings represented by blue circles, imidazole rings by red circles, γ -butyric acid linkers by curved lines (hairpins), β -alanine by diamonds, and N,N-dimethyl-1,3-propyldiamine by a half circle containing a plus sign.

To analyze the transcriptional effects of polyamide 1 on a genome-wide scale, we chose the DLD1 colon cancer cell line for study because it constitutively ex-

presses high levels of β -catenin/LEF-1 [8]. Because issues surrounding the ability of polyamide compounds to enter the nuclei of cells have been raised [7], the uptake of polyamides by DLD1 cells was tested. A model polyamide (2B) labeled with a fluorescein tag was prepared, and DLD1 cells were treated with this compound under a variety of concentrations and conditions. Fluorescence microscopy showed that effective uptake occurred at 100–150 μ M concentrations (Figure 3). Polyamide 2B was taken up into the cytosol and, more importantly, the nuclei of DLD1 cells, which suggests that unmodified polyamides should have access to the nucleus.

The differences in gene expression in DLD1 cells treated with polyamide 1 were examined 16 and 48 hr after treatment and over a concentration range of 30–120 μ M ligand 1 via Affymetrix U95A high-density oligonucleotide arrays containing probes for more than 11,000 genes (Table 1). Gene expression analysis reveals both downregulated and upregulated genes. In general, we observe related time and concentration dependencies for a subset of downregulated genes, suggesting that primary events involving transcriptional antagonism control their differential regulation. The majority of the upregulated genes appear later in time or at the highest concentrations of polyamide, suggesting that their induction is downstream of the primary transcriptional disruption.

Interestingly, the expression levels of the target gene CD1 do not appear to be affected. Rather, mRNA profiling data suggest that other genes within the transcriptome are more susceptible to polyamide 1. Genes displaying 3- to 6-fold downregulation include signal transduction genes such as tyrosine/tryptophan 3-monooxygenase activation protein, epsilon polypeptide (YWHAE or 14-3-3 ϵ protein), and a protein kinase C analog. Hydrolases, including epoxide hydrolase 1 and neuropathy target esterase, a gene implicated in neurodegeneration, are also downregulated upon treatment with 1. Interestingly, cell stress- and DNA damage-related genes such as DNA damage-inducible transcript 3 (DDIT3, also known as CHOP 10 or GADD153), delta sleep-inducing peptide, and regulatory subunit 15A of protein phosphatase 1 are also downregulated. These genes are members of a group of genes whose transcript levels increase after stressful growth arrest conditions or treatment with DNA-damaging agents; their behavior is opposite to what might be expected from the introduction of DNA binding compounds. Downregulation of calreticulin, a multifunctional protein that acts as a major Ca^{2+} binding protein and that has been implicated in

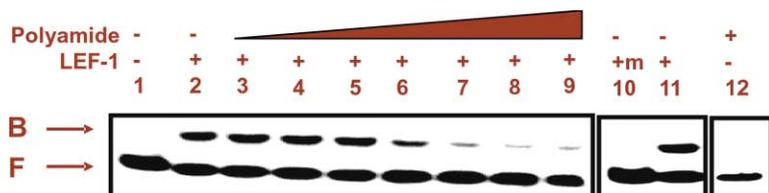


Figure 2. Autoradiogram of a Representative EMSA Showing the Titration of Polyamide 1 into the LEF-1/DNA Complex and the Inhibitory Effect of the Polyamide on LEF-1 Binding Positions corresponding to free probe (F) and bound probe (B) are indicated. The concentration of LEF-1 was constant (50 nM in lanes 2–9), and the concentration of the 43 bp probe was 50 pM. The polyamide concentrations were 50 nM, 100 nM, 500 nM, 1 μ M, 5 μ M, 500 μ M, and 750 μ M in lanes 2–9, respectively. Lanes 10 and 11 contain LEF-1 and ^{32}P -labeled mismatched and matched oligonucleotide sequences of the LEF-1 binding site in the cyclin D1 promoter. Lane 12 contains only polyamide 1 and labeled probe.

