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PRINCIPAL INVESTIGATOR: Yong-Dong Wang, Ph.D.

CONTRACTING ORGANIZATION: Health Research Incorporation
Buffaol, New York 14263

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6. AUTHOR(S)
Yong-Dong Wang, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Health Research Incorporation
Buffaol, New York 14263

E-MAIL:
gnodgnoy@yahoo.com

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Abnormal regulation of gene expression plays an important role in cancer. The first step in the regulation of gene expression requires the binding of transcription factor (TF) to its DNA response element in the gene promoter region. Therefore, interfering with TF-DNA complexes could be a powerful tool for blocking oncogene expression and elucidating how aberrant gene expression contributes to neoplastic phenotypes. The HER2/neu oncogene is amplified and transcriptionally upregulated in 25-30% of human breast cancers. This upregulation has been shown to depend on a highly conserved ETS binding site (EBS) and its upstream AP-2 binding site within the key regulatory region of the HER2/neu promoter. In this study, we investigated a new class of DNA minor groove binding ligands, hairpin pyrrole-imidazole polyamides, as potential TF-DNA inhibitor in gene expression. Several new polyamides were designed specifically targeted to these TF binding sites within HER2/neu promoter region. Polyamides represent a significant advancement in ligand design in that they can achieve a remarkable degree of sequence specificity and high affinity for predetermined DNA sequences. Our results indicate that the first generation of polyamides are potent inhibitors of TF-DNA complex formation and transcription under cell-free conditions.

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FOREWORD

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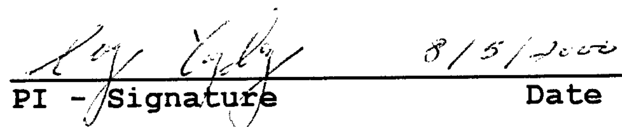

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INTRODUCTION:

The ability to preferentially block gene expression by interfering with oncogenic transcription factor (TF)-DNA complexes could be a powerful tool for elucidating how aberrant gene expression contributes to neoplastic phenotypes (1). One approach to inhibit these complexes is to target DNA binding agents to the TF DNA binding site (2, 3). This study investigates a new class of DNA minor groove binding ligands, pyrrole-imidazole polyamides, as transcription inhibitors targeted at TF promoter elements. Ets protein ESX and AP-2 DNA binding domains contained within the regulatory region of the HER2/neu proximal promoter were chosen as the targets. The protein binding to the HER2/*neu* gene promoter results in a deregulation and overexpression of this growth factor receptor proto-oncogene that is linked to human breast cancer (4, 5). Sequence specific DNA minor groove binding polyamides were synthesized that bind with high affinity ($K_a \leq 10^{-10}$ M^{-1}) to ESX or AP-2 DNA binding sites (6-8). As measured by electrophoretic mobility shift assay (EMSA), polyamides binding to these sites were one to two orders of magnitude more effective than distamycin at inhibiting formation of complexes between the purified proteins and the HER2/*neu* promoter probes. The polyamides also effectively inhibit the HER2/*neu* promoter-driven transcription measured in vitro with nuclear extract from the HER2/*neu*-overexpressing human breast cancer cell line, SKBR-3. However, the examination of these polyamides in SKBR-3 cell revealed a limited ability to interfere with cellular gene expression based upon Northern analysis of Her2/neu mRNA. While studying other possible cellular activities of the polyamides, it was found that the polyamides inhibited the cellular uptake of uridine. To enhance the cellular activity of the polyamides a number of analogs were created with the idea of improving their activity in cell. No significant improvement was detected in both cell-free and cellular condition. Further modifications of these compounds are planned.

BODY (PROGRESS REPORT):

Pyrrole and imidazole containing polyamides (PA) represent a new class of synthetic DNA binding ligands with remarkably high affinity and sequence-specificity. The code for their sequence-specific DNA recognition is based on a side-by-side pairing of the heterocyclic amino acid units within the minor groove of DNA. In this project, the activities of two polyamides, PA-2 and PA-10, were first tested both in cell-free and in whole cell system. PA-2 was designed to bind immediately downstream of the ESX core binding site while PA-10 was designed to bind the AP-2 binding site of the Her2/neu promoter.

The quantitative footprint titration analysis showed that PA-2 and PA-10 bind to their target sites with equilibrium association constant of 1.4×10^{10} M^{-1} and 8.7×10^{10} M^{-1} respectively. In this study, electrophoretic mobility shift assay was used to test the ability of each drug to inhibit its target binding site within the Her2/neu promoter. Incubation of PA-2 or PA-10 with a DNA oligonucleotide containing the binding site from Her2/neu promoter followed by the addition of ESX or AP-2 protein resulted in a concentration-dependent inhibition of transcription factor-DNA complex formation. Ten nM of PA-2 inhibited complex formation up to 95 % while 1 nM resulted in a detectable decrease in complex formation. Eight nM of PA-10 inhibited complex formation 95% while as little as 0.5 nM resulted in a detectable decrease in complex formation. The result showed that 2.2 nM of PA-2 and 1.2 nM of PA-10 are required to inhibit complex formation by 50%.

