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14. ABSTRACT This research proposal has two primary objectives which are to (1) increase FIU investigators' research expertise and competitive ability to succeed as independent breast cancer researchers; and (2) to execute research with the promise of identifying molecular causes of breast tumor resistance to anti-estrogen therapy. This research is of significant merit because of its clinical relevance to breast cancer. Secondly, the research accomplishments through the FIU/BFBCI training program will lead to FIU investigator publication(s) in peer-reviewed journals that will facilitate the further advancement of the FIU investigators. We proposed to investigate how reactive oxygen species (ROS)-induced redox signaling pathways in breast cancer cells may contribute to molecular mechanisms of antiestrogen resistance. Our hypothesis is that the conversion of breast tumors to a tamoxifen-resistant phenotype is associated with a progressive shift towards a pro-oxidant environment of cells as a result of oxidative stress. We postulate that excess ROS levels induce both CDC25A and change p27 phosphorylation promoting the loss of its inhibitory function and leading to antiestrogen resistance. We will investigate whether reducing the oxidative environment of breast cancer cells will restore the anti-proliferative action of tamoxifen and other antiestrogens by repressing CDC25A and altering p27 phosphorylation and restoring p27 function.					
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INTRODUCTION

This research proposal has two primary objectives which are to (1) increase FIU investigators' research expertise and competitive ability to succeed as independent breast cancer researchers; and (2) to execute research with the promise of identifying molecular causes of breast tumor resistance to anti-estrogen therapy. We proposed to investigate how reactive oxygen species (ROS)-induced redox signaling pathways in breast cancer cells may contribute to molecular mechanisms of antiestrogen resistance. Our hypothesis is that the conversion of breast tumors to a tamoxifen-resistant phenotype is associated with a progressive shift towards a pro-oxidant environment of cells as a result of oxidative stress. We postulate that excess ROS levels induce both CDC25A and change p27 phosphorylation promoting the loss of its inhibitory function and leading to antiestrogen resistance. We will investigate whether reducing the oxidative environment of breast cancer cells will restore the anti-proliferative action of tamoxifen and other antiestrogens by repressing *CDC25A* and altering p27 phosphorylation and restoring p27 function.

BODY

The training tasks and their progress:

To extend and enhance the FIU investigators' skills to increase their research expertise and competitive ability to succeed as independent breast cancer researchers, we proposed to conduct the following training tasks each year during the 4yr period of this project: i) To conduct onsite weekly lab meetings in which FIU investigators and trainees reported research data, trouble-shooted, and planned experiments; ii) To meet every other week onsite to facilitate coordination of the project; iii) To participate in the monthly BFBCI Scientist Seminars at the University of Miami to broaden FIU investigators knowledge of the most current clinical research in breast cancer; iv) To establish an Invited Expert Breast Cancer Research Seminar Series at the FIU campus. v) To promote breast cancer research at the FIU campus, FIU/BFBCI training program will sponsor an annual onsite Breast Cancer Workshop. vi) To participate in the grant writing workshop entitled "The Molecular Mechanisms of Breast Cancer"; and vii) To prepare a written quarterly progress report of ongoing activities, and compile them together to prepare the annual progress report.

In order to meet the objective of the first training task, we have been holding weekly lab meetings on every Monday at 9.00 am at the FIU campus. Since the start date of this grant, both pre-doctoral students and faculty participated in the weekly lab meetings in which we reported the results and from the interpretation of the data we planned experiments for the upcoming week. To achieve task 2, all three FIU investigators have been involved in biweekly discussions to facilitate the forward progress of this proposal at FIU. To achieve task 3, all three FIU investigators and three pre-doctoral student trainees have participated in BFBCI Scientist Seminars at the University of Miami since June 2007. To achieve task 4, Dr. Felty has initiated the process to start the breast cancer seminar series at FIU and identified the speakers. To achieve task 5, we plan to hold an annual breast cancer workshop in August 2008. As part of training task 6, one of our FIU faculty participated in the NIH grant training workshop. For training task 7 we have records of our activities in MS PowerPoint presentations.

Challenges: Immediately upon the receipt of the award notice, we could not initiate the project because FIU Grants and Contract office first needed to open the account for this grant and then issue a sub-contract award to UM. This took a month. Two of our pre-doctoral trainees were able to join this project in June 1, 2007, however, the third pre-doctoral trainee joined us in August 2007, because she had to move from Houston, TX.