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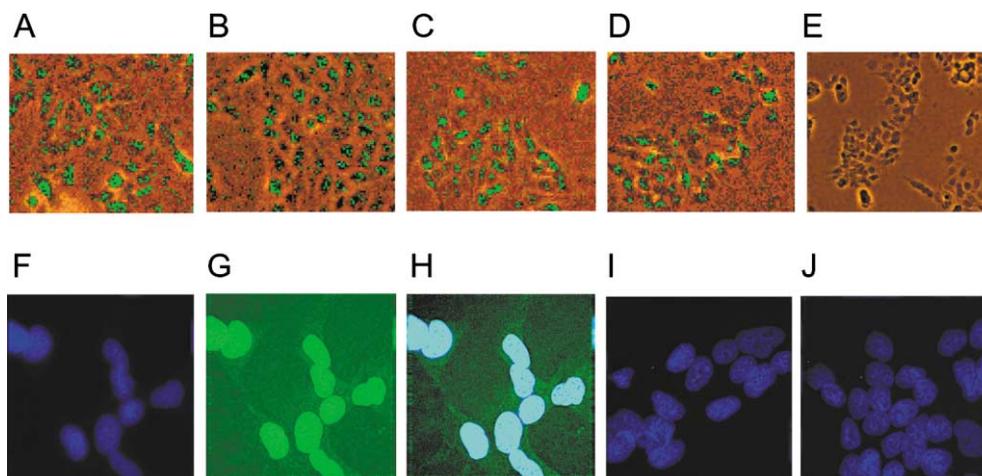


Figure 3. Fluorescence Microscopy Images of DLD1 Cells Treated with Fluorescent Polyamide 2B

The figure shows DLD1 cells incubated with (A) 150 μM 2B for 16 hr; (B) 100 μM 2B for 16 hr; (C) 150 μM 2B for 2 hr; (D) 100 μM 2B for 2 hr; and (E) 150 μM 2A for 16 hr. (F–H) DLD1 cells with nuclear staining (blue) were treated with 150 μM 2B (green). (F) Blue channel. (G) Green channel. (H) Overlay of blue and green channels. (I) Overlay of blue and green channels with 150 μM 2A. (J) Blue and green channels for cells not treated with polyamide.

gene transactivation through diverse mechanisms, is also observed.

The most consistently downregulated gene observed upon treatment of DLD1 cells with **1** under a variety of conditions is YWHAE. Expression changes for YWHAE were confirmed by quantitative PCR methods. YWHAE is a protein kinase-dependent activator of tyrosine and tryptophan hydroxylases (which are involved in L-dopa and melatonin biosynthesis [17]), as well as an endogenous inhibitor of protein kinase C [18]. Isoforms are involved in regulating the G2/M phase of cell cycle progression [18] through interactions with *cdc25*. YWHAE is also implicated in neurodegenerative diseases such as schizophrenia [19] and cancer [18].

In order to test whether these effects were specific for **1**, we also profiled polyamide **3** (our unpublished data). Although polyamide **3** is shorter and therefore has different sequence selectivity, it was profiled because it was identified in an independent screen of a small library of 6, 8, and 10 ring hairpin polyamides against the CD1-luciferase assay in HeLa cells (our unpublished data). The TCF binding site (Figure 1B) in the CD1 promoter contains a 1 bp mismatch of the binding sequence for compound **3**. Negligible overlap in the differentially regulated genes after treatment of DLD1 cells with **1** versus **3** is consistent with small changes in the polyamide structure leading to large differences in their effect on transcription.

To determine the effects of **1** on DLD1 cell proliferation, we performed 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) colorimetric assays for mitochondrial function (Promega, Madison, WI). Cell growth increased by approximately 20% at concentrations ≥ 120 μM in a dose-dependant manner. Previous studies in fission yeast have shown increased growth rates and enhanced sensitivity to DNA damage when a homologous gene to YWHAE is mutated and the DNA damage checkpoint is subverted [20]. In our case, enhanced growth rates may

occur through misregulation of DNA damage checkpoints via downregulation of YWHAE and/or other DNA-damage checkpoint genes (e.g., DDIT3, which is also involved in mediating apoptosis) that are downregulated by **1** (Table 1). To test this possibility, we examined the sensitivity of treated cells to DNA damage. Preincubation with polyamide and subsequent treatment with bleomycin leads to an approximately 25% decrease in growth rate, in a dose-dependent manner, with a visible decrease in cell number compared with cells treated with bleomycin alone (compared to untreated cells, bleomycin-treated cells had a 50% survival rate). Polyamide pretreatment causes DLD1 cells to be more sensitive to DNA damage agents, probably by downregulating DNA damage checkpoint genes. Although the changes in growth (or survival) are not as dramatic as those observed in mutants [20] in which DNA damage checkpoint genes are nonfunctional, the 2- to 5-fold changes observed in gene expression do lead to specific, observable cellular responses.

