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The purpose of this study is to develop novel DNA ligands that offer the potential for the treatment of human breast cancer. Our approach is to develop small, cell-permeable molecules that prevent the activation of genes involved in breast cancer cell proliferation by the DNA-binding protein estrogen receptor. A series of pyrrole/imidizole polyamides have synthesized in the laboratory of Dr. Peter Dervan at The California Institute of Technology and supplied to our laboratory. These polyamides were designed to bind the 6 bp half-site recognized by estrogen receptor. Standard DNase footprinting methods were used to measure the binding affinities of the synthetic ligands for their target sequences. A series of polyamides were screened for binding affinities and sequence specificity. We have used recombinant human estrogen receptor protein in DNA binding studies with the same target ERE sequences. Using DNase footprinting methods and gel mobility shift assays, we optimized conditions for ER-DNA interactions and we have shown that the ERE-binding polyamides inhibit ER binding to EREs. Future studies will examine whether these compounds are effective inhibitors of ER-dependent gene transcription in breast carcinoma cells in culture.

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INHIBITION OF ESTROGEN RECEPTOR-DEPENDENT GENE TRANSCRIPTION BY A DESIGNED LIGAND

TABLE OF CONTENTS

Page Number

(5) INTRODUCTION

The purpose of this study is to develop novel DNA ligands that offer the potential for the treatment of human breast cancer. The growth of many human breast carcinomas is regulated by the female hormone estrogen through the action of the estrogen receptor protein. The logic of our approach is to develop small, cell-permeable molecules that prevent the activation of downstream genes by the DNA-binding protein estrogen receptor. A series of pyrrole/imidizole polyamides have synthesized in the laboratory of Dr. Peter Dervan at The California Institute of Technology and supplied to our laboratory. These polyamides were designed to bind the 6 bp half-site recognized by estrogen receptor. Standard DNase footprinting methods were used to measure the binding affinities of the synthetic ligands for their target sequences. A series of polyamides were screened for binding affinities and sequence specificity. We have used recombinant human estrogen receptor protein in DNA binding studies with the same target ERE sequences. Using DNase footprinting methods and gel mobility shift assays, we optimized conditions for ER-DNA interactions and we have shown that the ERE-binding polyamides inhibit ER binding to EREs. Future studies will examine whether these compounds are effective inhibitors of ER-dependent gene transcription in breast carcinoma cells in culture.

(6) RESEARCH ACCOMPLISHMENTS: Inhibition of Estrogen Receptor-Dependent Gene Transcription by a Designed Ligand

Background:

Growth of many breast cancers is regulated by estrogens through the action of the nuclear estrogen receptor. Estrogen receptor (ER) is a member of the superfamily of nuclear receptors which bind small ligands such as the steroid hormones, vitamin D, thyroid hormone, and the retinoids (1). These receptors function in the nucleus as sequence-specific DNA-binding transcription factors. The nuclear receptors are comprised of structural domains involved in ligand binding, dimerization, DNA recognition and binding, and transcriptional activation. For ER, extracellular estrogens freely diffuse across the cell membrane and bind ER, leading to ER dimerization and transport to the nucleus. ER in the nucleus then binds to estrogen-response-elements (EREs) located upstream from a variety of estrogen-responsive genes. Many of these genes are involved in cell growth and differentiation, especially in the breast and female reproductive organs. Once bound to the promoter elements of these genes, the activation domain(s) of ER participate in multiple protein-protein interactions which ultimate lead to high levels of transcription by RNA polymerase II (2, 3).

Although estrogen stimulates the growth of many breast carcinomas, the targets of ER activation in breast cancer are largely unknown. Nonetheless, the antiestrogen tamoxifen clearly elicits its antiproliferative effects by competition with estrogen for binding cytoplasmic ER (4). A recent study has shown that tamoxifen does not inhibit the ER-DNA interaction (5). Thus, it is believed that the tamoxifen-ER complex does not undergo the necessary conformational change in the protein required for transcriptional activation (1). Although tamoxifen has prolonged survival in many postmenopausal ER-positive breast cancer patients, some ER-positive breast carcinomas do not respond to tamoxifen and others become tamoxifen-resistant during adjuvent therapy(4, 6). Moreover, tamoxifen can had adverse side-effects involving endometrial pathologies(7) and bone mineral density loss. Therefore, new chemotherapeutic agents would be of great benefit, especially in premenopausal women where tamoxifen has not been as beneficial. We are exploring whether superior inhibitors of ER-dependent gene transcription can be generated based on rational design of DNA-binding ligands. These small molecules will bind EREs with high affinity and sequence-specificity and will compete with the natural estrogen-ER complex for binding to the EREs of estrogen-responsive genes. In this way, these molecules will block activation of estrogen-responsive genes involved in breast cancer proliferation.

