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Transcription By a Designed Ligant

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13. ABSTRACT <i>(Maximum 200 words)</i> Antiestrogens, like tamoxifen, are currently being used for the treatment of many breast cancers, however, not without significant side effects. We proposed to use small molecules to inhibit binding of estrogen receptor to its DNA target sites, thereby blocking estrogen-responsive gene expression. We have shown that the small hairpin pyrrole/imidazole polyamides can be effective inhibitors of gene transcription in living cells. As the first objective, pyrrole-imidazole polyamides will be synthesized to bind sequences adjacent to the estrogen response element and their binding affinity will be determined by quantitative DNase I footprint (Continued on next page...)			
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experiments. For objective two we will determine whether these polyamides inhibit binding of the estrogen receptor to its recognition sequence. In objective three it will be determined whether the polyamides inhibit gene expression *in vivo*. The laboratory of Peter Dervan has synthesized a polyamide that specifically recognizes the estrogen response element of the pS2 gene. We have shown by quantitative DNase I footprinting experiments that this polyamide binds its recognition site with high affinity and high specificity. We are currently investigating whether this polyamide inhibits binding of recombinant estrogen receptor *in vitro*. Future studies will focus on the inhibition of transcription of the pS2 gene in living cells by this polyamide.

FOREWORD

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

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ANNUAL REPORT

INTRODUCTION

The growth of many breast cancers is regulated by the natural hormone estrogen. Estrogen forms a complex with estrogen receptor(ER) (1), which leads to dimerization of ER and transport to the nucleus. ER then binds to a specific DNA recognition site, the estrogen response element (ERE) present in the promoters of a number of genes involved in cell proliferation. Upon binding, ER activates transcription of these genes (2, 3).

Current breast cancer therapies include the use of antiestrogens. Among those, tamoxifen effectively inhibits cell proliferation by competing with estrogen for binding ER (4). However, tamoxifen does not inhibit DNA-binding of ER (5). Furthermore, some carcinomas do not respond or become resistant to tamoxifen (4) (6) and prolonged use may increase the risk of uterine cancer (7). Moreover, the beneficial effects of estrogen on bone tissue can be counteracted by these therapies (8). Therefore, new therapies based on a different approach would be of great benefit. We proposed to use small synthetic DNA-binding ligands to specifically inhibit binding of ER to ERE's, thereby blocking estrogen-responsive gene expression.

The small hairpin pyrrole-imidazole polyamides can be designed to bind specific DNA sequences with affinities and specificities comparable to or even higher than eukaryotic transcriptional regulators. Pairing rules have been developed that allow the design of compounds that bind to any predetermined sequence with subnanomolar affinities (9). Polyamides bind in the minor groove of DNA (10). Many transcription factors contact the DNA in the minor groove, and these are ideal candidates for polyamide inhibition. We have shown that a polyamide binding to the recognition site of zinc finger four of transcription factor TFIIIA, which binds the minor groove, inhibits DNA-binding of TFIIIA and transcription of 5 S RNA genes (which are regulated by TFIIIA) in vitro and in

living *Xenopus* cells (11). We have also shown that polyamides can inhibit DNA-binding proteins that predominantly make major groove contacts, provided that the protein makes additional contacts in the minor groove or the phosphate backbone (12). The structure of the DNA-binding domain of ER has been solved by NMR (13) and X-ray crystallography (14), and consists of two zinc-binding domains similar to zinc finger motifs. DNA is contacted predominantly in the major groove, with additional phosphate backbone and minor groove contacts (15). Thus, ER is possibly a good target for inhibition by minor groove binding polyamides.