We performed extensive promoter analyses in an effort to identify potential polyamide binding sites linked to transcriptional regulation. For the approximately 11,000 genes probed by the high-density oligonucleotide arrays, the genomic sequence 3 kb upstream of the translational start site was searched for all possible binding sites of **1** (5'-WWGWWCWW-3', W = A or T). We identified 37,380 sites with an average of approximately three sites per 3 kb promoter. Several of the downregulated genes have a larger than average number of binding sites in the 3 kb promoter regions. In order to try to determine whether there are common transcription factor binding sites within the promoter regions of the downregulated genes, we used the AlignACE and ScanACE algorithms designed by the Church group [21] to perform pattern searching. We searched for conserved sequence motifs in various clusters of downregulated genes as well as for isolated sequences containing each polyamide binding site flanked by 30 base pairs on both

Table 1. Genechip Analysis of Changes in Gene Expression in DLD1 Cells after Treatment with Polyamide 1

Downregulated Genes for Polyamide 1				Upregulated Genes for Polyamide 1					
UG	Gene Name	Fold Change			UG	Gene Name	Fold Change		
		16 hrs	48 hrs	Number of Binding Sites			16 hrs	48 hrs	Number of Binding Sites
		60 μ M	90 μ M	120 μ M	60 μ M	90 μ M	120 μ M	30 μ M	30 μ M
Signal transduction, Schizophrenia related									
Hs.79474	tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, ϵ polypeptide	-2.8	-2.6	-4.5	-4.6	N/A			
Hs.334330	calmodulin 3 (phosphorylase kinase, delta)	---	---	-2	0				
Hs.2499	protein kinase C-like 1	-3.6	---	---	-3.5	0			
Hydrolases									
Hs.89649	epoxide hydrolase 1, microsomal	---	-2.6	---	-3.8	4			
Hs.74573	similar to vaccinia virus HindIII K4L ORF	-2.3	---	---	---	3			
Hs.5038	neuropathy target esterase	---	-4.1	---	---	2			
Stress, Proliferation Related									
Hs.337761	DNA-damage-inducible transcript 3	---	---	-4	---	3			
Hs.75450	delta sleep inducing peptide, immunoreactor	---	---	-2.4	---	4			
Hs.76556	protein phosphatase 1, regulatory subunit 15A	---	---	-2	---	3			
Hs.100602	MAD (mothers against decapentaplegic, Drosophila) homolog 7	-2.2	-2.1	---	---	0			
Trafficking and Transport Related									
Hs.90535	syntaxin binding protein 2	-3.8	---	---	---	2			
Hs.16488	calreticulin	---	---	-3.8	-3.6	2			
Hs.111024	solute carrier family 25, member 1	---	---	---	-2.2	1			
Other									
Hs.11244	hypothetical protein	---	---	-2.4	---	0			
Hs.239018	RAB11B, member RAS oncogene family	---	---	-2	-2.8	4			
Hs.228059	tripartite motif-containing 28	---	---	---	-2.4	1			
Hs.115232	splicing factor 3a, subunit 2, 66 kD	-2	---	---	-2.2	2			
Hs.25817	BTB (POZ) domain containing 2	---	---	---	-2.1	0			
Cyclin D1									
Hs.82932	Cyclin D1	---	---	---	---	1			
Genes Involved in transcription									
Hs.46423	H4 histone family, member G	4.8	---	3.4	---	1			
Hs.25647	v-fos FBJ murine osteosarcoma viral oncogene homolog	---	2.4	6.5	---	0			
Hs.74088	early growth response 3	---	2.1	3.4	---	0			
Hs.37078	v-crk avian sarcoma virus CT10 oncogene homolog	---	---	4.1	---	0			
Genes Involved in Signal Transduction									
Hs.75498	small inducible cytokine subfamily A, member 20	---	2.0	2.7	---	6			
Hs.110571	growth arrest and DNA-damage-inducible, beta	---	---	3.3	---	2			
Hs.260523	neuroblastoma RAS viral (v-ras) oncogene homolog	---	---	2.6	---	2			
Hs.1666	guanine nucleotide binding protein, α 13	---	---	2.5	---	0			
Hs.120996	serine/threonine kinase 17b	---	---	2.5	---	7			
Hs.2128	dual specificity phosphatase 5	---	---	2.3	---	N/A			
Hs.22868	protein tyrosine phosphatase, non-receptor 11	---	---	2.2	---	N/A			
Hs.112378	LIM and senescent cell antigen-like dom. 1	---	---	2	---	6			
Other									
Hs.119597	stearoyl-CoA desaturase	---	---	6	---	1			
Hs.283664	aspartate beta-hydroxylase	---	---	2.7	3.2	4			
Hs.318885	superoxide dismutase 2, mitochondrial	---	---	2.4	---	N/A			
Hs.75511	connective tissue growth factor	---	---	2.3	---	4			
Hs.77910	3-hydroxy-3-methylglutaryl-CoA synthase 1	---	---	2.2	---	N/A			
Hs.37288	nuclear receptor subfamily 1, gp D, 2	---	---	2.2	---	N/A			
Hs.227730	integrin, alpha 6	---	---	2.1	---	2			
Hs.111429	dynactin 4	---	---	2	---	4			
Unknowns									
Hs.324504	W27675:36b3 Homo sapiens cDNA cDNA DKFZp586J0720	---	---	2.3	---	N/A			
Hs.128653	hypothetical protein DKFZp564F013	---	---	2	---	5			

