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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Although aromatase inhibitors (AIs) 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. 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. Unlike the well-studied role of hypoxia-regulated HIF-1α in a variety of cancers, nonhypoxic regulation of HIF-1α and its role in cancer remains largely unclear. Here we have investigated HIF1α in AI resistance. Results indicate that basal nonhypoxic HIF-1α protein expression 1) is higher in AI resistant cells than in their AI-sensitive parental cells, 2) is regulated by HER2, and in turn 3) regulates cancer stem cell markers and cancer stem cell characteristics that may contribute to drug resistance. Lastly, EZN-2968, a specific HIF-1 RNA antagonist currently in phase 1 clinical trials has shown potential in treating aromatase inhibitor resistance.

15. SUBJECT TERMS
None provided.

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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.

BODY

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 successful, as shown in **Figure 1a.** The three cell lines expressed HER2, ERα, and HIF-1β protein at expected levels. LTLTCa and MCF-7/HER2 cells expressed 4-fold higher HER2 protein than MCF-7Ca cells. ERα was expressed in both MCF-7Ca and MCF-7/HER2 cells, but not in LTLTCa cells. HIF-1β, known to be constitutively expressed in cells (Semenza et al), was expressed equally in the three cell types. Correlating with HER2 expression and regardless of ERα and HIF-1β, HIF-1α was expressed higher in LTLTCa and MCF-7/HER than MCF-7Ca cells.*

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 to accomplish this task were successful as shown in **Figure 1b**. MCF-7/HER2 cells were treated with either vehicle or HER2 kinase inhibitor lapatinib and/or ERα antagonist ICI182,780 for 24 h. Western blot analyses for HER2, phosphorylated (p-) and total-ERK1/2 (p-ERK), ERα, HIF-1α, and β-actin were done. Both inhibitors were confirmed to inhibit their target: lapatinib inhibited activation of the MAPK pathway (p-ERK) and ICI182,780 inhibited ERα expression. Inhibition of HER2 activity decreased HIF-1α expression (0.1-fold vs. vehicle, p <0.001). Interestingly, ICI182,780 alone also reduced HIF-1α expression (0.4-fold vs. vehicle), but not to the extent of lapatinib alone or lapatinib and ICI182,780 in combination (0.1-fold each vs. vehicle).*

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 to accomplish this task were successful, as shown in **Figure 2**. In these experiments, protein phosphorylation is detected as a change in protein migration after λ-phosphatase treatment. In LTLTCa and MCF-7/HER2 cells, phosphorylation of HIF-1α was not detected. Phosphorylation was detected in MCF-7/HER2 cells treated with HIF-1α stabilizer CoCl2. These results suggest that HIF-1α is either not phosphorylated under basal conditions, or its phosphorylation is undetectable by λ phosphatase.*

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 to accomplish this task were successful, as shown in **Figure 2**. As expected ICI182,780 and lapatinib decreased total HIF-1α. However, no change in phosphorylation state was detected with treatment from either inhibitor.*

Additional experiments: *To further confirm 1) HER2's role in regulating nonhypoxic expression of HIF-1 in breast cancer cells and 2) HER2 and nonhypoxic HIF-1's involvement in drug resistant breast cancer, additional experiments were conducted. First, since HIF-1α protein expression can be affected by cell density/confluency (Fang et al. Experimenetal Cell Research. 2007; Paltoglou and Roberts. Oncogene. 2005), protein expression in LTLTCa and MCF-7Ca cells at both ~50% and 95% confluencies were also analyzed. LTLTCa cells had higher levels of HIF-1α protein than MCF-7Ca cells under nonhypoxic conditions regardless of cell density (**Figure 3a**). Furthermore, while MCF-*

