Award Number: DAMD17-03-1-0433

TITLE: Proteomic Analysis of Genistein Mammary Cancer Chemoprevention

PRINCIPAL INVESTIGATOR: Coral A. Lamartiniere, Ph.D.

CONTRACTING ORGANIZATION: University of Alabama at Birmingham
Birmingham, AL 35294-0111

REPORT DATE: July 2005

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;
Distribution Unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.
**REPORT DOCUMENTATION PAGE**

**REPORT DATE (DD-MM-YYYY)**: 01-07-05

**REPORT TYPE**: Annual

**DATES COVERED (From - To)**: 07/01/04-06/30/05

**TITLE AND SUBTITLE**: Proteomic Analysis of Genistein Mammary Cancer Chemoprevention

**AUTHOR(S)**: Coral A. Lamartiniere, Ph.D.

E-Mail: Coral@uab.edu

**PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**: University of Alabama at Birmingham
Birmingham, AL 35294-0111

**SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**DISTRIBUTION / AVAILABILITY STATEMENT**: Approved for Public Release; Distribution Unlimited

**SUBJECT TERMS**: Genistein, Breast Cancer Chemoprevention, Proteomics, Rats

**SECURITY CLASSIFICATION OF:**
- a. REPORT: Unclassified
- b. ABSTRACT: Unclassified
- c. THIS PAGE: Unclassified

**LIMITATION OF ABSTRACT**: Unclassified

**NUMBER OF PAGES**: 9

**NAME OF RESPONSIBLE PERSON**

**TELEPHONE NUMBER (include area code)**: 301-619-7325

---

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18
Abstract

Using 2-D gels and mass spectrometry we have discovered that GTP-cyclohyrolase 1 protein expression is significantly increased in mammary glands of 21 day old rats, but not in 50 day old rats following prepubertal only exposure to the phytoestrogen genistein. By evaluating related metabolic pathways we found up-regulated P-ERK-1, but no significant short-term changes in the tyrosine hydroxylase and INOS protein expressions in mammary glands of 21 day old rats. At day 50, there was significant up-regulation of tyrosine hydroxylase and VEGF-R2. This and previous work suggests that early postnatal (prepubertal) exposure to genistein enhances cell proliferation and cell differentiation and gland maturation. This unique development maturation leads to a new biochemical "blue-print" whereby the cells have reduced EGF-signaling and VEGFR2 that render the mature mammary gland less proliferative and susceptible to chemically induced mammary cancer initiation, angiogenesis and for cancer progression. This study demonstrates the usefulness of proteomics for the discovery of novel pathways that may be involved in cancer prevention. Our ongoing work is to collect proteins from interstitial fluid surrounding mammary glands of rats, and to identify and characterize the major proteins that are modulated by DMBA.
# Table of Contents

Cover

SF 298

Contents

Introduction 4

Body 4

Key Research Accomplishments 6

Reportable Outcomes 6

Conclusions 7

References 7

Appendices 8
INTRODUCTION
Breast cancer is the most common malignancy diagnosed in American women. Even with improved technology for early detection and aggressive therapeutics, most often the disease is incurable once it is discovered. We believe that prevention rather than therapy is the desired future against cancer, and that innovative approaches and new technology will be the key to breakthroughs. Towards this, our laboratory has been studying how developmental alterations to the mammary gland can program against this cancer. More specifically, we have demonstrated that prepubertal only, and prepubertal plus adult, exposure(s) to dietary genistein, a phytoestrogen component of soy, confer(s) a long-term protective effect against dimethylbenz(a)anthracene (DMBA)-induced mammary cancer in rats (1). Consistent with our findings are epidemiological reports that Asian women exposed to a diet high in soy during adolescence have a lower incidence of breast cancer (2, 3). We have hypothesized that genistein exerts its chemopreventive actions by postnatally programming developmental modifications to genes/proteins that render the mammary gland less susceptible to cancer. The objective of this proposed research is to identify regulatory proteins responsible for conferring breast cancer protection using innovative aims and technology.