The fold change (Δ) for differentially expressed genes is listed with unigene (UG) accession numbers. Δ values greater than 3 are underlined. The number of polyamide binding sites within the 3 kb promoter regions of the differentially regulated genes is given.

Table 2. Results from Proximity-Searching Algorithm that Determines the Distance between Human Transcription Factor Binding Sites to the Closest Match Polyamide Binding Site

Proximity between Transcription Factor and Polyamide 1 Binding Sites					
UG	Gene	Identifier Factor	Transcription Factor	distance, bp	Position
Hs.337761	DNA damage-inducible transcript 3	HSSCLASE_02	PEA3	11	1009
		HSS\$TERT_03	Sp1	21	2774
		HSSGAST_01	unknown	29	1239
		HSS\$EGFR_18	window 8	65	1637
Hs.76556	protein phosphatase 1, regulatory (inhibitor) subunit 15A	HSS\$TERT_03	Sp1	21	2774
		HSS\$A4_01	promoter of Alzheimer's disease amyloid A4 precursor gene	28	782
Hs.16488	calreticulin	HSS\$APOB_06	C/EBPalpha	93	296
		HSS\$DG_03	PYR	26	1996
		HSS\$INOS_01	FOXF1	51	2692
Hs.90535	syntaxin binding protein 2	HSS\$CD2_06	3' enhancer, CD2E6	73	2058
		HSS\$CD2_06	3' enhancer, CD2E6	264	1867
Hs.228059	tripartite motif-containing 28	HSS\$TERT_03	Sp1	87	1168
Hs.239018	RAB11B, member RAS oncogene family	HSS\$ACT2_01	AP-1	102	2163
		HSS\$HOX4C_01	HOXD9,10	138	2269
		HSS\$CD2_06	3' enhancer, CD2E6	146	1563
		HSS\$ACT2_01	AP-1	242	1819
Hs.75450	delta sleep-inducing peptide, immunoreactor	HSS\$AAC_10	Sp1	172	2101
		HSS\$EGFR_18	window 8	194	2498
		HSS\$EGFR_18	window 8	251	2555
		HSS\$AAC_10	Sp1	357	1916

The unigene (UG) accession numbers for each gene downregulated by the corresponding polyamide is listed as well as the TransFac site identifier. The distance (8 bp or longer) between the binding sites is reported in the distance column and represents the distance between the first nucleotide (5') of each. The position of the polyamide binding site is reported in the position column and is relative to the translational start site (ATG).

the 5' and 3' sides. In both analyses, no common patterns were found other than those consistent with the polyamide binding sites. Pattern searching, with the AlignACE and ScanACE algorithms [21], did not identify homologous sequences consistent with a common binding motif (other than that for 1) within the promoters of the downregulated genes. This suggests that the genes are downregulated by independent mechanisms involving antagonism of a number of different transcription factors.