*7Ca cells still had little or no HIF-1α protein at 95% confluency, LTLTCa cells exhibited a significant increase in HIF-1α. These results suggest that 1) LTLTCa cells basally express higher levels of HIF-1α than MCF-7Ca cells, and 2) they are more sensitive to inducers of HIF-1α expression, such as cell density/confluency. Second, since lapatinib is a HER2/EGFR1 dual kinase inhibitor, the effects of a more specific HER2 inhibitor trastuzumab (a HER2 monoclonal antibody) on nonhypoxic HIF-1 expression was also determined. As shown in **Figure 3b**, trastuzumab decreased HIF-1α protein expression along with HER2 expression and activation of the MAPK pathway (p-ERK), 0.6-, 0.4-, and 0.5-fold vs. vehicle (p<0.05), respectively. Third, expression of HER2 and HIF-1 was also analyzed in another aromatase inhibitor (AI)-resistant cell line. Exemestane-resistant AC1-ExR cells were developed in the laboratory of Dr. Angela Brodie in a similar manner as letrozole-resistant LTLTCa cells. Although AC1-ExR cells retained ERα, they still had higher levels of HER2 and HIF-1α compared to their exemestane-sensitive, parental AC1 cells (**Figure 1c**), and despite retaining ERα.*

Current Conclusions: *Current findings are in agreement with previous results (refer to grant project narrative). Further evidence is provided that 1) HIF-1α expression under nonhypoxic conditions correlates with aromatase inhibitor (letrozole and exemestane) resistance; 2) nonhypoxic HIF-1α expression is regulated by HER2 independently from and regardless of ERα; and 3) nonhypoxic HIF-1α is not or minimally phosphorylated.*

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: HIF-1α was inhibited using HIF-1α siRNA from Qiagen and EZN-2968 from Enzon Pharmaceuticals. 1 uM Qiagen HIF-1α siRNA and 10 uM EZN-2968 were selected as concentrations based on recommendations from Qiagen and Enzon. At these concentrations, Qiagen HIF-1α and EZN-2968 were found to be effective in reducing HIF-1α mRNA and protein expression in LTLTCa cells by 48 h (**Figure 4a-b**) without significantly affecting overall cell viability (**Figure 4c**). Thus, any effects on cancer stem cell characteristics observed would likely not be due to an apoptotic effect of the siRNAs on the overall LTLTCa cell population.*

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 successful, although they had to be modified. Prior to starting these experiments, the UV laser on the LSR I flow cytometer used for side population experiments using Hoechst 33342 dye broke. Side population analyses were, therefore, conducted instead using Vybrant Dyecycle Violet stain (Invitrogen) and the LSR II. Treatment of LTLTCa cells with either Qiagen's HIF-1α siRNA or EZN-2968 for 48 h, significantly decreased side population percentage compared to negative control siRNA (19.2% vs. Qiagen negative control siRNA 23.5% and 16.7 vs. Enzon negative control siRNA 9.4%, respectively) (**Figure 5**).*

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 successful. LTLTCa cells were treated with negative control siRNA or HIF-1α siRNA (Qiagen or EZN-2968) for 48 h, prior to being subjected to flow cytometry analysis for CD44 and CD24 positivity Increasing CD44+ vs.

*CD24+ is indicative of a more cancer stem cell phenotype. Similar to previous findings, LTLTCa cells are both CD44+ and CD24+. EZN-2968 significantly decreased the ratio of CD44+:CD24+ LTLTCa cells compared to negative siRNA (27.4 vs. 46.1 of Enzon negative control, p <0.05; **Figure 6**). Interestingly, Qiagen HIF-1α siRNA had no significant effect on CD44+:CD24+ ratio.*

5.b.iii) Effect of EZN-2968 on mammosphere formation of LTLTCa cells will be determined.
*Experiments: Experiments to accomplish this task were successful, as observed . Qiagen HIF-1α siRNA treatment decreased mammosphere formation in LTLTCa cells (101 mammospheres/20,000 cells ± 18), while treatment with the HIF-1α stabilizer CoCl2 increased formation (500 mammospheres /20,000 cells ± 20) compared to negative control-treated siRNA (**Figure 7**). At the time of this experiment, EZN-2968 was not available. The effect of EZN-2968 on mammosphere formation will be done shortly.*

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: These experiments will be conducted shortly. Dr. Stuart Martin (UMB), who has conducted extensive research into microtentacles, has been modifying the technique to improve the visualization and scoring of microtentacles.
5.b.v) Effect of EZN-2968 on cytoskeletal components of microtentacles (vimentin and detyrosinated tubulin) will also be assessed by immunofluorescence
Experiments: These experiments will be conducted shortly. With delays in accomplishing task 5.b.iv, this task has also been delayed.

