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Award Number: DAMD17-99-1-9148

TITLE: Breast Cancer Therapeutics, Environmental Estrogens, and  
the Estrogen Receptor (ER); Characterization of the  
Diverse Ligand Binding Properties of the ER

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REPORT DATE: July 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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DTIC QUALITY INSPECTED 4

20001124 050

# REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

<b>1. AGENCY USE ONLY (Leave blank)</b>		<b>2. REPORT DATE</b> July 2000	<b>3. REPORT TYPE AND DATES COVERED</b> Annual (1 Jul 99 - 30 Jun 00)	
<b>4. TITLE AND SUBTITLE</b> Breast Cancer Therapeutics, Environmental Estrogens, and the Estrogen Receptor (ER); Characterization of the Diverse Ligand Binding Properties of the ER			<b>5. FUNDING NUMBERS</b> DAMD17-99-1-9148	
<b>6. AUTHOR(S)</b> Deborah Wuttke, Ph.D.				
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> University of Colorado Boulder, Colorado 80309  E-MAIL: Deborah.Wuttke@Colorado.EDU			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b> None				
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for public release; distribution unlimited			<b>12b. DISTRIBUTION CODE</b>	
<b>13. ABSTRACT (Maximum 200 Words)</b>  The estrogen receptor (ER) is found in the nucleus of several tissues, including breast, bone, liver, the organs of the reproductive system, and the cardiovascular system. The ER binds several types of compounds, including compounds that are quite distinct from its natural ligand. Estrogens bind to and activate the ER, which leads to the stimulation of transcription of genes containing an estrogen responsive element (ERE). Antiestrogens and partial antiestrogens bind tightly to the ER but fail to activate transcription; these compounds are currently in widespread use for the treatment of breast cancer. In addition, a variety of compounds introduced into the environment by human activity have also been found to act as estrogen mimics and alter reproductive function and development. The goals of this project are to understand, on a molecular level, how the ER binds estrogens, anti-estrogens, and estrogen mimics present in the environment, how this binding triggers activity, and how mutations in the ER discovered in breast cancer patients affect ER activity. Towards this end, we have expressed and purified the ligand binding domain of the estrogen receptor, and studied complexes with estradiol, the natural ligand, and tamoxifen, a partial antiestrogen in use as a breast cancer therapeutic using high-resolution heteronuclear NMR spectroscopy.				
<b>14. SUBJECT TERMS</b> Breast Cancer, Estrogen Receptor, Nuclear Magnetic Resonance Spectroscopy, Estradiol, Tamoxifen			<b>15. NUMBER OF PAGES</b> 8	
			<b>16. PRICE CODE</b>	
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)  
Prescribed by ANSI Std. Z39-18  
298-102

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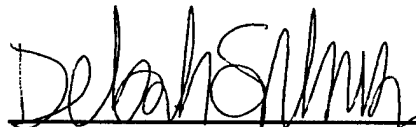
N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

  
PI - Signature

6/30/00  
Date

**Annual Report for Breast Cancer Therapeutics, Environmental Estrogens,  
and the Estrogen Receptor (ER): Characterization of the Diverse Ligand  
Binding Properties of the ER**

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**TABLE OF CONTENTS**

Front Cover	1
Standard Form (SF) 298	2
Foreword	3
Table of Contents	4
Introduction	5
Body	5
Key Research Accomplishments	7
Reportable Outcomes	7
Conclusions	7
References	8
Appendices	8