In summary, we have initiated all training tasks we proposed to carry out in the first year. And we will continue to participate and complete all the tasks proposed.

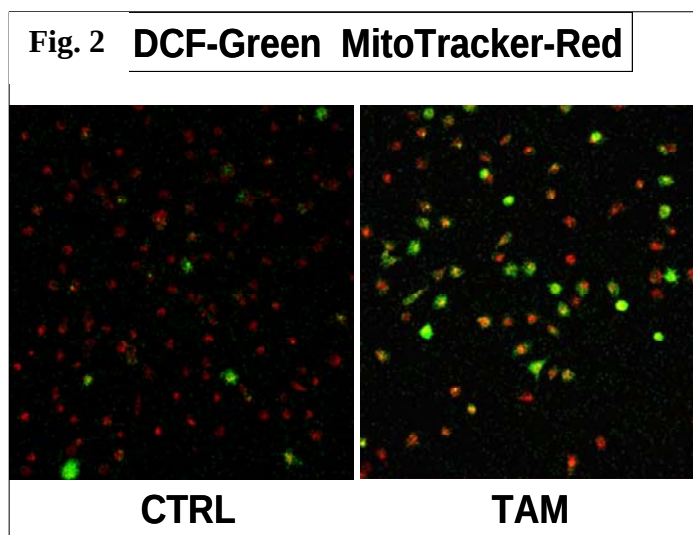
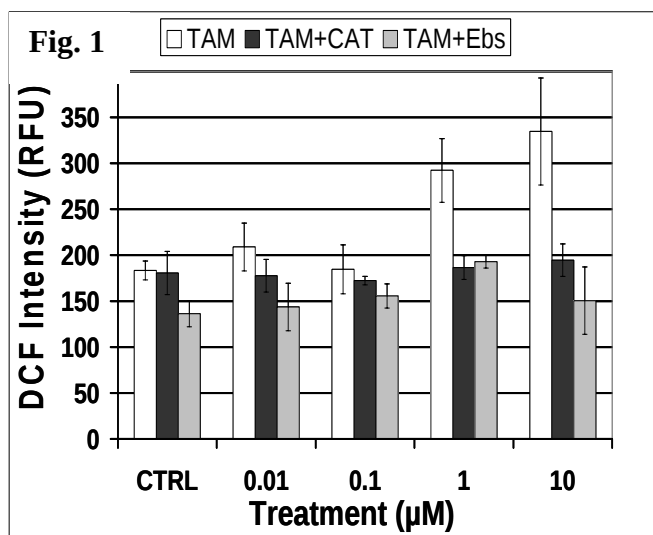
Research Purpose and Scope: We proposed to investigate how reactive oxygen species (ROS)-induced redox signaling pathways in breast cancer cells may contribute to molecular mechanisms of antiestrogen resistance. Our **hypothesis** is that the conversion of breast tumors to a tamoxifen-resistant phenotype is associated with a progressive shift towards a pro-oxidant environment of cells as a result of oxidative stress. We postulate that excess ROS levels induce both *CDC25A* and change p27 phosphorylation promoting the loss of its inhibitory function and leading to antiestrogen resistance. We will investigate whether reducing the oxidative environment

of breast cancer cells will restore the anti-proliferative action of tamoxifen and other antiestrogens by repressing *CDC25A* and altering p27 phosphorylation and restoring p27 function.