Additional experiments: To further determine nonhypoxic HIF-1's role in aromatase inhibitor resistance, an additional experiment analyzing the effect of HIF-1α inhibition on LTLTCa cell sensitivity to the growth inhibitory effects of letrozole were done. As shown in Figure 7, inhibition of HIF-1α via siRNA for 48 h (the same timepoint in which cancer stem cell characteristics were inhibited) significantly decreased percent LTLTCa cell viability after 6 days of treatment with letrozole, compared to negative control siRNA.

Current Conclusions: Current findings suggest that nonhypoxic HIF-1 does play a role in the cancer stem cell phenotype of LTLTCa cells. Specific inhibition of HIF-1α via siRNA, either from Qiagen or EZN-2968, significantly decreased cancer stem cell characteristics (side population percentage, CD44+:CD24+ ratio, and mammosphere formation), and this correlated with a later effect on overall LTLTCa cell resistance to the growth inhibitory effects of letrozole. These current findings also suggest the potential effectiveness of EZN-2968 in prolonging or restoring sensitivity of breast cancer cell tumors to letrozole. Such effects will be studied in subsequent years of this grant.

Specific Aim 3: To identify HIF-1 target genes that serve as markers of letrozole resistance. (1-1.5 years)
Although this task was not scheduled to start in Year 1 of the grant, initial experiments were conducted 1) since task 5.b.iv and 5.b.v were being delayed, and 2) to provide data for a published journal article (Gilani et al. Breast Cancer Res Treatment, 2012).
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 will start shortly.

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 started and will continue In addition to BCRP, VEGF mRNA expression was analyzed in MCF-7Ca vs. LTLTCa cells by real time RT-PCR. No significant difference in VEGF mRNA levels was observed between the two cell types (**Figure 8**).*

7.c) Genes of particular interest include:

      7.c.i)   Genes associated with cancer stem cell maintenance: BCRP, Oct-4, kit ligand, JARID1B

      7.c.ii) Genes associated with EMT: Twist, Snail

      7.c.iii) Genes associated with microtentacles: vimentin (also includes Twist)

      7.c.iv) Genes associated with invasion: endothelin 1, fibronectin, MMP-2 and -4

      7.c.v) Genes associated with metastasis: angiopoietin-like 4, CXCR4

      7.c.vi) vascular endothelial growth factor (VEGF), a well-known HIF-1 target gene

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 will start shortly.

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 started and will continue. As expected, HIF-1α inhibition via siRNA (either Qiagen or EZN-2968) decreased HIF-1α and BCRP mRNA (**Figure 4**). It also decreased expression of GAPDH (Higashimura et al. Arch Biochem Biophys. 2011), another known HIF-1 target gene; BMI-1, another stem cell marker (Wu et al. Bioscienc Rep. 2011); and TWIST, an epithelial-mesenchymal transition marker and transcription factor known to regulate BMI- 1 (Wu et al. Bioscienc Rep. 2011). mRNA expression of Nanog, another stem cell marker was only decreased by EZN-2968. Interestingly, EZN-2968 resulted in greater decreases overall in the mRNAs studied.*

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 been started and will continue. The importance of cancer stem cell marker and efflux transporter protein BCRP on cancer stem cell characteristics of LTLTCa cells has been studied using either specific pharmacological inhibitors (K0143 and fumitremorgen C) or siRNA. Results were reported in a published journal article by Gilani et al. Figures 1a, 4, and 5 demonstrated that inhibition of BCRP reduced side population percentage and mammosphere formation. Figure 8 further demonstrated a temporal correlation between HER2 protein expression, BCRP protein expression, and development of letrozole resistance in MCF-7Ca xenograft tumors treated long-term with letrozole.