## INTRODUCTION

The estrogen receptor (ER) is found in the nucleus of several tissues, including breast, bone, liver, the organs of the reproductive system, and the cardiovascular system. The ER binds several types of compounds, including compounds that are quite distinct from its natural ligand. Estrogens bind to and activate the ER, which leads to the stimulation of transcription of genes containing an estrogen responsive element (ERE). Antiestrogens and partial antiestrogens bind tightly to the ER but fail to activate transcription; these compounds are currently in widespread use for the treatment of breast cancer. In addition, a variety of compounds introduced into the environment by human activity have also been found to act as estrogen mimics and alter reproductive function and development. The estrogenic behavior of these compounds has proven difficult to predict from their structures; many of these hormone mimics bear little structural resemblance to natural estrogens. The molecular level details of the conformational changes that allow the ER to tightly bind a diverse array of compounds and result in diverse patterns of gene activation are not understood. The goals of this project are to understand, on a molecular level, how the ER binds estrogens, anti-estrogens, and estrogen mimics present in the environment, how this binding triggers activity, and how mutations in the ER discovered in breast cancer patients affect ER activity. A complete structural understanding of how various ligands interact with the ER, and are able to elicit different responses, will assist in identifying compounds with therapeutic benefit for treating breast cancer. In addition, our studies will contribute to understanding the effects environmental estrogens have on breast cancer, and in identifying estrogenic activity in compounds before they are introduced into the environment.

## BODY

The large numbers of compounds which bind the ER will require the use of an innovative and rapid approach. We are investigating ligand binding to the ER using multidimensional nuclear magnetic resonance spectroscopy (NMR) studies of complexes of these compounds with the ligand binding domain (LBD) of the ER. The ER-LBD is uniformly isotopically labeled with  $^{13}\text{C}$  and  $^{15}\text{N}$  and ligand binding will be followed by acquiring heteronuclear single quantum coherence (HSQC) spectra, which are exquisitely sensitive to molecular conformation. Also, we will study the structural effects of several point mutations isolated from breast cancer cell lines. We will investigate the ligand binding differences between  $\text{ER}\alpha$  and  $\text{ER}\beta$ , the two natural variants of the ER. These differences may play an important role in the tissue specific activity of partial antiestrogens. Analysis of our NMR data will be significantly aided by the present availability of the crystal structures of the ER bound to an estrogen and an antiestrogen. The use of NMR spectroscopy as an efficient tool for screening for compounds with estrogenic or antiestrogenic activity, or with activity specific to either  $\text{ER}\alpha$  or  $\text{ER}\beta$  will be explored.

Progress towards the tasks in the statement of work are as follows:

### *Technical Objective 1:*

Prepare and purify unlabeled LBD-ER <sub>2</sub>	2 Months
Identify solution conditions suitable for NMR experiments	1 Months
Collect and evaluate preliminary homonuclear NMR data	1 Month

The tasks associated with technical objective 1 have been completely achieved. The LBD-ER was prepared by expression in BL21(DE3) *E. coli* by induction with IPTG from a pET plasmid. Complete purification was achieved using an estradiol affinity column that afforded protein of high purity and activity in one step. In addition, the purified protein is highly stable to proteolysis, a problem that rendered protein purified by other strategies inactive within a few days of preparation. A complete buffer screen revealed optimal buffer conditions (20mM d-Tris, 200mM NaCl, 1mM d-DTT, 0.02% sodium azide) that allowed us to prepare samples with concentrations of up to 500  $\mu$ M. Homonuclear NMR data obtained on these samples revealed features consistent with a protein of the anticipated molecular weight. The optimum temperature for data collection was determined to be 30°C. The anticipated molecular weight of the estradiol bound complex was confirmed by light scattering data.

*Technical Objective 2:*

Prepare and purify ligand complexes of $^{15}\text{N}$ -labeled ER from minimal growths	2 Months
Collect and evaluate $^{15}\text{N}$ - $^1\text{H}$ HSQC NMR data at 500 MHz	1 Month
Collect and evaluate $^{15}\text{N}$ - $^1\text{H}$ HSQC NMR data at 600 MHz	1 Month
Prepare and purify ligand complexes of $^{13}\text{C}$ , $^{15}\text{N}$ -labeled ER from minimal growths	2 Months
Collect and evaluate $^{13}\text{C}$ and $^{15}\text{N}$ - $^1\text{H}$ HSQC NMR data at 500 MHz	1 Month
Collect and evaluate $^{13}\text{C}$ and $^{15}\text{N}$ - $^1\text{H}$ HSQC NMR data at 600 MHz	1 Month
Prepare and purify ligand complexes of isotopically labeled ER from minimal growths	3-9 Months
Collect and evaluate $^{13}\text{C}$ and $^{15}\text{N}$ - $^1\text{H}$ HSQC NMR data of complexes at optimum NMR conditions	6-12 Months
Investigate deuteration strategies	6-9 Months