The structure of the minimal DNA binding domain (DBD) of ER has been solved by nuclear magnetic resonance (NMR) (8) and X-ray crystallographic (9)

methods and has been shown to consist of two zinc-binding domains, each of which is comprised of four cysteine residues involved in chelation of a single zinc atom. Unlike the classical cys2-his2 zinc finger motif found in numerous other DNA-binding transcription factors, the nuclear receptor zinc fingers are folded into a single structural unit. It is also well established that ER binds DNA as a homodimer. From the crystal structure of a ERDBD-DNA complex, it has been shown that amino acids in the helical region of the amino-terminal zinc-binding domain participate in specific base and phosphate contacts. Base recognition is achieved through direct and water-mediated hydrogen bonds to functional groups in the major-groove of DNA. Additional phosphate contacts are formed with amino acids in the helical segment of the second zinc-binding domain. Full DNA binding affinity by ER, however, requires an amino acid sequence immediately adjacent to the minimal DBD(10). This region of the protein is involved in dimerization and stabilizes the protein-DNA complex through additional DNA contacts. Each monomer recognizes and binds a 6 bp half site within a palindromic sequence, with a three bp spacing between half-sites.

The Pyrrole-Imidazole Polyamides, developed in the laboratory of Dr. Peter B. Dervan at the California Institute of Technology, represent the only class of small molecules developed to date that can bind *predetermined* DNA sequences (11). DNA recognition depends on side-by-side pairings of pyrrole (Py), imidazole (Im) and β-alanine amino acids in the minor groove. An Im/Py pair targets a G•C base pair, while Py/Im targets a C•G base pair. Py/Py, β-alanine/β-alanine or β-alanine/Py pairs are degenerate and target both A•T and T•A base pairs (12). Recent studies have shown that a 3-hydroxypyrrole/Py pair can discriminate between T•A and A•T base pairs and specifically recognize T•A (13). These pairing rules are supported by direct NMR structural studies (14) and by X-ray crystallography (15, 16). The Py-Im polyamides have affinities and specificities comparable to those of natural eukaryotic DNA-binding transcription factors: for example, an eight ring hairpin polyamide, which targets a six base pair sequence, binds with a dissociation constant of 0.03 nM (11). Moreover, two eight-ring pyrrole-imidizole polyamides differing in sequence by a single amino acid bind specifically to respective six base-pair target sites which differ in sequence by a single base pair. The replacement of a single nitrogen atom with a C-H can regulate specificity and affinity by two orders of magnitude.

Since a six base-pair sequence would be highly redundant in the human genome (occurring at random once every 4 kilobases or 500,000 times in the human genome), polyamides have been synthesized to recognize much longer sequences, ranging from nine to thirteen base pairs (17). Recently, an eight ring polyamide has been designed to target a 16 bp site and binding is again observed with subnanomolar affinity. In molecules of this class, sequence selectivity is achieved by binding as a "slipped" antiparallel dimer (18). Since a 16 base pair sequence would be predicted to occur at random once every ~4 billion base pairs, target sites of this size should occur only once in the human genome. Such

molecules thus have the potential to act as specific inhibitors of gene transcription *in vivo* and as human therapeutic agents. Our studies with model systems have shown that polyamides interfere with the binding of sequence-specific transcriptional activator proteins and with components of the basal transcription machinery and thus block transcription of target genes. Most importantly, a designed polyamide has been shown to inhibit transcription of a specific gene in living cells and thus these compounds must be both cell permeable and once inside the cell they must be able to transit the nuclear envelope and bind their target sites within chromatin. These findings form the basis for the proposal that these designed ligands may be useful agents in the treatment of human breast cancer.