Current Conclusions: *Current findings provide additional evidence of a HER2-nonhypoxic HIF-1-BCRP signaling pathway in LTLTCa cells, as HIF-1α siRNA EZN-2968 significantly decreased HIF-1α and BCRP in LTLTCa cells. Other cancer stem cell markers were also affected. Lastly, similar to the effects of HIFα siRNAs, BCRP siRNA decreased cancer stem cell characteristics in LTLTCa cells.*

KEY RESEARCH ACCOMPLISHMENTS

- HER2-activated PI3K/Akt pathway regulates HIF-1α expression in aromatase inhibitor (letrozole and exemestane)-resistant breast cancer cells.
- EZN-2968, a HIF-1α inhibitor (siRNA) currently under phase 1 clinical trials, can effectively inhibit HIF-1α in LTLTCa cells and cell characteristics it is involved in.
- Nonhypoxic HIF-1 is involved in regulating cancer stem cell characteristics in letrozole-resistant breast cancer cells.
  - Specific inhibition of HIF-1α by siRNA decreased expression of cancer stem cell markers, such as breast cancer resistance protein (BCRP), BMI-1, Nanog, and TWIST (an epithelial mesenchymal transition factor and regulator of BMI-1).
  - Specific inhibition of HIF-1α by siRNA decreased cancer stem cell characteristics (i.e., mammosphere formation, CD44/CD24 positivity, and side-population percentage)
- BCRP is involved in regulating cancer stem cell characteristics in letrozole-resistant breast cancer cells.

REPORTABLE OUTCOMES

Abstracts and Presentations:

- Rabia Gilani, <u>Armina Kazi</u>*, Amanda Schech, Saranya Chumsri, Preeti Shah, Angela Brodie. The involvement of HER2, HIF-1, and BCRP in cancer stem cell characteristics of letrozole-resistant breast cancer cells. Abstract #2303 peer-reviewed and presented at American Association of Cancer Researchers (AACR) 2012 Annual Meeting. *co-first author and presenting author

Manuscripts:

- Accepted:
  - Rabia A. Gilani*, <u>Armina A. Kazi</u>*, Preeti Shah, Amanda J. Schech, Saranya Chumsri, Gauri Sabnis, Anil K. Jaiswal, Angela H. Brodie. The Importance of HER2 signaling in the tumor initiating cell population in aromatase inhibitor resistant breast cancer. Breast Cancer Research and Treatment. 2012 Oct;135(3):681-92.*co-first author

- Submitted:
  - <u>Armina A. Kazi</u>, Rabia A. Gilani, Amanda J. Schech, Saranya Chumsri, Gauri Sabnis, Angela H. Brodie. Nonhypoxic regulation and role of hypoxia-inducible factor 1 in aromatase inhibitor resistant breast cancer. Submitted to Breast Cancer Research. October. 2012

Employment

- Obtained tenure-track assistant professor position Loyola Univerisity Maryland

CONCLUSIONS

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 other genes, 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 et al. Oncogene. 2010). 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 study provides evidence that this HER2-PI3K/Akt pathway-HIF-1 signaling mechanism can occur endogenously in HER2+ cells 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). 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.

Overall, this current study provides evidence that nonhypoxic HIF-1α is an important factor in letrozole resistant breast cancer cells. 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.

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SUPPORTING DATA: Figure legends and Figures