BODY

Specific Aim 1) To identify proteins that are differentially expressed in mammary glands of rats treated ± the carcinogen, DMBA, and the chemopreventive agent, genistein.

As previously reported and approved in last year's report, we treated female rats prepubertally (500 µg genistein/g BW, subcutaneously on days 16, 18 and 20 postpartum) with genistein and investigated the proteome in mammary glands of 21 and 50 day old female Sprague Dawley rats. Mammary glands were subjected to 2-D gel separation and then the stained gels were scanned via a BioRad flat bed densitometer and analyzed with the Progenesis (nonlinear) 2-D gel software system. Differentially regulated protein spots were excised from the gels, destained and prepared for tryptic fragmentation. Tryptic digests of each protein were analyzed using a Voyager MALDI TOF and the spectra produced were matched against a non-redundant protein database using MASCOT to determine the protein's identity. One protein that was determined to be differentially up-regulated from prepubertal genistein treatment was guanosine triphosphate cyclohydrolase one (GTP-CH1) in mammary glands of 21 day old rats, but not in 50 day old rats.

RESULTS for Tasks a-c and f-h. Now, we report that P-ERK-1 was also up-regulated in mammary glands of 21 day old rats only. In 50 day old rats, mammary tyrosine hydroxylase protein expression was up-regulated and now we report that vascular endothelial growth factor receptor two (VEGFR-2) was down-regulated. This and previous work suggests that early postnatal (prepubertal) exposure to genistein enhances cell proliferation by up-regulating GTP-CH1 and the EGF-signaling pathway, and hence cell differentiation and gland maturation. This unique developmental maturation leads to a new biochemical “blue-print” whereby the cells have reduced EGF-signaling and VEGFR2 that render the mature mammary gland less proliferative and susceptible to chemically induced mammary cancer initiation, angiogenesis and for cancer progression (Figure 1). Therefore, genistein acts through a diverse and coordinated effect of signaling mechanisms and pathways that likely account for the cellular changes responsible for its chemopreventative action. This study demonstrates the usefulness of proteomics for the discovery of novel pathways that may be involved in cancer prevention.
Figure 1. Proposed Genistein Action in the Mammary

In regards to Tasks d and e of Aim 1, we have subjected the mammary glands of 75 day old rats treated at day 50 with and without the carcinogen, DMBA, to 2-D gels and mass spec and have already identified one protein to be significantly down-regulated. This protein is guanine deaminase, an enzyme involved in nucleic acid synthesis. We are presently constructing a virtual pathway to carry out functional proteomics.

Also in the third year, we will analyze mammary glands of rats exposed prepubertally with and without genistein and with and without DMBA at day 50.

Task i. The 2005 AACR meeting was attended and data from Aim I was presented.

Task j. Biostatistical and bioinformatic analyses of the data are in progress., One manuscript has been accepted for publication in Journal of Proteome Research (Modeling Biological Variability in 2-D gel Proteomic Carcinogenesis Experiments), and another (Proteomic Discovery of Genistein Action in the Rat Mammary Gland) is in preparation.

Specific Aim 2. To collect proteins from interstitial fluid surrounding mammary glands of rats, and to identify and characterize the major proteins that are modulated by DMBA and genistein.

Task a) After agonizing with design and building of our own ultrafiltration probes, we have found commercially available probes that we can purchase from Bioanalytical Systems, Inc. The MF-7026 ultrafiltration probes, with a molecular weight "cut-off" of 30,000, allow us to collect mammary interstitial fluid.
Task b-f) We have bred and treated female offspring prepubertally with and without genistein (protocol C, approved last year) and at day 50 with and without DMBA. Ultrafiltration probes are being placed in the mammary glands of rats and we have been able to collect interstitial fluid.