Progress Report:

1. Design and synthesis of pyrrole/imidizole polyamides: Based on the pairing and recognition rules described above, polyamides have been synthesized to target the sequence of natural EREs. For example, the human pS2 gene promoter (10, 19) has the following ERE sequence:

5'-CTTCCCCCTGCAA**GGTCAC**G**GTGGCC**ACCCCGTGAGCC-3'

where the half-sites are in bold the central nucleotide is underlined. The pS2 gene is an estrogen-responsive gene transcribed in the breast and has an imperfect palindromic ERE (10, 19). A Py-Im polyamide to target the first half-site element (5'-AGGTCA-3') would be Im-Im-Py-Py-γ-Im-Py-Py-Py-β-Dp (**1**, where Py denotes pyrrole, Im denotes imidizole, γ denotes γ-aminobutyric acid, β denotes β-alanine and Dp denotes dimethylpropylamide). Based on the pairing rules described above, this compound is predicted to bind the sequence 5'-WGGWCW-3', where W = A or T. A second DNA target is contained in plasmid 2ERE-B/N-CAT, generously given to our lab by Dr. L. Krauss (University of California, San Diego). This plasmid contains two EREs of the following sequence:

5'-CAAAGTC**AGGTCAC**A**GTGACCT**GATCA-3'

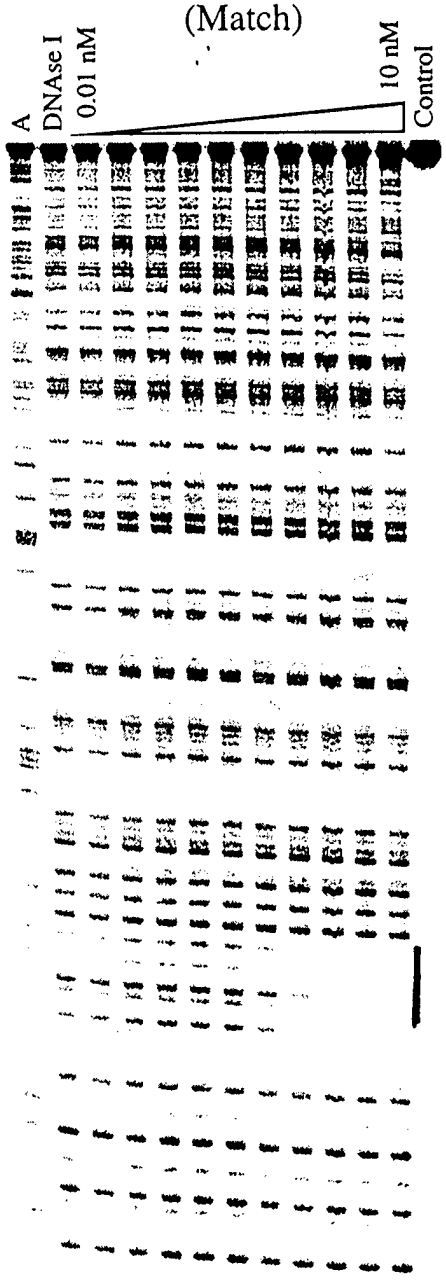
Each half site in this ERE contains a binding site for polyamide 1 (5'-WGGWCW-3'). A mismatch polyamide **2** of sequence Im-Im-Py-Py-γ-Py-Py-Py-Py-β-Dp (where the Im to Py change is underlined) is a single atom substitution. A second single atom mismatch polyamide **3** for the ERE is Im-Py-Py-Py-γ-Im-Py-Py-Py-β-Dp , binds the sequence 5'-WGWWCW-3'. A binding site for polyamide **3** is found immediately adjacent to the downstream half-site in the 2ERE plasmid. Chemical synthesis was carried out by the solid phase methods described by Baird and Dervan (20) and the identity and purity of the compounds was established by HPLC, ^{1}H-NMR and mass spectrum analysis (MALDI-TOF-

MS). These polyamides were synthesized in the laboratory of Dr. Peter Dervan at Caltech and provided to the Gottesfeld laboratory at The Scripps Research Institute.

2. DNA binding assays: Conventional DNase I footprinting assays have been used to monitor the binding affinity of the designed polyamides for the EREs. Briefly, a restriction fragment containing the ERE sequence was isolated from plasmid DNA and singly end-labeled (either by the 3' fill-in reaction with the Klenow fragment of DNA polymerase and α-^{32}P-dNTPs or by 5' end-labeling with γ-^{32}P-ATP and T4 polynucleotide kinase). The labeled DNA (at subnanomolar concentration) is incubated with various concentrations of the polyamide and, after sufficient time for the reaction to reach equilibrium, the complexes are digested with DNase I under single-hit enzyme conditions. Regions of protection, and hence binding, by the polyamide are determined by analysis of the digestion products on a DNA sequencing gel. Phosphorimage analysis of the data also yields an apparent association constant for the binding reaction. A single site of protection will demonstrate the specificity of binding. The polyamide described above, Im-Im-Py-Py-γ-Im-Py-Py-Py-β-Dp, binds its target sequence with an apparent association constant (K_a) of 1 X $10^9 M^{-1}$, and the mismatch molecule, Im-Im-Py-Py-γ-Py-Py-Py-Py-β-Dp , has a K_a of <1 X $10^8 M^{-1}$ for the same sequence. Figure 1 shows the results of such a quantitative footprint titration for the match polyamide and for a single atom substitution mismatch. A binding model is also shown below the footprint gels, in which filled circles represent imidazoles and open circles represent pyrroles. The single mismatch is highlighted on the right.

