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14. ABSTRACT

During the 2013-2014 period of the grade evidence is provided that HER2 and HIF-1alpha expression are temporally correlated in vivo in letrozole-treated tumor xenografts. In addition, further evidence is provided that nonhypoxic HIF-1 is involved in regulating cancer stem cell characteristics in letrozole-resistant breast cancer cells, as specific inhibition of HIF-1alpha by EZN-2968 decreased cancer stem cell characteristics (i.e., microtentacle expression. However, HIF-1alpha does not seem to be associated with the vimentin promoter in LTLTCa cells, though it regulates its expression. Which suggests that HIF-1alpha may regulate this vimentin through an intermediary protein.

15. SUBJECT TERMS

Breast cancer, aromatase inhibitors (ex. letrozole), drug resistance, cancer stem cells, nonhypoxic regulation

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INTRODUCTION

This research aims to understand the factors and molecular mechanisms involved in drug-resistant breast cancer, specifically aromatase inhibitor resistant breast cancer. Although aromatase inhibitors (AIs; i.e., letrozole) have been shown to be highly effective in treating estrogen receptor positive (ER+) breast cancer, a significant percentage of patients either do not respond to AIs or become resistant to them. Previous studies suggest that resistance to AIs involves a switch from dependence on ER signaling to dependence on growth factor-mediated pathways, such as human epidermal growth factor receptor-2 (HER2). The mechanism by which HER2 is involved in AI resistance remains mostly unclear. It is, therefore, important to elucidate the HER2-mediated pathway that contributes to AI resistance, and to identify other relevant factors involved that can be used as biomarkers of AI resistance or targets for therapy. One such factor may include HIF-1, a heterodimeric transcription factor made up of an inducible alpha (α) subunit and a constitutively expressed beta (β) subunit. HIF-1 regulates genes important for cell survival, metabolic adaptation, and angiogenesis. Oxygen (O_2) tension is a well-known regulator of HIF-1α, but other factors independent of O_2 can also regulate it. Thus, the purpose of this study is to determine the potential role of nonhypoxic HIF-1 in aromatase inhibitor resistant breast cancer, and whether it could be used as a diagnostic marker and therapeutic target. In vitro breast cancer cell studies, as well as and in vivo xenograft tumore studies will be conducted to test this hypothesis. In addition, EZN-2968, a specific RNA antagonist that specifically targets HIF-1α and is currently in phase 1 clinical trials will be investigated as a potential therapeutic drug.

KEY WORDS:

Breast cancer, letrzole, aromatase inhibitor resistance, hypoxia inducible factor 1 (HIF-1)

ACCOMPLISHMENTS—Overall project summary

Specific Aim 1: To determine the role of ERα and HER2 in the regulation of nonhypoxic HIF-1α expression and activity in letrozole-resistant breast cancer cells (LTLTCa cells).

Task 1: HER2, ERα, HIF-1α, HIF-1β, and β-actin protein expression in MCF-7Ca, LTLTCa, and MCF-7/HER2 cells under basal, nonhypoxic conditions will be determined by western blot analysis.
Experiments: *Experiments to accomplish this task were reported in the 2012 annual summary and published in Kazi et al. 2014 (Appendix 1).*

Task 2: Effect of inhibiting ERα and/or HER2 on HIF-1α protein expression in MCF-7/HER2 cells will be determined by western blot analysis
Experiments: *Experiments for to accomplish this task were reported in the 2012 annual summary and published in Kazi et al. 2014 (Appendix 1).*

Task 3: HIF-1α phosphorylation state in LTLTCa cells will be determined via λ-phosphatase treatment of protein followed by western blot analysis.
Experiments: *Experiments for this task were accomplished and explained in 2012 annual summary.*

Task 4: Effect of inhibiting ERα and/or HER2 on HIF-1α phosphorylation state in MCF-7/HER2 cells will be studied via λ-phosphatase treatment of protein followed by western blot analysis.
Experiments*: Experiments for this task were accomplished and explained in 2012 annual summary.*

Specific Aim 2: To define HIF-1α's role in letrozole-resistant cell phenotype and in xenograft growth.