Figure 1. Protein expression in HER2+ cells and exemestane-resistant cells.
A, MCF-7Ca (M), SUM149 (S), MCF-7/HER2 (MH), and LTLTCa (LT) cells were plated in their respective passage media. Total protein was extracted and HER2, phosphorylated- and total-ERK, ERα, HIF-1$^{\alpha}$, BCRP, and β-actin protein were analyzed by Western blot. Densitometry results are expressed as fold-change compared to MCF-7Ca after normalization to ERK (mean ± SEM, n = 6 independent cell samples/group; * vs. MCF-7Ca, p <0.05; , † vs. MCF-7Ca, p <0.001). Dashed lines indicates omitted lane in between M and MH of the same blots. B, MCF-7/HER2 cells were treated with either vehicle (V), 1 μM lapatinib (Lap), 100 nM ICI 182,780 (ICI), 1 μM lapatinib+100 nM ICI 182,780 (Lap+ ICI) for 24 h. Total protein was extracted and HER2, phospho- and total-ERK1/2, ERα, HIF-1α, and β-actin were analyzed by Western blot. Densitometry results are expressed as fold-change compared to vehicle-treated cells after normalization to β-actin (mean ± SEM, n = 4 independent cell samples/group; * vs. vehicle, p < 0.001). C, AC1 (AC1) and AC1-ExR (ExR) cells were plated in their respective passage media. Total protein was extracted and HER2, phosphorylated- and total-ERK, ERα, HIF-1α, and β-actin protein were analyzed by Western blot.

Figure 2. Phosphorylation state of nonhypoxic HIF-1α in LTLTCa and MCF-7/HER2 cells.
LTLTCa cells were treated with vehicle while MCF-7/HER2 cells were treated with vehicle, 100 μM CoCl2, 1 μM lapatinib, or 100 nM ICI 182,780 for 24 h. Whole cell protein was then extracted and subjected to λ phosphatase digestion, prior to analysis of HIF-1α protein by western blot apaparatus. Representative blots are shown.

Figure 3. The effects of cell density/confluency and of HER2 inhibition on HIF-1α protein expression in LTLTCa cells.
A, LTLTCa and parental MCF-7Ca cells were plated and cultured in their respective passage media at 1X or 2X density. Total protein was extracted when 2X density plates reached ~90-95% confluency, and consequently 1X density plates reached ~50-60% confluency. HIF-1$^{\alpha}$ and β-actin protein were analyzed by Western blot. Densitometry results are expressed as fold-change compared to MCF-7Ca cells after normalization to β-actin. (mean ± SEM, n = 6 independent cell samples/group; vs. MCF-7Ca, p < 0.01). B, LTLTCa cells were treated with either PBS vehicle or 500 ug/ml trastuzumab for 24 h-96 h. Total protein was extracted and HER2, phospho- and total-ERK1/2, ERα, HIF-1α, and β-actin were analyzed by Western blot. Densitometry results are expressed as fold-change compared to vehicle-treated cells after normalization to β-actin (mean ± SEM, n = 3 independent cell samples/group; * vs. vehicle, p < 0.05).

Figure 4. Effect of HIF-1α siRNA on mRNA expression in LTLTCa cells.
A, LTLTCa cells were treated with either negative control siRNA or HIF-1α siRNA (Qiagen or EZN-2968) for 48 h. Total mRNA was extracted and HIF-1α, BCRP, GAPDH, Nanog, BMI-1, and TWIST mRNA, and 18S rRNA were analyzed by real-time RT-PCR. Real-time results are expressed as the fold-change in mRNA levels compared with negative control after normalization to 18S rRNA (mean ± SEM, n = 4 samples/group). B, LTLTCa cells were plated in passage media and then treated with either negative control siRNAs (NC, Qiagen or Enzon),Qiagen HIF-1α siRNA 1 (H1), Qiagen HIF-1α siRNA 2 (H2), or EZN-2968 (2968) for HIF-1α for 48 h. Total protein was extracted and HIF-1α and β-actin protein were analyzed by Western blot. Densitometry results are expressed as fold-change compared to negative control after normalization to β-actin (mean ± SEM, n = 6 independent cell samples/group; * vs. negative, p <0.05). C, Viability of cells was measured by MTT assay after 48 h treatment with negative control siRNAs, Qiagen HIF-1α siRNA, or EZN-2968 and a

subsequent 6 day treatment with 1 μM letrozole. Results are expressed as percent of negative control siRNA. (mean ± SEM, n = 5 samples/group).