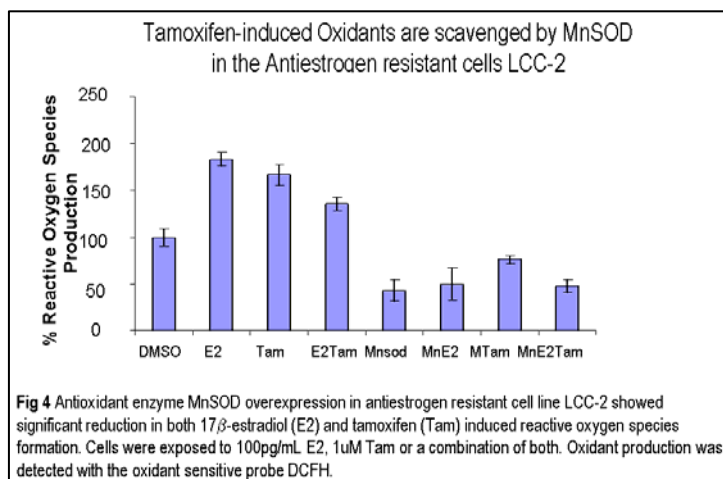
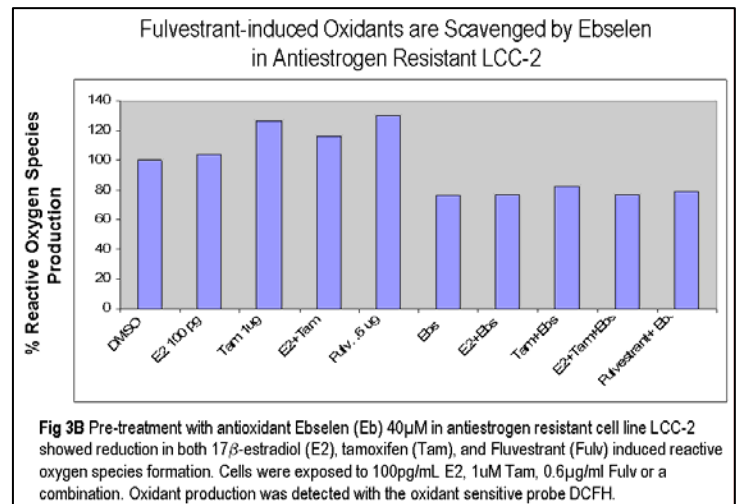
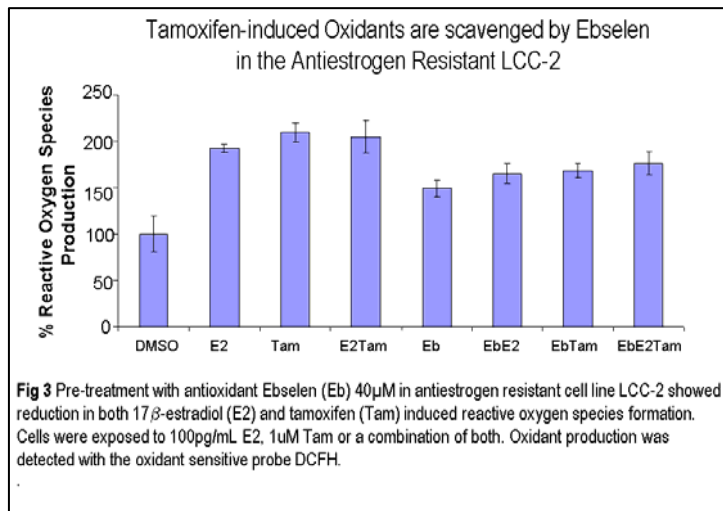
Tasks to be accomplished in the first 12 months of this project were:

- i) To test whether the ER⁺ antiestrogen resistant MCF-7^R, LY-2 and LCC-2 breast cancer cell lines regain sensitivity to the inhibitory growth effects of antiestrogen when oxidant levels are reduced by:(a) co-treatment with antioxidants or overexpressing antioxidant enzymes; (b) raised or restored levels of GSH by glutathione modifiers or overexpressing GSH restoring enzymes; and (c) raised or restored levels of Trx by thioredoxin modifiers or overexpressing Trxn.
- ii) To perform comparative studies on the antiestrogen resistant MCF-7^R, LY-2 and LCC-2 cells using tamoxifen and fulvestrant (ER blockers) to study restoration of antiestrogen action in these resistant cells by ROS modifiers.