Task 5: Effect of HIF-1α inhibition on LTLTCa cell characteristics in vitro will be investigated

5.a) Dose experiment to determine the most effective, yet non-toxic dose of EZN-2968 that can inhibit HIF-1α protein expression in LTLTCa cells will be done by MTT assay and western blot analysis.
Experiments: *Experiments to accomplish this task for BCRP were completed and reported in the 2012 annual summary and in abstract #95 at the annual AACR meeting (2013).*

5.b) Effect of EZN-2968 on cancer stem cell characteristics of LTLTCa cells will be studied:

5.b.i) Effect of EZN-2968 on side population percentage in LTLTCa cells will be determined by Hoecsht 33342 dye efflux and flow cytometry.
Experiments*: Experiments to accomplish this task were completed and reported in the 2012 annual summary and in abstract #95 at the annual AACR meeting (2013).*

5.b.ii) Effect of EZN-2968 on CD44 and CD24 positivity of LTLTCa cells will be determined by flow cytometry.
Experiments: *Experiments to accomplish this task were completed and reported in the 2012 annual summary and in abstract #95 at the annual AACR meeting (2013).*

5.b.iii) Effect of EZN-2968 on mammosphere formation of LTLTCa cells will be determined.
Experiments: *Experiments to accomplish this task have been completed and reported in the 2013 annual summary.*

5.b.iv) Effect of EZN-2968 on microtentacle scoring in LTLTCa cells will be determined by transfection with vector expressing membrane-localized GFP followed by fluorescence microscopy.
Experiments: *Experiments to accomplish this task have been completed. LTLTCa cells treated with EZN-2968 for 72 h significantly inhibited the basal number of cells exhibiting microtentacles, as well as the cobalt chloride-induced number of cells exhibiting microtentacles* ***(Figure 1****).*

5.b.v) Effect of EZN-2968 on cytoskeletal components of microtentacles (vimentin and detyrosinated tubulin) will also be assessed by immunofluorescence
Experiments: *Experiments to accomplish this task have been completed and reported in the 2013 annual summary.*

Task 6: Effect of HIF-1α inhibition on LTLTCa cell characteristics in vivo in xenograft models will be investigated.
This task has been delayed to no-cost extension Year 4 of the grant due because EZN-2968 is no longer available through Enzon Pharmaceuticals. This task will now be accomplished using a different HIF-1α inhibitor.

Current Conclusions: *Current findings indicate that characteristics of camcer progression and metastasis, such as microtentacle formation, are regulated by nonhypoxic HIF-1 expression. Thus, providing further support for the importance of the HER2-HIF-1 pathway in the aromatase inhibitor resistant phenotype.*

Specific Aim 3: To identify HIF-1 target genes that serve as markers of letrozole resistance. (1-1.5 years)
Task 7: To compare expression between in MCF-7Ca and LTLTCa cells of known HIF-1 targets involved in cancer

7.a) To compare protein expression of known HIF-1 targets in MCF-7Ca and LTLTCa cells by western blot analysis.
Experiments: *Experiments to accomplish this task have been completed and reported in the 2012 and 2013 annual summaries, respectively, and in Gilani et al. 2012.*

7.b) To compare mRNA expression of known HIF-1 targets in MCF-7Ca and LTLTCa cells by real-time RT-PCR analysis.
Experiments: *Experiments to accomplish this task have been completed and reported in the 2012 and 2013 annual summaries and in Kazi et al. 2014 (Appendix 1).*

Task 8: To determine whether HIF-1α binds to promoters of genes overexpressed in LTLTCa cells by chromatin immunoprecipitation.
Experiments: *Experiments to accomplish this task for BCRP were completed and reported in the 2012 and 2013 annual summaries and in Kazi et al. 2014. Experiments to accomplish this task for vimentin have been completed. A potential hypoxia response element (HRE) 5'-GCGTG-3' was located at nucleotide position 1082 on the vimentin 5'-flanking sequence and untranslated region (Rittling and Baserga. 1987). Chromatin immunoprecipiation (ChIP) was then performed to analyze HIF-1 binding to a region of the promoter that included this sequence. Although input samples showed successful isolation of the promoter region by ChIP, HIF-1 binding to this region under normoxic conditions was not detected. (Figure 2). These results may indicate that HIF-1 indirectly regulates vimentin expression by regulating another transcription factor. Both TWIST (Sun et al. 2009) and Snail (Zhang et al. 2013) transcription factors are known to be direct HIF-1 target genes. In turn, both Snail (Kaufhold et al. 2014) and Twist (Yang et al. 2004) are known to regulate vimentin expression..*