The tasks associated with technical objective 2 have been completely achieved. Growth of uniformly  $^{15}\text{N}$  labeled protein progressed smoothly based on the expression and purification scheme developed for unlabeled protein.  $^{15}\text{N}$ - $^1\text{H}$  HSQC NMR data were collected at 500 and 600 MHz. Significant improvement of data quality was observed at higher field strengths and with the addition of TROSY pulse sequences. Based on these data, we determined that data should be collected at as high of a field strength as possible; towards this end we are pursuing collaborations with researchers with access to an 800 MHz spectrometer. In addition, TROSY-based pulse sequences will be used exclusively to study this system given the large enhancement. A tamoxifen-bound complex was prepared to compare tamoxifen to estradiol-bound forms of the protein. Significant chemical shift differences were noted between these two complexes, as anticipated, illustrating the validity of our strategy.

In order to interpret the chemical shifts in the context of available structures, resonance assignments need to be completed. Towards this end, uniformly  $^{13}\text{C}$ ,  $^{15}\text{N}$  and  $^2\text{H}$  labeled protein has been prepared. The  $^{13}\text{C}$  and  $^{15}\text{N}$  labels will allow assignment of the backbone resonances via their direct scalar couplings. Complete deuteration is employed to further reduce linewidths by eliminating the dominant mechanism of line broadening, relaxation via  $^1\text{H}$ - $^1\text{H}$  dipolar couplings. To achieve this result, growth in  $\text{D}_2\text{O}$  has been optimized. Initial efforts aimed at obtaining triple resonance data for resonance assignments indicate that further optimization, either through preparation of higher concentration samples or data collection at higher field, will be necessary.

In addition to the work described above, CD denaturation studies have been conducted on the estradiol and tamoxifen complexes. These data have allowed us to compare the thermodynamic stabilities of these complexes.

Future work on this project will be focused on Technical objectives 3 and 4. If it is not feasible to obtain resonance assignments using the fully labeled protein, selective labeling strategies allowing uniform incorporation of only one amino acid type or selective labeling of the C-terminus will be employed.

## KEY RESEARCH ACCOMPLISHMENTS

- Highly purified LBD-ER has been prepared.
- Conditions for NMR data collection have been optimized.
- $^{15}\text{N}$ - $^1\text{H}$  HSQC NMR data on estradiol and tamoxifen complexes has been obtained.
- Uniformly  $^{13}\text{C}$ ,  $^{15}\text{N}$  and  $^2\text{H}$  labeled protein has been prepared.
- The process of resonance assignment has been initiated.

## REPORTABLE OUTCOMES

*Posters presented:* The work described in this progress report has been presented at several meetings as either a talk or poster. These include (1) The University of Colorado Annual Retreat (Winter Park, Colorado) (2) Environmental Hormones: Past, Present, Future (Tulane University, New Orleans, LA) (3) The University of Colorado Biophysics Supergroup Meeting and (4) The EPA-STAR Award Annual Meeting (Washington, DC).

*Graduate Student Training:* One graduate student, Dana Warn, has been trained on this research project. She has been primarily responsible for protein expression and purification protocols, and NMR spectroscopy. Her stipend and tuition has been provided through an EPA-STAR fellowship.

*Undergraduate Training:* Andrea Wismann, an undergraduate at the University of Colorado, Boulder, conducted independent research on this project from August, 1999 through June 2000. Through this research experience, she learned protein expression and purification strategies, as well as circular dichroism spectropolarimetry.

## CONCLUSIONS

In the first year of funding, good progress has been made towards the stated goals. Highly purified, stable samples have been obtained. Complexes of LBD-ER with estradiol and the partial antiestrogen tamoxifen have been prepared and characterized by high resolution NMR spectroscopy. Preliminary experiments aimed at complete resonance assignment have been

initiated. Future work towards technical objectives 3 &4 has been undertaken. In addition, alternate strategies for obtaining assignment information are being developed.

**REFERENCES**

No references are included in this report.

**APPENDICES**

No appendices are attached.