**Title:** In Utero Estrogen Exposure Increases Antiestrogen Resistance by Inducing EMT

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**Abstract:**

Endocrine therapies such as antiestrogens (AE) are widely used in estrogen receptor positive (ER+) breast cancer, due to their efficacy and limited toxicity. However, the clinical reality is that of the ~50% of ER+ patients that initially respond to these therapies, many will acquire resistance and recur. The causes of antiestrogen resistance are currently unclear. Our recent preclinical study found that maternal exposure to excess estrogens during pregnancy increases the risk that AE resistance in ER+ mammary tumors in the offspring. This increase may be caused by epigenetic mechanisms, such as an increase in DNA methyltransferases (DNMT) and methylation, and histone deacetylases (HDAC) and histone modifications. In this study, our preliminary data show not only an increase in TAM resistance in rats exposed to in utero estrogen, but concurrent treatment with DNMT and HDAC inhibitors inhibited this resistance, suggesting that epigenetic changes driven by estrogenic exposures early in life may preprogram the breast to develop tumors with a higher propensity to develop antiestrogen resistance.

**Subject Terms:**

- In Utero
- Estrogen Exposure
- Antiestrogen Resistance
- EMT
- Endocrine Therapies
- Antiestrogens (AE)
- Breast Cancer
- ER+
- Resistance
- Recurrence
- Epigenetic Mechanisms
- DNA Methyltransferases (DNMT)
- Methylation
- Histone Deacetylases (HDAC)
- Histone Modifications
- TAM Resistance
- Rats
- Preclinical Study
- Maternal Exposure
- Estrogenic Exposures
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1. Introduction

It is well known that in utero estrogenic exposures influence cancer risk in offspring [1-3]. We recently found that in utero exposure to the synthetic estradiol (ethinyl estradiol, EE2) increased rat mammary cancer risk in daughters, granddaughters, and great-granddaughters. [4] This multigenerational increase in risk was accompanied by persistent changes in DNA methylation as well as an increase in DNA methyltransferase expression, suggesting that elevated hormone levels during pregnancy induce epigenetic changes in the offspring of several subsequent generations [4]. Recently, our laboratory developed a preclinical rat model that recapitulates the clinical picture of Tamoxifen (TAM; an antiestrogen) resistance [manuscript submitted]. Using this DMBA-driven model we showed that in utero EE2 exposed rats are significantly more resistant to TAM vs. control rats. Importantly, we have recently conducted a pilot experiment in in utero EE2 exposed rats using the HDAC inhibitor valproic acid (VA) and the DNMT inhibitor hydralazine (H) as 2nd line therapy and showed a decrease in TAM resistance in these animals compared to controls. Taken together, these data suggest that in utero EE2 exposures increases TAM resistance, likely through epigenetic alterations, and the increased risk may be reversible with 1st line VA and H combined with TAM treatment. In this DOD award, I proposed that using VA and H combined with TAM treatment as a 1st line therapy in in utero EE2 exposed rats may be able to reverse the increased risk of TAM resistance. The central purpose of this study is to determine how epigenetic alterations preprogramed by in utero EE2 exposures drive resistance to TAM and whether this is reversible with drugs that reverse these epigenetic changes (VA and H). Finally, in years 2 and 3, we aim to identify an epigenetic signature that can predict which women will benefit from combination therapy as well as which molecular mechanisms play a role in in utero EE2 induced increases in TAM resistance.

2. Keywords
Breast cancer, in vivo model, antiestrogen resistance, Tamoxifen, epigenetics

3. Research Accomplishments:
Specific Aim1: Determine whether VA and H can prevent the increased TAM resistance observed in in utero estrogen exposed mammary tumors.