Although TWIST and Snail were not upregulated LTLTCa vs. MCF-7Ca cells (refer to 2013 annual summary), their functions as transcription factors may be upregulated in LTLTCa cells. Investigation of regulation of TWIST and Snail function by nonhpoxic HIF-1 in aromatase inhibitor resistant breast cancer cells will be conducted in a separate future project.

Task 9: To determine whether EZN-2968 decreases expression of known HIF-1 targets in LTLTCa cells.
Experiments: *Experiments to accomplish this task have been completed and reported in the 2012 and 2013 annual summaries and in abstract #95 at the annual AACR meeting (2013).*

Task 10: To determine importance of identified HIF-1 target genes in letrozole-resistant cells by inhibiting target genes via specific pharmacological inhibitors or siRNA followed by in vtiro cancer cell assays (as specific aim 2)
Experiments: *Experiments to accomplish this task have completed and were reported in the 2012 and 2013 annual summaries and published in Gilani et al. 2012.*

Conclusions: *Findings suggest that the HER2-nonhypoxic HIF-1 signaling pathway in LTLTCa cells*

ACCOMPLISHMENTS—Key accomplishemts from 2013-2014)

Completion of in vitro studies in the SOW

- Evidence is provided that HER2 and HIF-1α expression are temporally correlated in vivo in letrozole-treated tumor xenografts.
- Further evidence is provided that nonhypoxic HIF-1 is involved in regulating cancer stem cell characteristics in letrozole-resistant breast cancer cells.
  - Specific inhibition of HIF-1α by EZN-2968 decreased cancer stem cell characteristics (i.e., microtentacle expression).
- HIF-1α is not associated with the vimentin promoter in LTLTCa cells.

IMPACT

Prior to this study, AI resistance was associated with increased dependence on growth factors and decreased dependence on ERα. However, the role that such molecular changes play in AI resistance and the mechanism by which they elicit their effects were not known. Results from this study demonstrated that nonhypoxic expression of HIF-1 mediates HER2's effects on letrozole-resistance. Specifically, HER2-activated PI3K/Akt pathway increases HIF-1α protein synthesis in LTLTCa cells. HIF-1α, in turn, upregulates expression of BCRP and vimentin, and contributes to letrozole resistance and stem cell characteristics of LTLTCa cells.

Nonhypoxic regulation of HIF-1 expression and activity in LTLTCa cells is due to HER2-activated PI3K/Akt pathway. This is consistent with findings by others indicating hypoxia independent upregulation of HIF-1α in cancer cells by loss of function of tumor suppressor genes and gain of function of oncogenes (Semenza. Trends Pharmacol Sci. 2012). The oncogene HER2/neu, in particular has been previously associated with nonhypoxic HIF-1 (Laughner et al. Mol Cell Biol. 2001; Li et al. Cancer Research. 2005). Laughner et al. and Li et al have demonstrated that transfection of HER2 into NIH/3T3 cells or activation of HER2 in MCF-7 cells led to activation of the PI3K/Akt pathway, and subsequent increased HIF-1 expression via protein synthesis and HIF-1 transcriptional activity. Our current results provide additional evidence that this HER2-PI3K/Akt pathway-HIF-1 signaling mechanism can occur endogenously in HER2+ cells in vitro and in vivo (xenograft tumors) and has physiological relevance (i.e., regulation of cancer stem cell characteristics) as well as potential clinical implication (i.e., AI resistance).