3. Polyamide inhibition of ER binding to the ERE. Recombinant human ERα was used in gel mobility shift experiments and in DNase footprinting assays to determine the relative affinity of the protein for the various DNA sites under study. These assays used either a radiolabeled restriction fragment or labeled double stranded synthetic oligonucleotide. Conditions for high affinity ER-ERE binding were determined, and a K_a of approximately 1 X $10^9 M^{-1}$ was observed for this interaction with a 500 bp restriction fragment isolated from the 2ERE plasmid. This protein-DNA complex was then challenged with the high-affinity-binding polyamide and mismatch molecules. Polyamides were added to the DNA at various concentrations to test for the efficiency of inhibition. These experiments utilized the restriction fragment from the 2ERE plasmid as well as the pS2 ERE sequence in the form of a 38 bp oligonucleotide whose sequence is shown above. This latter duplex DNA was radiolabeled with T4 polynucleotide kinase and γ-^{32}P-ATP using standard procedures. Data are shown below for the 2ERE sequence, but comparable results were obtained with the pS2 ERE oligonucleotide.

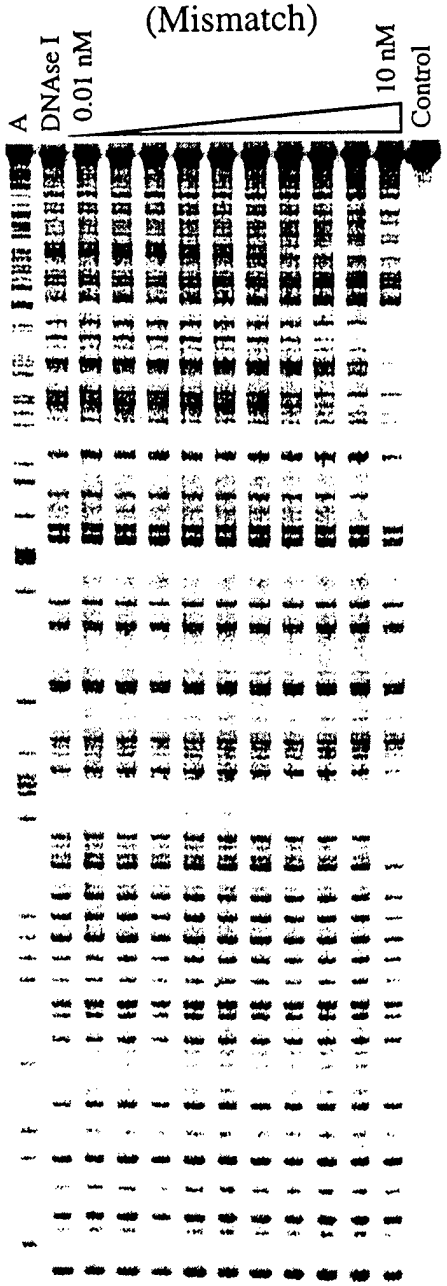
ImImPyPy-γ-ImPyPyPy-β-Dp
(Match)



5′-A G G T C T-3′
3′-T C C A G A-5′

$K_a = 1 \times 10^9\ M^{-1}$

ImImPyPy-γ-PyPyPyPy-β-Dp
(Mismatch)