Polyamide binding sites close to or overlapping with known transcription factor binding sites are likely to be relevant with respect to transcriptional antagonism. Therefore, we mined the TransFac Professional V 5.2

database (BioBase) for all experimentally determined transcription factor binding sites within human genes to identify proximity relationships between them and polyamide 1 binding sites within the promoter regions of the downregulated genes. We searched for unique proximal pairs of polyamide and transcription factor binding sites 8 bp or longer in the promoter regions of the downregulated genes as opposed to the approximately 11,000 other genes represented on the high-density oligonucleotide arrays (results for downregulated genes are given in Table 2). Searches were limited to consensus sequences 8 bp or longer because shorter sequences occur too frequently on a genome-wide scale. The results are indicative of possible interactions that

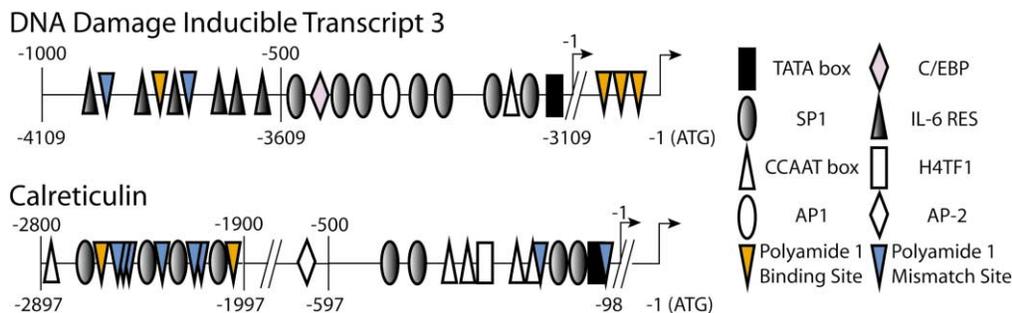


Figure 4. Schematic of the Promoter Regions of DDIT3 and Calreticulin. Known transcription factor and polyamide 1 binding sites are indicated.

lead to transcriptional antagonism. On a genomic level we identified several transcription factor binding sequences that directly overlapped with polyamide binding sites such as WT-1, SF-1, AR, HNF4 α , B, E, LEF-1, and URE. Unfortunately, nothing is known of their involvement in the transactivation of the genes we see downregulated.

In order to evaluate our proximity-searching algorithm, we investigated representative promoter regions of genes that are downregulated by 1 and whose transcription factor binding sites are known. Induction of expression of DDIT3 is known to involve a variety of transcription factors, including AP1, TATA box binding protein, C/EBP, and IL-6 responsive element (IL-6 RE) as well as SP1 and PEA3 [22], which were identified in proximity searches (Table 2). Upon inspection of these sites within the promoter sequence of DDIT3, two other proximity relationships were identified. An upstream polyamide 1 binding site was found to be located 19 and 26 bp away from two IL-6 RE binding sites (Figure 4). We investigated single mismatch polyamide binding sites, which compared with match sites typically show between 10- and 100-fold lower affinity for polyamides [7], and found an additional two single base pair mismatch sites located 6 and 33 bp away from two other IL-6 RE sites. The transactivation of calreticulin, a gene we observe downregulated in DLD1 cells upon treatment with high doses of 1, involves factors including TATA box binding protein, CCAAT box binding protein, SP1, and AP-2, among others [23]. We have identified two polyamide binding sites within close proximity to two Sp1 binding sites, 14 and 141 bp away. Interestingly, two single mismatch polyamide 1 binding sites directly overlap with both the TATA box motif (5'-TAT AAAAGTGCAA-3', -22) and a CCAAT box motif (5'-TAGACCAATT-3', -93) as well as two other single mismatch sites within 5 bp of SP1 sites (5'-TTGAACAG(C)-3', -2035 and -2339). The possible relevance of the mismatch sites we have identified in representative promoter sequences suggests further complications with predicting genomic targets through rational design. Inadequacies in our understanding of global mechanisms for gene transactivation only highlight the importance of using functional genomics to discover the downstream effects of polyamides on cells.

Significance

Previous studies have shown that imidazole/pyrrole polyamides can modify the expression of a number of genes [7]. Here we show that transcript profiling can be used to identify the preferred targets of polyamides in vivo. In this particular instance, expression of the target gene was not significantly affected, although it is unclear whether the behavior of polyamide 1 will be typical of other polyamides. It is encouraging that the expression of only a small set of genes is altered given the large number of possible binding sites in the genome. In light of our limited knowledge of transcriptional regulation mechanisms, predicting which genes will be affected by polyamide binding may, in general, be a difficult task. Moreover, because the genome-

wide effects are related to polyamide identity, structure-activity studies on a genomic level may provide generalized rules for regulating gene expression with these molecules. A combination of cellular and genomic screens of libraries of polyamides of different sizes and sequence may help to elucidate these rules, which should facilitate their use in cell biology and as potential therapeutics.