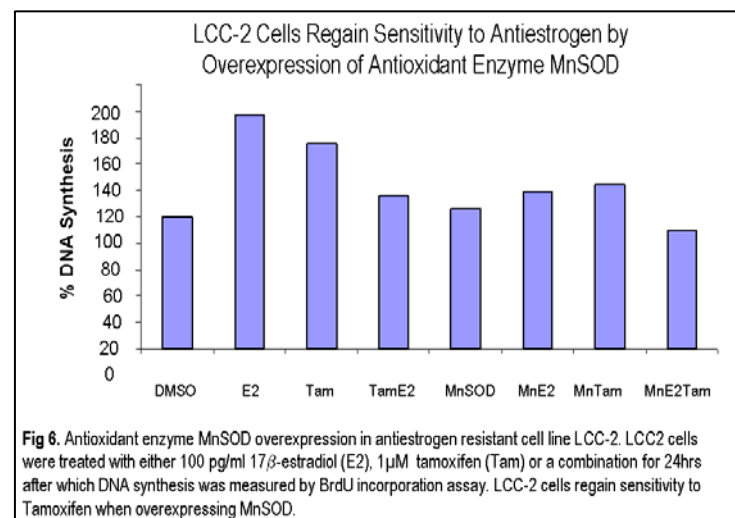
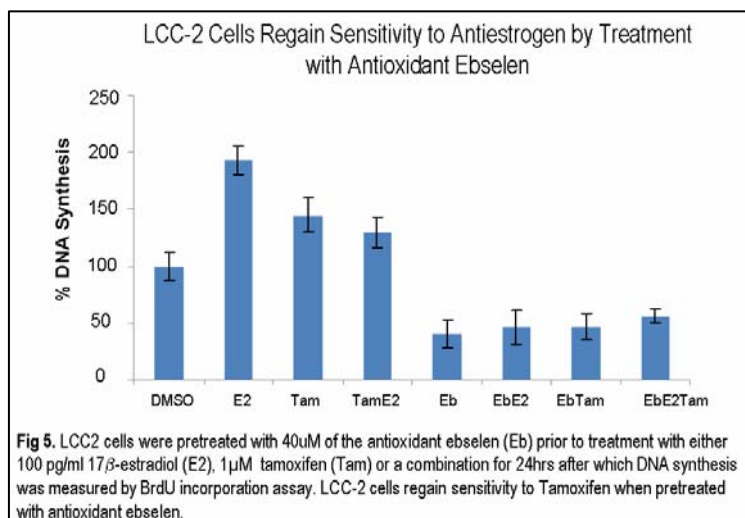
Progress of Proposed Research: Although the primary mechanism of antiestrogen action is believed to be through the inhibition of ER activation, research over the years has indicated that additional, non-ER-mediated mechanisms exist. Recently, we have shown that the growth of breast cancer cells was inhibited by antioxidants. Our findings showed that estrogen-induced ROS promote G1 progression and the early G1 gene *cyclin D1* through signaling phosphorylation of CREB and AP-1. Our first objective was to demonstrate that indeed antiestrogens counteract estrogen actions by controlling ROS formation. Our study confirms previous findings that tamoxifen exposure to MCF-7 increases ROS formation; based on the increased oxidation of the oxidant detecting probe DCFH in tamoxifen treated cells (**Fig 1, 2**). We confirmed this finding using confocal microscopy showing that Tamoxifen indeed increases intracellular level of ROS (**Fig 2**).