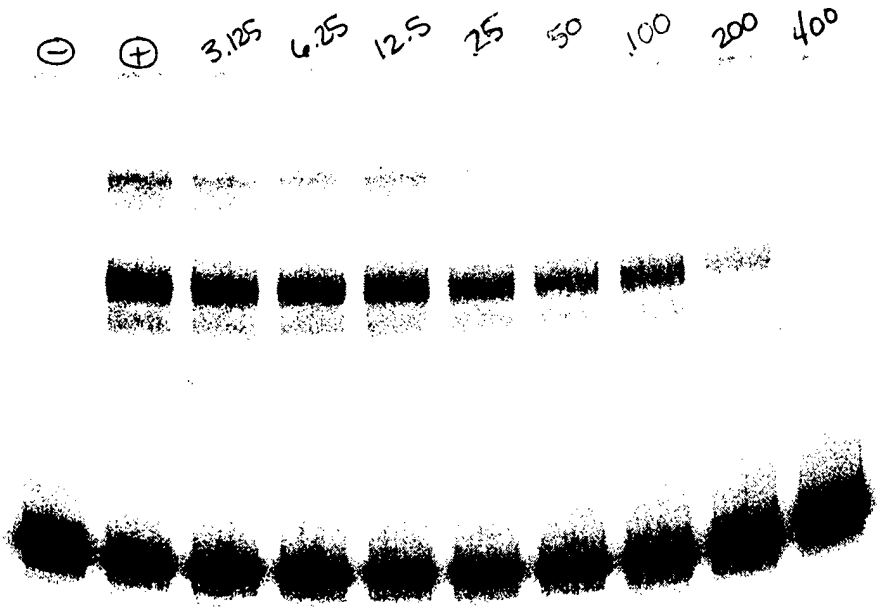
5′-A G G T C T-3′
3′-T C C A G A-5′

$K_a < 10^8\ M^{-1}$

In the experiment shown in Figure 2A, increasing concentrations of the match polyamide were added to the DNA prior to the addition of a constant amount of ERα. The amount of ERα used in this experiment (2.4 nM final concentration) was sufficient to bind approximately 50% of the input DNA. Clearly, with addition of increasing amounts of the match polyamide, the fraction of ERα-bound DNA decreases. The observed K_i was at approximately 20 nM polyamide. This value was comparable to the concentration of total polyamide binding sites in the reaction. We also performed order of addition experiments, in which the DNA was incubated with protein prior to the addition of polyamide or both reactants were added to the DNA simultaneously. Figure 2B shows the results of an experiment where the 2ERE restriction fragment was incubated with ERαprotein prior to addition of the polyamide. Within the error of our determinations, we find that order of addition does not affect the outcome of the experiment, suggesting that ERα does not hinder polyamide access to the minor groove and, once bound, the polyamide inhibits ERα binding. These data are shown graphically in Figure 3. The mechanism whereby a minor groove-binding polyamide inhibits the binding of a major groove-binding protein is currently under investigation. In control experiments, we have established that similar concentrations of mismatch polyamides do not inhibit ERα-ERE interactions (data not shown).

Figure 2. Inhibition of ERα binding with polyamide **1**. In A the DNA was preincubated with the indicated concentrations of polyamide for 15 minutes prior to the addition of ERα to a final concentration of 2.4 nM. In B, the order of addition was reversed. Samples were subjected to nondenaturing gel electrophoresis and the phosphorimage is shown.