Task g) Now, we are investigating the use of chromatofocusing, 1-D gel separation, coupled with mass spectrometry for protein enrichment and identification. Presently, we are investigating the resolution capabilities with a chromatofocusing column from GE HealthCare (formally AmershamBioscience) and an automated gradient mixer and HPLC system (a BioRad "demo"). To date we have demonstrated that we can get peak separation using very small pH ranges (i.e. 4-5.6), collecting peak fractions as opposed to time or volume collection. In order to overcome issues of sample pooling we have begun to use the Experion automated electrophoresis system from BioRad. This "lab on a chip" technology allows us to use very small loading volumes (4 μl) to perform protein separation. The computer assisted analysis provides accurate information as to protein concentration and protein mass to look at alterations in expression levels of protein bands between groups. Since we have created a discreet group of enriched proteins by filtering out larger proteins and separating the mixture by chromatofocusing (separation by charge) there are a finite number of bands visible within each collected fraction. The Experion’s sensitivity and concentration measurement allow us to look for changes in protein levels without the a priori knowledge of the specific protein. While we are unable to recover the same sample for further examination/identification we have sufficient sample left from the FPLC system to run a traditional 1-D gel using higher loading concentrations and to identify our peak of interest by subsequent band excision and MALDI-TOF/MS. The advantage of the initial use of the use of this system over traditional means is evident in the low sample volume required, resulting in no need to pool samples to acquire quantifiable data. It is our intent to analyze the proteins from mammary interstitial fluid by LC-MS/MS from rats treated ± genistein and DMBA.

Task h) Tissue slices from mammary gland of 75 and 100 day old rats exposed to DMBA (at day 50) have been submitted for histomorphological evaluation.

Task i. The 2005 AACR meeting was attended.

Task j) Methods development and data gathering is in progress.

**KEY RESEARCH ACCOMPLISHMENTS**

- We have developed reproducible 2-D gel and biostatistical methodologies to evaluate the proteome of mammary glands.
- Prepubertal genistein treatment up-regulates GTP-CH1 in mammary glands of 21-, but not 50-, day old female rats.
- Prepubertal genistein treatment up-regulates P-ERK-1 in mammary glands of 21-, but not 50-, day old female rats.
- Prepubertal genistein treatment up-regulates tyrosine hydroxylase in mammary glands of 50, but not 21, day old female rats.
- Prepubertal genistein treatment down-regulates VEGFR-2 in mammary glands of 50, but not 21, day old female rats.
- In the 21 day old animals we found no significant changes in BH4 and iNOS protein expressions.

**REPORTABLE OUTCOMES**


CONCLUSION
To date, we have discovered that GTP-CH1 expression is significantly increased shortly following exposure to genistein. However, this was not a permanent effect because by day 50, in the absence of genistein, there was no difference between the treatment groups. By evaluating related metabolic pathways, we have been able to identify down-stream targets and evaluate changes in these proteins in response to the changes of GTP-CH1. In the 21 day old animals we found no significant short-term changes in the tyrosine hydroxylase and iNOS protein expressions. However, at the 50 day time point there was significant up-regulation of tyrosine hydroxylase and VEGF-R2 expression. Since this difference is measurable 30 days after the final genistein treatment, we postulate that some underlying programming effect on protein expression is manifested in the long-term expression profile of this downstream target. This and previous work suggests that early postnatal (prepubertal) exposure to genistein enhances cell proliferation by up-regulating GTP-CH1 and the EGF-signaling pathway, and hence cell differentiation and gland maturation. This unique developmental maturation leads to a new biochemical “blue-print” whereby the cells have reduced EGF-signaling and VEGFR2 that render the mature mammary gland less proliferative and susceptible to chemically induced mammary cancer initiation, angiogenesis and for cancer progression. Therefore, genistein acts through a diverse and coordinated effect of signaling mechanisms and pathways that likely account for the cellular changes responsible for its chemopreventative action. This study demonstrates the usefulness of proteomics for the discovery of novel pathways that may be involved in cancer prevention.

REFERENCES


7. Basu, S. et. al. The neurotransmitter dopamine inhibits angiogenesis induced by vascular 

APPENDICES

None