RESULTS

Objective 1, to be carried out during the first year: Polyamides will be designed to bind the 6 base-pair half-site recognized by ER (16, 17). Binding affinities of these compounds to their target sequences will be measured by DNase I footprinting. The target sequences will include the EREs from several estrogen responsive genes previously shown to be stimulated in breast carcinoma cells. So far we have focused on an ERE of following sequence composition: 5'-A G G T C A C A G T G A C C T-3', the two half-sites of the palindrome are underlined. According to the pairing rules, a polyamide (designated CMV-1) of sequence composition ImImPyPy- γ -ImPyPyPy- β -Dp is predicted to bind the sequence 5'-A G G T C A/T-3'. This polyamide was synthesized in the Dervan lab by solid phase methods (18) and the purity was verified by a combination of HPLC, 1H-NMR and MALDI-TOF spectroscopy. In quantitative DNase-I footprint experiments we found that CMV-1 binds the sequence 5'A G G T C T-3' with a dissociation constant K_d of 1×10^{-9} M, while a mismatch polyamide, designated 216, which differs only in the replacement of one imidazole by a pyrrole, binds the same sequence with much lower affinity ($K_d = > 10^{-8}$ M). Furthermore, we have shown that CMV-1 also binds a concatemer containing four repeats of the ERE palindrome (obtained from Lee Krauss, UCSD) with very high affinity, while the mismatch, polyamide 216, displays no binding to this sequence.

Objective 2: We will express and purify recombinant human estrogen receptor protein for use in binding studies with the same ERE target sequences. We have purchased purified, recombinant ER α from Panvera. This protein is functionally active, because it is expressed in the baculovirus expression system which produces a protein that is post-translationally modified similar to the one found in mammalian cells. We are currently optimizing DNA-binding conditions for ER using DNase-I footprint experiments and gel mobility shift experiments. We will next test in the same assays whether polyamide CMV-1 inhibits ER binding to a concatemer of ERE, while mismatch polyamide 216 should have no effect on ER binding to the ERE.

Objective 3: If the ERE-specific polyamide inhibits ER binding, we will evaluate the effects of the polyamides on ER-dependent transcription in cultured human breast cancer cell lines. To this end we will use cotransfection experiments with a plasmid containing an ERE flanking a minimal promoter linked to a reporter gene (17), and another plasmid expressing ER. In an alternative approach, we will test the effect of polyamides on an endogenous, ER-responsive gene expressed in a breast carcinoma cell-line (19). For the first part of this objective, we have obtained (from L. Krauss) a reporter plasmid containing two EREs, spaced by about 15 base-pair, upstream of the distal promoter of the rat progesterone receptor gene, followed by the chloramphenicol-acetyltransferase (CAT) gene. However, we have found in studies that involved inhibition of transcription from the HIV-1 promoter by polyamides, that transient expression assays gave less reliable and reproducible results than experiments which targeted endogenous genes. Therefore we will focus on the second approach that uses a breast carcinoma cell-line, MCF-7, which expresses an ER-responsive gene, pS2 (19). This cell-line will be grown in our laboratory and both match and mismatch polyamides will be added to the culture medium for various amounts of time. The expression of the pS2 gene will then be monitored by quantitative reverse transcriptase polymerase chain reaction (RT-PCR).

Preliminary work has been done that will allow rapid progress on this objective. In addition to a laminar flow hood, we now have purchased a CO₂ incubator that we are using for cell culture, and we have established growth conditions for several human breast cancer cell lines. We have developed an RT-PCR protocol that allows us to detect and quantitate changes in the level of specific mRNAs being transcribed. This approach has successfully been used in our laboratory in the context of a separate project, which studies the effects of polyamides on the HER2/neu (c-erb B2) oncogene, which is overexpressed in human breast cancer cells. We have shown that specific polyamides block DNA-binding of the TATA-box-binding protein TBP to the Her2/neu promoter and inhibit transcription of the HER2/neu gene in vitro and in vivo in a human breast cancer cell-line which overexpresses this gene. The techniques that were developed for this project will be applicable for the most part to the ER project as well.

CONCLUSIONS

A small imidazole/pyrrole polyamide and a corresponding mismatch polyamide have been synthesized by solid phase methods. This polyamide specifically binds with high affinity to the hexanucleotide repeat of a typical estrogen response element. The mismatch polyamide, which differs only in the replacement of one imidazole by a pyrrole, does not bind the ERE under the conditions used. We have obtained recombinant ER α , and are currently establishing optimal DNA-binding conditions. We have established culture conditions for human breast cancer cell-lines, and we have worked out an RT-PCR protocol that allows us to quantitate changes in specific mRNAs being transcribed.

We anticipate that these studies will represent an important first step in the development of novel therapeutic agents for the treatment of breast cancer.

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