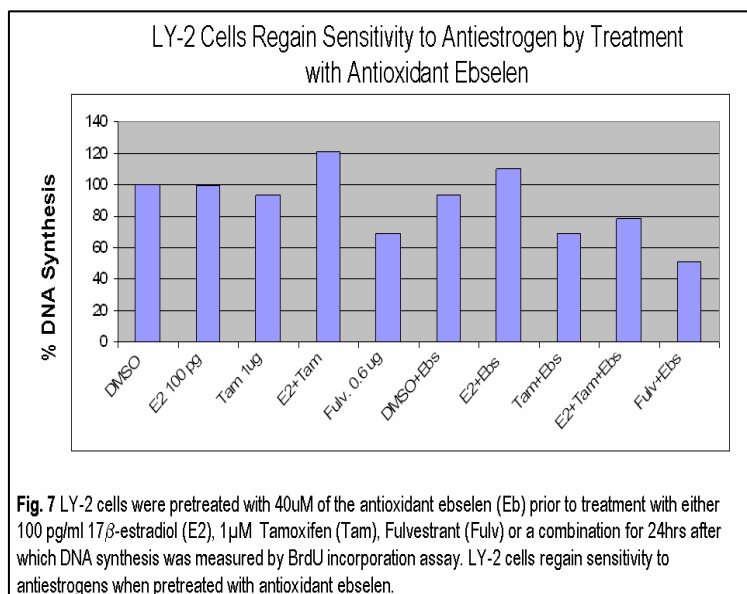


To test whether antiestrogen resistant breast cancer cell lines regain sensitivity to inhibitory effects of antiestrogen when oxidant levels are reduced, we first confirmed whether E2 and Tam induced oxidants can be reduced by ROS modulators. As shown in **Fig 3** and **4**, ROS production is inhibited in antiestrogen resistant breast cancer cell line LCC-2 when exposed to ebselen or by overexpression of the antioxidant enzyme MnSOD.



Next, we determined whether these same ROS modulators could restore sensitivity of antiestrogen resistant breast cancer cell lines. DNA synthesis was measured using the BrdU incorporation assay. As shown in **Fig 5** and **6**, the growth inhibitory effect of tamoxifen is regained in LCC-2 cells by treatment with the antioxidant ebselen as well as by the overexpression of the antioxidant enzyme MnSOD. We performed comparative studies with the antiestrogen resistant breast cancer cell line LY-2. As shown in **Fig. 7**, LY-2 cells regain sensitivity to the growth inhibitory effect of antiestrogens Tamoxifen and Fulvestrant when treated with the antioxidant ebselen.





Challenges and difficulties encountered:

Training: Immediately upon the receipt of the award notice, we could not initiate the project because FIU Grants and Contract office first needed to open the account for this grant and then issue a sub-contract award to UM. This took a month. Two of our pre-doctoral trainees were able to join this project in June 1, 2007, however, the third pre-doctoral trainee joined us in August 2007, because she had to move from Houston, TX. Two of our pre-doctoral trainees are progressing satisfactorily while one trainee has had difficulty with both didactic coursework as well as research performance. To overcome this deficiency we plan to replace her with a post-doctoral trainee which was proposed in the original grant.

Research: Even though most of the research techniques are routinely performed in the PI's and Co-PI's laboratories at FIU, the pre-doctoral trainees need to become familiar with these techniques and standardize them. Since mastering these techniques can take time for a pre-doctoral student, it is one of the reasons for the slow research progress in the beginning. An additional challenge was that both FIU and UM laboratories had fungal infections in the cells which slowed down some of work-related to the antiestrogen-resistant cell lines. However, we have overcome these difficulties and we are working at full capacity to complete the research proposed in Year 1. Still the MCF-7R cell line is not available from Dr. Slingerland's lab due to fungal infection so we have only reported progress using two antiestrogen resistant cells LY-2 and LCC-2. The lack of progress from one of the pre-doctoral trainees has also slowed down research accomplishments and we are hoping to overcome this by replacing this trainee with a post-doctoral fellow as described in the training section.

KEY RESEARCH ACCOMPLISHMENTS

- Confirmed that ROS modulators scavenge oxidants in antiestrogen resistant cell lines LCC-2 and LY-2.
- Antiestrogen resistant cell lines LCC-2 and LY-2 regained sensitivity to the growth inhibitory effects of antiestrogens Tamoxifen and Fulvestrant by treatment with ROS modulators.

REPORTABLE OUTCOMES

Two abstracts were accepted for poster presentation at the 2008 Era of Hope Meeting:

1. Penny, R., Felty, Q., *Slingerland, J, and Roy, D. Ebselen co-treatment counteracts the effects of antiestrogen on estrogen-induced growth of breast cancer cells as well as restores the growth inhibitory effects of antiestrogen in resistant cells.
2. Garba, N.A., Penny R, Okoh, V., Felty, Q., Slingerland, J*, and Roy, D. Reversible Inactivation of CDC25A by Estrogen and Antiestrogen-Induced Reactive Oxygen Species may be Involved in the Phosphorylation of P27.

CONCLUSION

These initial results show support towards our hypothesis, with increased ROS and cell proliferation in breast cancer cells upon treatment with antiestrogens, and the cotreatment with ebselen or MnSOD overexpression restore the growth inhibitory effects of antiestrogen in resistant cells. As the overall knowledge of ROS and p27, as well as new results based on the hypothesis, is being expressed in these experiments, it is evident that further investigation into the hypothesis is necessary and justified.