A



B

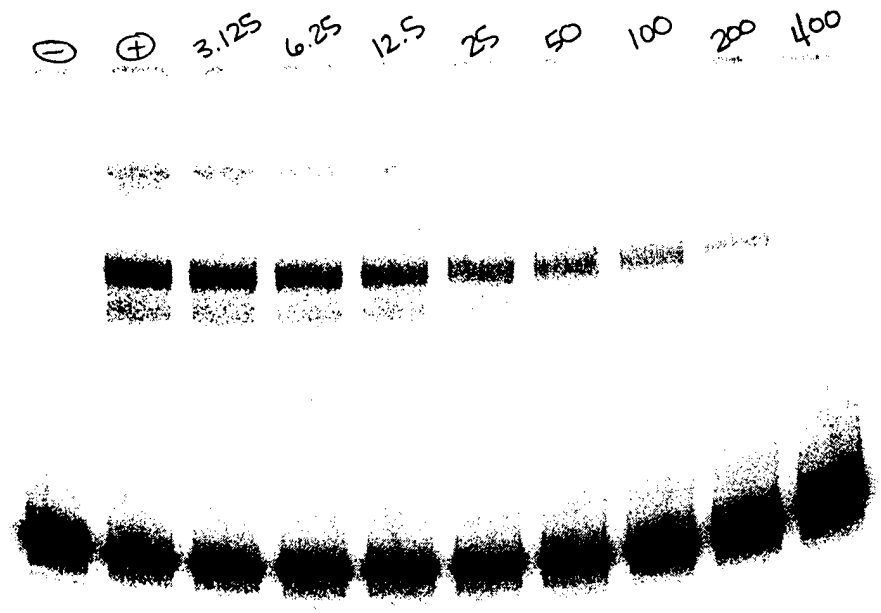
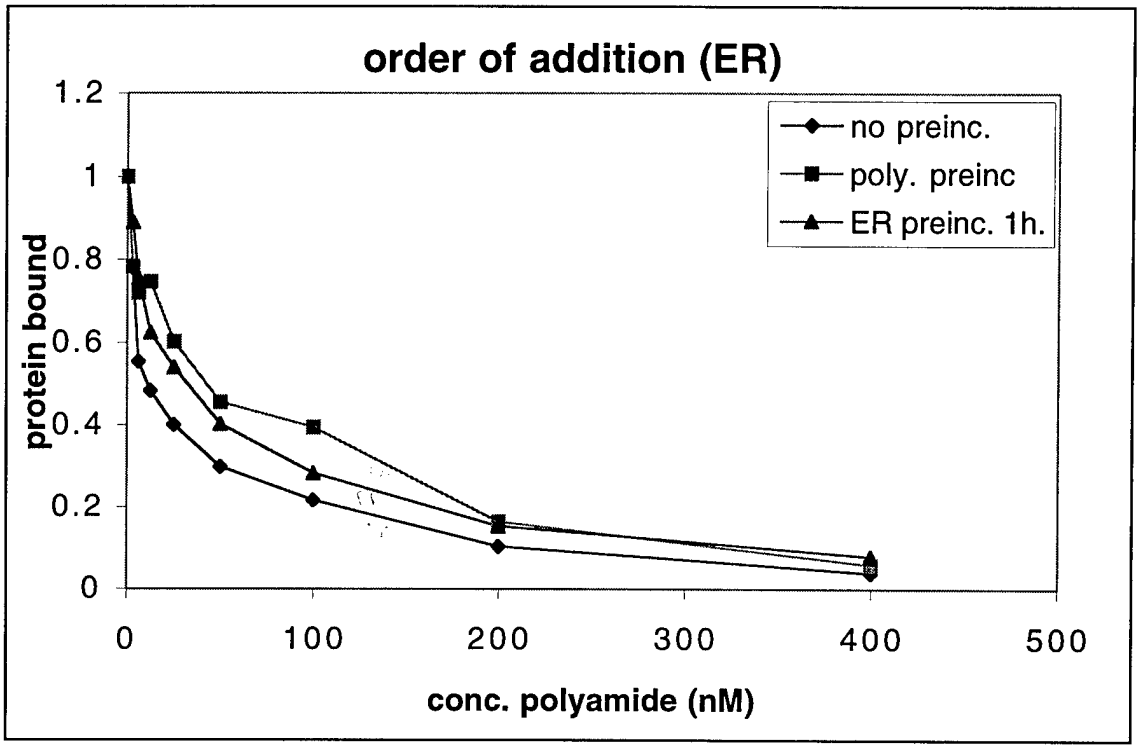


Figure 3. Graphical representation of gel mobility shift data.



(7) KEY RESEARCH ACCOMPLISHMENTS:

- A pyrrole-imidazole polyamide was synthesized that binds the estrogen response element (ERE) in the pS2 promoter with high affinity
- Mismatch polyamides were also synthesized as controls
- The match, but not mismatch, polyamide inhibits estrogen receptor-ERE interactions
- Polyamides disrupt preformed ER-ERE complexes

(8) REPORTABLE OUTCOMES:

A manuscript describing our results will be submitted for publication in the near future.

(9) CONCLUSIONS:

The studies supported by this grant represent a new approach to the discovery of potential therapeutic agents for the treatment of human breast cancer. The pyrrole/imidizole polyamides are a new class of DNA-sequence specific ligands, with affinities for specific DNA sequences that approach or exceed those of natural eukaryotic transcription factors (11). Our collaborative studies with the Dervan laboratory are the first to demonstrate that these compounds inhibit transcription factor-DNA interactions *in vitro* and that these polyamides are permeable to living cells in culture and will disrupt transcription complexes on their target genes *in vivo*. These findings suggest to us that similar pyrrole/imidizole polyamides can be designed to target the EREs of estrogen-inducible genes involved in breast cancer proliferation. These reagents will have the potential for use in the treatment of ER-positive human breast carcinomas.

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