Inherent upregulation of HIF-1α protein expression under nonhypoxic conditions is another novel finding in AI-resistant breast cancer. There is precedence for associating HIF-1 expression with drug resistance in different cancer cell types, including chronic myeloid leukemia cells (Zhao et al. Oncogene. 2010), gastric cancer cells (Liu et al. Mol Cell Biochem. 2007), non-small cell lung cancer cells (Song et al. Cancer Chemother Pharmacol. 2006), and even breast cancer cells (Flamant et al. Mol. Cancer 2010). However, these previous cases involved hypoxia-induced, HIF-1α rather than the nonhypoxic HIF-1. Our findings are also consistent with previous clinical evidence that HIF-1α is associated with letrozole resistance. Generali et al demonstrated that increase p-MAPK and HIF-1α protein expression were significant determinants of primary letrozole resistance in breast cancer patients. In contrast, increased ERα and decreased p-MAPK were significant determinants of response to letrozole treatment (Generali et al. J Clin Oncol 2009). The protein expression patterns observed by Generali et al. are similar to what is observed in letrozole-resistant LTLTCa and –sensitive MCF-7Ca cells, respectively (Figures 1 and 6 of 2012 annual summary). Although these clinical findings involve de novo letrozole resistance, they still correlate with, and likely pertain to, our laboratory's results on acquired letrozole resistance. These results combined suggest that HIF-1 is involved in both de novo and acquired AI resistance, and therefore could be therapeutically targeted to prevent and treat resistance to letrozole and the other AIs. Lastly, this study indicates that HIF-1 may contribute to letrozole resistance by mediating HER2's effects on target genes, such as BCRP and vimentin. HIF-1 regulated vimentin, in particular, has now been associated with letrozole-resistant cells and their expression of microtentacles and invasion and migration ability (Figures 2-5 and 6 of 2013 annual summar). However, HIF-1 regulation of vimentin appears to be indirect, as recent ChIP analysis indicated that HIF-1 protein does not bind to the vimentin promoter (Figure 2).

Overall, this current study provides further evidence that nonhypoxic HIF-1α is an important factor in letrozole resistant breast cancer cells, particularly their ability to form microtentacles (Figure 1) that may contribute to their progression and metastasis. Based on this, a proposed model of acquired AI-resistance may involve the following scenario: under nonhypoxic conditions, when the breast cancer cell population and tumor size have been reduced by letrozole treatment and prior to significant tumor hypoxia, a switch from ERα- to

growth factor (i.e., HER2)-mediated signaling occurs, which leads to increased HIF-1α expression and activation of HIF-1 target genes that contribute to AI resistance. Consequently, inhibition of HIF-1 expression and/or activity would prolong cancer cell sensitivity to AIs and prevent recurrence and metastasis. Indeed, a number anti-cancer drugs in clinical use are also known to inhibit HIF-1 (Semenza. Oncogene. 2010). They include HER2 inhibitor, trastuzumab (Laughner et al. Mol Cell Biol. 2001) and lapatinib. Furthermore, as demonstrated in this study that HIF-1 is regulated mainly via PI3K/Akt pathway, inhibition of downstream affecter of this pathway using mTOR inhibitors such as rapamycin, temsirolimus/CCI-779 and everolimus/RAD-001, can also be considered (Laughner et al. Mol Cell Biol. 2001; Majumder et al., Nat Med. 2004; Faivre et al. Nature Reviews Drug Discovery. 2006; Thomas et al.Nat Med. 2006). Novel to this study, EZN-2968, a specific HIF-1α mRNA inhibitor (siRNa), which has previously been shown to reduce cancer cell viability and xenograft tumor growth and is currently under phase I clinical trial (Greenberger et al. Mol Cancer Ther. 2008), appears to be a promising drug to prevent and treat aromatase inhibitor resistance.

CHANGES/PROBLEMS

Unfortunately, EZN-2968 is no longer available from Enzon Pharmaceuticals. An alternative drug that can inhibit HIF-1α and shown to be effective in vivo, is now being explored as a substitute for EZN-2968 in in vivo xenograft experiments of this grant.

SUPPORTING DATA: Figure legends and Figures

Figure 1. Effect of CoCl2 and EZN-2968 on microtentacle formation in LTLTCa cells.
LTLTCa cells were plated in passage media and then treated with 100 μM cobalt chloride (CoCl2) and/or 10 μM HIF-1α -specific LNA EZN-2968 for 48 h. Cells were then collected, stained with Cell Mask Orange, and resuspended in Ibidi slides for microtentacle scoring. Results are expressed as percent of cells counted with microtentacles per 100 total cells counted (i.e., frequency). (n =4 samples per treatment; a, $p< 0,05$ vs. vehicle).