Experimental Procedures

Synthesis of Polyamides

Polyamides were synthesized with a combination of solution and solid-phase methods as previously described [24]. The identity and purity of polyamides were confirmed by a combination of techniques that include analytical HPLC, MALDI-TOF mass spectroscopy, and NMR.

Surface Plasmon Resonance Measurements

The biotinylated oligonucleotide LEF1 (5'-biotin-GGCCGATGATC TATGCTATAGCATAGATCATCGGCC-3') was purchased from Sigma. Streptavidin-functionalized SA sensorchips were obtained from Biacore. Immobilization of the hairpin DNA onto the sensorchips, measurement of response curves in the presence of polyamide 1 (concentrations ranging from 1 nM to 20 μ M), and data analysis were performed as previously described [15]. We determined that there were mass transfer effects for interactions of the polyamide DNA binders with hairpin DNA-derivitized surfaces; therefore binding experiments were conducted so that responses at equilibrium (R_{eq}) could be accurately determined, and the K_d values were determined by plotting the ratio of R_{eq}/R_{max} versus concentration of the polyamide according to the Langmuir equation (Equation 1).

$$\frac{R_{eq}}{R_{max}} = \frac{\frac{1}{K_d} \cdot [polyamide]}{1 + \frac{1}{K_d} \cdot [polyamide]} \quad (1)$$

Real-Time PCR

PCR primers and TaqMan probe (5'-GAAAAGCATGGTGCTGGTA ACA-3' [forward primer], 5'-CTAACCTGCAGTGGGAGAGTAA-3' [reverse primer], and 5'-TCCGTGGCTGCTCATTCTTGCCTAC-3' [probe]) were designed for YWHAE according to the Primer Express version 1.5 software (Applied Biosystems, Foster City, CA) and purchased from Applied Biosystems. TaqMan probes were labeled with 6-carboxy-fluorescein (FAM) as the reporter dye and 6-carboxy-tetramethyl-rhodamine (TAMRA) as the quencher dye. Quantitative PCR experiments were performed as previously described [25].

Electrophoretic Mobility Shift Assay

EMSA were performed with 50 nM LEF-1 protein. For studies of protein-DNA interactions, LEF-1 was incubated with 32 P-labeled duplex oligonucleotide probes. Probes containing either the wild-type LEF binding site (5'-CTCTGCCGGGCTTTGATCTTTGCTTAACA ACA-3') or the mutated LEF-1 binding site at nucleotides 75 and 74 from AT to GC (5'-CTCTGCCGGGCTTTGGCCCTTTGCTTAACA ACA-3'). Equimolar amounts of two complementary oligonucleotides were combined and end-labeled with [γ - 32 P]ATP and T4 polynucleotide kinase. The labeled oligonucleotides were annealed and used in gel mobility shift experiments at a final concentration of 1 fmol/20 μ l (50 pM). Binding reactions were carried out analogously to those outlined in previous reports [11].

Microarray Hybridization

RNA extraction and hybridization on oligonucleotide microarrays (U95A GeneChip; Affymetrix Inc., Santa Clara, CA) were performed as described previously [26], with the exception that the arrays were hybridized at 50°C for 16 hr. GeneChip hybridization data were processed and scaled as described previously [27, 28].

Cell Culture and Fluorescence Microscopy

DLD1 cells were cultured at 37°C in 5% CO₂ in RPMI medium 1640 (Gibco) supplemented with 10% (vol/vol) heat-inactivated FBS (Gemini Bioproducts). DNA binder 1 was used at 30, 60, 90, and 120 μM concentration in the medium. Cells were harvested 16 hr after treatment with 60, 90, and 120 μM polyamide or 48 hr after treatment with 30 μM 1. For fluorescence microscopy, cells were grown on ethanol/HCl-washed 22 mm glass coverslips. Cells on coverslips were rinsed in 1 × PBS, then fixed with 4% paraformaldehyde in PBS and again washed with 1 × PBS. Cells were examined with a confocal microscope (Zeiss). For fluorescence microscopy a total of 4 × 10⁵ DLD1 cells were seeded onto 22 mm square coverslips in 6-well plates. The growth medium was replaced with fresh RPMI medium, which contained fluorescently labeled DNA binder 2B at a final concentration of 100 μM. After 16 hr, the coverslips were pried from their wells and dip-rinsed in phosphate-buffered saline. Cells were then fixed for 10 min with 3.7% formaldehyde and permeabilized with 0.2% Triton X-100. For nuclei visualization, staining was performed with a cell-permeable fluorochrome (bisbenzimidazole-BIS). MTS assays were performed as previously described [29].

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