Reportable Results: The major goal of year 1 of this grant was to conduct an in vivo experiment to address whether using VA and H in combination with TAM as a first line therapy in in utero EE2 exposed rats can prevent the development of TAM resistance. Briefly, pregnant Sprague Dawley dams (Harlan, USA) were fed a modified AIN93G control diet (soybean replaced with corn oil), that contained either 0 (control, C; n=12) or 0.1 ppm EE2 (n=12) between gestation day 10 and 20; after that all dams were fed control AIN93G diet. Pregnant dams gave birth to an average of 8 pups each in both treatment groups. All pups were weaned on postnatal day (PND) 21, and continued on the control diet. In utero EE2 exposed rats exhibited significantly earlier vaginal opening than control exposed rats (p=0.005), consistent with an accelerated sexual maturation in the EE2 exposed rats [Figure 1] as we have previously seen [1].
Mammary tumors were induced in the female offspring on PND 50 in both in utero EE2 and control animals by oral gavage of 10 mg of 9,12-dimethylbenz[a]anthracene (DMBA) (Sigma, St. Louis, MO) in 1 ml of peanut oil. Rats were examined for mammary tumors by palpation every week, starting three weeks post-DMBA. Tumor growth was measured using a caliper and the length and width of each tumor was recorded. Once the rats had palpable mammary tumors that reached a defined size (longest diameter 13mm), drug treatments were started. The study had three treatment arms: 1) Tamoxifen citrate (TAM, administered via diet, resulting in ca. 15 mg/kg daily dose), 2) TAM + HDAC inhibitor VA and the DNMT inhibitor H as concurrent 1st line treatment (TAM dose = arm 1; VA and H administered via drinking water, resulting in ca. 1.16 g/kg and 5 mg/kg daily doses, respectively), and 3) VA and H alone. In utero EE2 and control animals were divided evenly amongst these three groups.

Drug treatments were begun once the first tumor in an individual animal reached 13 mm in diameter therefore, the treatment start varied from animal to animal depending upon the rate of growth of the tumors. Control rats were treated with TAM for an average of 19.5±2.273 weeks, while EE2 rats were treated with TAM for 15.73±2.273 weeks [Table 1]. EE2 rats were treated with TAM+VA & H for 14.86±2.07 weeks and control animals for 18.14 ±1.96 weeks. EE2 animals were treated with VA and H alone for 7.85 ± 1.50 weeks and control rats for 13 ±1.98 weeks. Treatment length was variable as it was dependent on the response, and thus rats with more quickly growing (resistant) tumors had to be euthanized according to the requirements of our IACUC. Overall the control rats had a longer mean treatment time vs. EE2 rats for all treatments, suggesting that the tumors in the EE2 animals grow at a faster rate. Further, the shortest duration of treatment was in the EE2 rats treated with VA and H alone.

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>Mean Treatment (EE2)</th>
<th>Treatment Range (EE2)</th>
<th>Mean Treatment (Ctrl)</th>
<th>Treatment Range (Ctrl)</th>
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<tr>
<td>TAM</td>
<td>15.73 ±2.27</td>
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<td>19.5 ±2.27</td>
<td>2–35 weeks</td>
</tr>
<tr>
<td>TAM+VA and H</td>
<td>14.86 ±2.07</td>
<td>2–34 weeks</td>
<td>18.14 ±1.96</td>
<td>4–34 weeks</td>
</tr>
<tr>
<td>VA and H</td>
<td>7.85 ±1.505</td>
<td>1-22 weeks</td>
<td>13 ±1.98</td>
<td>1-24 weeks</td>
</tr>
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</table>

Table 1: Mean time of treatment and range in in utero exposed EE2 and control rats.
Animals were followed for ~40 weeks post-DMBA treatment. During the follow-up, animals in which tumor burden approximated 10% of total body weight were sacrificed, as required by our Institution. Rats were housed in a temperature- and humidity-controlled room under a 12-hour light-dark cycle. All animal procedures were approved by the Georgetown University Animal Care and Use Committee, and the experiments were performed following the NIH guidelines for the proper and humane use of animals in biomedical research. At the completion of the study, the remaining rats were euthanized.

Treatment responses were classified as (1) complete response (CR, tumor disappeared), partial response (PR, tumor stopped growing and/or began to regress), and de novo resistant (R, tumor continued to grow). Some tumors exhibiting a CR (undetectable for at least 4 weeks), subsequently recurred at the original location and began rapidly growing (to at least 13mm in diameter) and thus were assigned an acquired resistant (AR) classification.