Figure 2. Binding of HIF-1$^{\alpha}$ to vimentin promoter in LTLTCa cells.
LTLTCa cells were plated in their respective passage media and allowed to attach under normal cell culture conditions (20% O2), prior to being treated with either vehicle or 10 μM EZN-2968 (EZN) for 48 h. Cells were then subjected to chromatin immunoprecipitation. Protein-chromatin complexes were crosslink with formaldehyde, collected, and subjected to immunoprecipitation with either normal mouse serum (negative control, NC) or HIF-1α antibody. DNA that was collected in the process was purified and analyzed by PCR analysis for human vimentin promoter region that contains potential hypoxia response element. Input refers to DNA from collected protein-chromatin complexes that were not subjected to immunoprecipitation. Shown is a representative gel of n =4 samples per treatment and IP condition.

REPORTABLE OUTCOMES

Abstracts and Presentations:

- Armina Kazi, Gauri Sabnis, Qun Zhou, Akina Tamaki, Spencer Todd, Saranya Chumsri, Amanda Schech, Preeti Shah, Angela Brodie. HER2 regulated miRNA expression in letrozole resistant breast cancer. Abstract #7633 peer-reviewed and presented at American Association of Cancer Researchers (AACR) 2014 Annual Meeting.

Manuscripts:

- Published:
  - Armina A Kazi, Rabia A Gilani, Amanda J Schech, Saranya Chumsri, Gauri J Sabnis, Preeti Shah, Olga Goloubeva, Shari Kronsberg and Angela H Brodie. Nonhypoxic regulation and role of hypoxia-inducible factor 1 in aromatase inhibitor resistant breast cancer. Breast Cancer Research. 2014.

Employment:

- Have maintained tenure-track assistant professor position Loyola University Maryland

New research collaborations that will be used to apply for R15 in 201.

- In meeting with project mentor Dr. Stuart Martin (UMB) to discuss research progress, I have continued with research collaboration with his laboratory investigating the role of hypoxia on microtentacle formation in breast cancer cells.
- I have continued with a clinical project at Greenbaum Cancer Center investigating the response of postmenopausal obese and overweight patients with ER+ breast cancer to neoadjuvant aromatase inhibitor therapy. My role in this clinical trial is to analyze HIF-1 and miRNA (particularly miRNAs that are associated with HER2 and HIF-1) profiles in patient samples.

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APPENDIX (refer to Kazi et al. Breast Cancer Research. 2014)

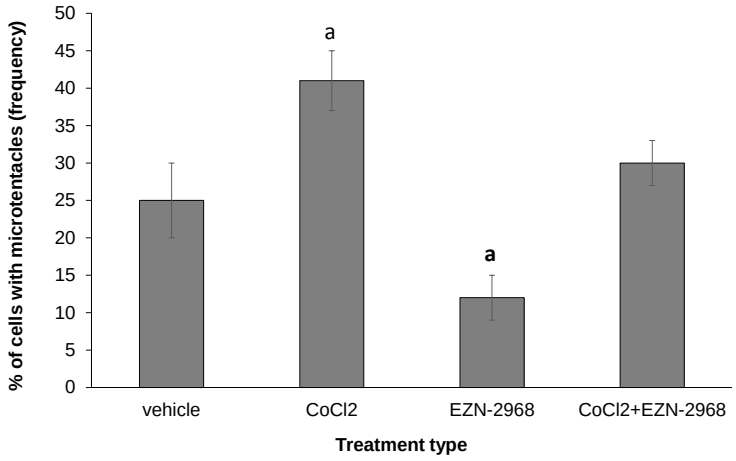

Figure 1.

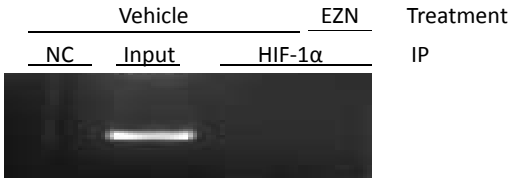

Figure 2.