To determine whether the effects of drugs on transcription factor-DNA complex formation resulted in an ability to influence biological function, *in vitro* transcription assays were performed. The plasmid DNA containing the Her2/neu promoter was used as a template and SKBR-3 nuclear extracts as transcription machinery, resulting in a 760 base transcript. Drugs were incubated with DNA template prior to the addition of nuclear extracts and nucleotides. The result demonstrates the ability of drugs to block synthesis of the 760 base transcript in a concentration-dependent manner. For example, 0.5 μM PA-10 inhibited transcript synthesis by 95% while 0.1 μM blocked the transcript less than 30% compared with the untreated control. Drug concentrations of 2 μM for distamycin, 0.5 μM for PA-2, and 0.2 μM for PA-10 were required to inhibit transcription by 50%. There was some evidence of the production of partial transcripts when higher drug concentrations were used. Comparison of the abilities of the polyamides with that of distamycin to inhibit transcript synthesis reveals that the potency of former compounds are about one order of magnitude greater. The very high affinity constant of polyamides likely contributes to the difference in activity.

Since polyamides show strong activities in cell-free conditions, we next evaluate the effectiveness of polyamides as Her2/neu transcription inhibitions in the whole cell system. Northern analysis and RNA synthesis assays were performed. AP-2, AP-10 along with several other drugs were tested in the Northern analysis. Treatment with 50 μM of distamycin for 48 hours decreases the Her2/neu mRNA of SKBR-3 cells by 50% while the mRNA of GAPDH is probed at the same time as comparison. However, no significant

change of Her2/neu mRNA was detected after up to 20 μM polyamide treatment. Meanwhile, the cytotoxicity assay failed to detect any cellular activity of polyamides. The result in our lab also strongly indicated that majority of the polyamide was located in the nucleus. It is unclear why the polyamides are located in the nucleus but do not show any functional activity.

In the process of the RNA synthesis assays, we have developed a more sensitive condition to detect the drug effects. When the traditional method for this assay was applied, the result showed that some drugs including distamycin and polyamides not only inhibit uridine incorporation, but also decrease uridine uptake at a comparable level. This observation makes it difficult to study the effect of RNA synthesis by using the uridine incorporation assay. The separation of the effects for uridine uptake from incorporation was greatly improved by adding 25 μM of cold uridine in the medium. The high uridine concentration at a saturated level for the uridine uptake helps to eliminate the drug effect on the uridine uptake.

There is a detectable polyamide cellular activity when nucleoside uptake assay was performed. Out of fifteen polyamides tested, nine of them inhibit nucleoside uptake, while six of them do not. When the chemical structures between these two groups were compared, it was found that all polyamides with internal β-alanin such as PA-10 don't inhibit nucleoside uptake, while polyamides without internal β-alanin such as PA-2 inhibit nucleoside uptake. This observation is interesting, but at this point we are not sure how this can be contributed to the project.

To understand factors in cellular environment that might interfere the polyamide-DNA binding, we take advantage of a fluorescent polyamide, PA-22. By using the fluorescent compound, we can detect the polyamide-DNA complex directly on an agarose gel. The fluorescent signal of polyamide-DNA complex is polyamide and DNA dose-dependent, and is competed by the parent polyamide, PA-2. This simple method allowed us to easily test the factors that interfere polyamide-DNA binding in cell-free condition. Factors that don't interfere polyamide-DNA binding include temperature (4°C - 70°C), pH (4 - 9.5), salt (up to 1M Na^+, 0.1M K^+, 10mM Ca^+), tRNA (up to 5μg/μl), BSA (up to 1%), and some cellular component such as polysome or nuclear extract. Factors that interfere polyamide-DNA binding include some detergents such as sarkosyl and SDS. These results will guide us to understand the basic pharmacological aspects of polyamide-DNA binding.

KEY RESEARCH ACCOMPLISHMENTS:

1. Sequence specific DNA minor groove binding polyamides bind with high affinity to ESX or AP-2 DNA binding sites in the HER2/neu promoter region.

2. As measured by mobility shift assay, polyamides inhibited formation of complexes between transcription factors and their DNA binding sites within the HER2/neu promoter region.

3. The polyamides effectively inhibit the HER2/neu promoter-driven transcription by using nuclear extract from the HER2/neu-overexpressing human breast cancer cell line, SKBR-3.

4. These polyamides showed a limited cellular activity based upon Northern analysis of HER2/neu mRNA or cytotoxicity assay.

5. Polyamides having the structure without internal β-alanine inhibit the cellular nucleoside uptake.

6. We conclude that polyamides have the potential to be designed specifically to block cellular proteins that are implicated in human breast cancers.

REPORTABLE OUTCOMES:

Chiang, S.-Y., Wang, Y.-D., Burli, R., Gawron, L., Scott, G.K., Benz, C.C., Dervan, P. and Beerman, T.A. (2000) Era of Hope Proceedings II, 729.

CONCLUSIONS:

We conclude that the first generation of polyamide are potent inhibitors of TF-DNA complex formation and transcription under cell-free conditions and that polyamides have the potential to be designed specifically to block cellular proteins that are implicated in human breast cancers. The information provided in this study can be utilized to improve polyamide specificity and effectiveness as selective transcription inhibitors. Modification of these compounds is under investigation to enhance their cellular activities.

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