Rats exposed to EE2 in utero showed significantly different response patterns to TAM (p<0.001) with more de novo TAM resistance compared to control rats (p=0.018) [Figures 2 and 3]. Control rats exhibited 29% CR, 33% PR and 38% R while the EE2 rats showed 18% CR, 22% PR and 60% R, respectively. Local recurrence of CR tumors (AR) was more than 2 times as common in rats exposed to EE2 in utero (63%) than control rats (30%) (p<0.001) [Figure 4] Further, the average length of CR in EE2 animals ~50% less than in control animals (5 vs 11 weeks, respectively; p=0.029). Taken together, these data suggest that rats exposed to EE2 in utero were more likely to develop both de novo and acquired TAM resistant tumors than control rats.

The concurrent use of VA and H with TAM as a first line therapy markedly altered the response profile of the in utero EE2 exposed rats. While EE2 rats showed 60% R when treated with TAM alone, the addition of VA and H to TAM reduced R to 40% (p=0.002)
[Figures 2 and 3]. In addition, this combined therapy reduced AR from 63% in this group to 16% (p<0.001). [Figure 4]. This suggests that it may be the epigenetic changes preprogrammed by EE2 exposure in utero partially driving the TAM resistance in these animals. The addition of VA and H to TAM treatment did not affect the average duration of CR in either the EE2 or control exposed rats.

Importantly, in those animals not exposed to EE2 in utero (control rats), the addition of VA and H led to a significantly worse outcome, increasing R from 38% to 53% (p=0.02) and reducing CR by more than half (p<0.001) [Figures 2 and 3]. Further, AR more than doubled from 30% to 63% in the control treated animals (p<0.001) [Figure 4]. This data may have important clinical implications, as DNMT and HDAC inhibitors are currently being studied in the clinic for their potential to improve therapeutic outcomes in breast and other cancers [5-7]. Based on the results from this experiment it may be critical to determine whether there has been an estrogenic exposure in utero that might influence whether a patient will have a worse outcome with combination therapy. In Aims 2 and 3, we will attempt to find an epigenetic signature that can predict which women will benefit from a combination of TAM plus HDAC and DNMT inhibitors.

Pretreatment biopsies were collected from each treatment group prior to commencing drug treatment. These will be used to create conditionally reprogrammed cell lines in Aim 2 (year 2) from each of the treatment groups and treatment outcomes. Upon sacrifice, mammary glands, tumors, blood, liver, spleen, brain, ovary, uterus and lung were collected from each animal. We froze tissue/blood samples from each animal for isolation of protein, RNA and DNA for potential use in subsequent Aims of this project. Further, we paraffin embedded mammary glands, tumors and organs for histopathology and immunohistochemical analysis (years 2-3). As the in vivo study just concluded this past week, tumor histopathology has not yet been conducted. Therefore, the results above are preliminary and final results available after histopathological classification of the tumors will be included in the report for Year 2.

References:


**Training and Professional Development:**

In year 1 of this grant, my training goals included auditing relevant courses, attending weekly seminars, and learning new laboratory techniques. I achieved all of these goals. I am currently auditing a graduate level course at Georgetown entitled “Cancer Epigenetics”. Next semester, I plan to audit a course on Cancer Systems Biology. I routinely attend both faculty and visiting lecturer seminars within the Lombardi Comprehensive Cancer Center at Georgetown. Further, I participate in weekly lab meetings in both the Clarke and Hilakivi-Clarke labs (my co-mentors). I also attend joint lab meetings with our math modeling and systems biology collaborators from Virginia Tech. I recently had the opportunity to collaborate with another PI at Georgetown, Dr. Priscilla Furth, which resulted in authorship on a paper published in Carcinogenesis this year. I also have learned a variety of scientific techniques this year. During my predoctoral work I conducted in vivo experiments in nude mice. This year, I gained experience using a carcinogen-induced in vivo rat model. I also began learning how to conditionally reprogram cells taken from my rat tumors (a technique required for year 2 of this grant).

In addition to meeting my stated training and professional development goals for this year, I also had the opportunity to teach a Cancer Research Techniques class (Tumor Biology graduate level course) specifically on cell culture and animal models. Further, I have been mentoring a Georgetown University undergraduate student who is a Howard Hughes research scholar and supervising her Senior thesis research project related to antiestrogen resistance. Even though she is not working on my project, I have exposed her to the preclinical animal model being used in my project. This will likely help her in her future research, as she will start an MD/PhD program
next year, where she will focus on cancer research. Finally, I was awarded a Georgetown University Medical Center Faculty Research Recognition Award this year for my research related to this grant.

4. Impact

Given the rate of obesity in this country (high fat diets resulting in elevated circulating estrogen levels), the prevalence of bisphenol A in our drinking and food containers, as well as the millions of women who took diethylstilbestrol while pregnant before it was banned, it is likely that the number of women exposed to increased levels of estrogenic chemicals in the womb is extremely high. If these women might have an increase in Tamoxifen (TAM) resistant estrogen receptor positive (ER+) breast cancer, as our data suggest, then it is critical that we find effective treatment regimens, as women with resistant (recurrent) ER+ tumors are the most likely to die of their disease. The data provided from the in vivo study from year 1 of this project show that in utero EE2 exposure increases both de novo (never responds to treatment) and acquired resistance (recur despite treatment) to TAM. This increased resistance may be caused by epigenetic mechanisms that change gene levels without altering the gene sequence (not a mutation of the gene). These epigenetic changes are inheritable and thus, are passed down from generation to generation. In this study, combining drugs that inhibit or reverse epigenetic changes (valproic acid and hydralazine) significantly reduced both de novo and acquired resistance in the in utero EE2 exposed rats. Importantly, the valproic acid and hydralazine in combination with TAM led to far worse outcomes than TAM alone in the control group. Therefore, it is necessary to find an epigenetic signature that might be able to predict whether a patient has been exposed to elevated estrogen in utero (to be addressed in Years 2 and 3). Thus, this data combined with the successful completion of the next two Aims (Years 2 and 3) of this grant has the potential to identify those women at highest risk of developing TAM resistant breast cancer, as well as to inform important clinical treatment decisions.

5. Changes/Problems

Nothing to report.

6. Products

Nothing to report.

7. Participants and Other Collaborating Organizations

Kerrie Briggs Bouker – PI – No changes

No collaborating organizations or updated funding - Nothing to report/no changes.
8. Special Reporting Requirements

Nothing to report.

9. Appendices

Current Curriculum Vitae (3 pages)
BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2. Follow this format for each person. DO NOT EXCEED FOUR PAGES.

NAME
Kerrie Briggs Bouker

POSITION TITLE
Postdoctoral Fellow

eRA COMMONS USER NAME (credential, e.g., agency login)

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)

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A. Personal Statement

My research has primarily focused on the hormonal and cytokine regulation of transcription factors and tumor suppressors. I have extensive experience with molecular and cell biology techniques, including the use of RNA. Further, I have experience with in vivo models of breast cancer, including xenograft, transgenic and DMBA models. I did not train in Dr. Hilakivi-Clarke’s lab during my predoctoral work but we have collaborated in the past, and as such have an excellent working relationship. Further, specific to this application, I have previous experience collaborating with clinicians and working in the public health arena. In 2003, I took family and medical leave to raise my two children, one of which has a rare autoimmune condition. I continued to be involved in science during that period as a part time free-lance science writer/editor. Now, with my daughter’s medical condition under control I have re-entered the laboratory. In January 2012, I returned to Georgetown University as a volunteer to reacquaint myself with laboratory work. I am currently a postdoctoral fellow in Dr. Hilakivi-Clarke’s laboratory where my research is focused on early life exposures and their relationship to breast cancer risk and antiestrogen resistance.

B. Positions and Honors


- August 1997 - April 2003: Predoctoral student, Georgetown University, Interdisciplinary Program in Tumor Biology, Department of Oncology, Lombardi Comprehensive Cancer Center.


- January 2012 – March 2013: Scientist (Volunteer), Georgetown University, Department of Oncology, Lombardi Comprehensive Cancer Center.
April 2013-present: Postdoctoral Fellow, Georgetown University, Department of Oncology, Lombardi Comprehensive Cancer Center.

2014: Georgetown University Medical Center Faculty Research Recognition Award, 2014

C. Selected Peer-reviewed Publications


D. Research Support

**Current Research Support**

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<td>Interferon Regulatory Factor-1 as a Mediator of Responsiveness to Antiestrogens and Cytotoxic Drugs in Breast Cancer.</td>
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