Figure 5. Effect of HIF-1α and BCRP siRNA on side population percentage of LTLTCa cells.
LTLTCa cells were treated with A) 1 μM Qiagen negative control siRNA or HIF-1α siRNA or 10 μM Enzon negative control siRNA or EZN-2968; or B) BCRP inhibitors 1 μM K0143 or Qiagen BCRP siRNA for 48 h. Cells were stained with Vybrant DyeCycle violet. Cells were then acquired using BD LSRII and analyzed. Plots show representative side population fractions as designated by polygons and quantified as percentages (n=3, p< 0.01).

Figure 6. Effect of HIF-1α siRNA on CD44:CD24 positivity of LTLTCa cells
LTLTCa cells were treated with A) 1 μM Qiagen negative control siRNA or B) HIF-1α siRNA or with 10 μM Enzon negative control siRNA or EZN-2968 for 72 h. Cells were stained with APC-conjugated CD44 and PE-conjugated CD24 antibody. Cells were then acquired using BD LSRII and analyzed by FlowJo software. Plots show representative CD44:CD24 positivity percentage ratios (n=3, p< 0.05).

Figure 7. Effect of HIF-1α and/or BCRP siRNA on mammosphere formation and cell proliferation in LTLTCa cells.
A, LTLTCa cells were plated in passage media and then treated with negative control siRNA, HIF-1α siRNA, BCRP siRNA, or 100 μM CoCl2 for 48 h. Cells were then collected and resuspended in mammosphere media on low-attachment cell culture wells. Results are expressed as number of mammospheres counted per 20,000 cells plated (mean ± SEM, n = 6 samples/group; * vs. vehicle, p < 0.01; † vs. control, negative control, and CoCl2). BCRP siRNA confirmed to decrease BCRP expression (0.35- and 0.15-fold vs. negative control, p <0.01; data not shown). B, Viability of cells was measured by MTT assay after 48 h treatment with negative control or Qiagen HIF-1α siRNA and subsequently 6 day treatment with increasing doses of letrozole. Results are expressed as percent of 0 uM letrozole (vehicle) (mean ± SEM, n = 4 samples/group; * vs. 0 uM letrozole, p < 0.05).
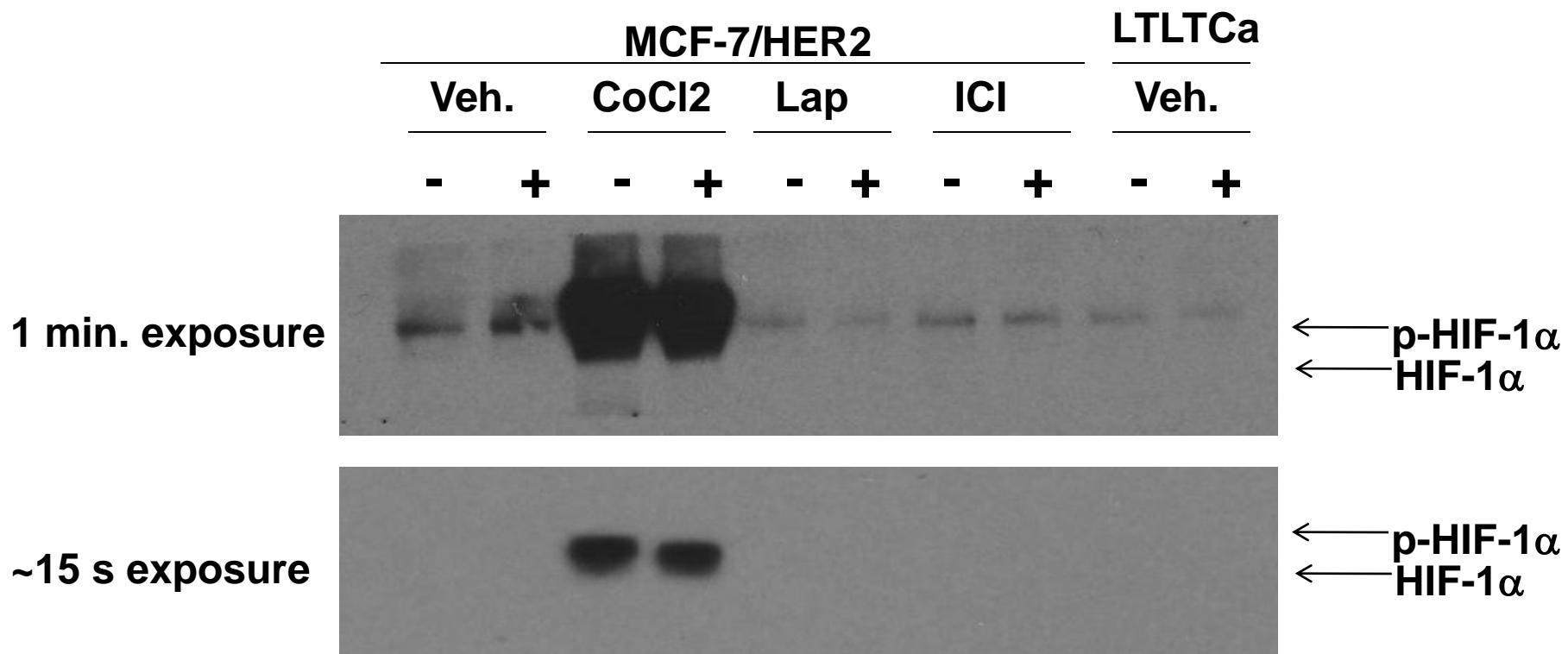
Figure 8. Comparison of BCRP protein and mRNA expression and stability in LTLTCa and MCF-7Ca cells
LTLTCa and parental MCF-7Ca cells were plated and cultured in their respective passage media under normal cell culture (nonhypoxic) conditions. A, Total RNA was extracted and BCRP mRNA, VEGF mRNA, and 18S rRNA were analyzed by real-time RT-PCR analysis. Results are expressed as the fold-change in mRNA levels compared with MCF-7Ca cells after normalization to 18S rRNA (mean ± SEM, n = 6 samples/group; * vs. MCF-7Ca, p < 0.01). C, LTLTCa and MCF-7Ca cells were treated with 0.5 ug/ml actinomycin D for 0-24 h. Total RNA was extracted and BCRP mRNA was analyzed by real-time RT-PCR analysis. Results are expressed or as percent mRNA of 0 h actinomycin D. (means ± SEM, n = 6 per group; † MCF-7Ca vs. LTLTCa, p <0.001).
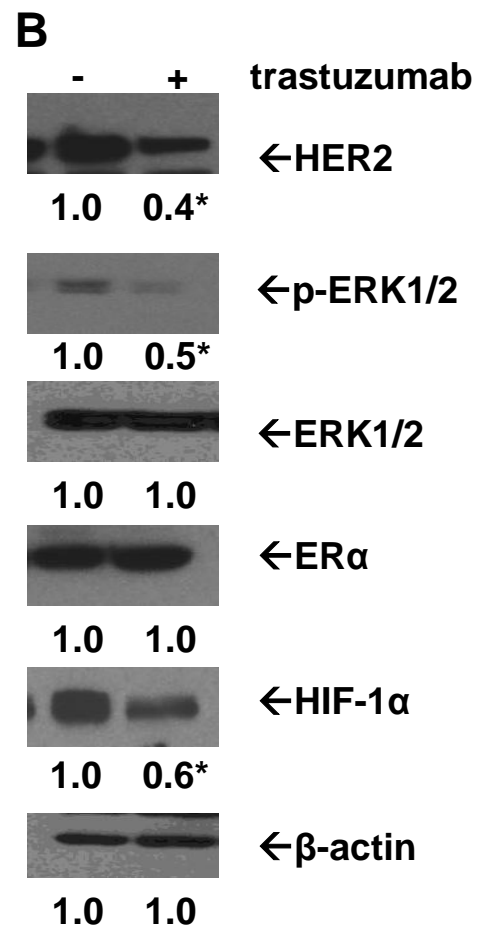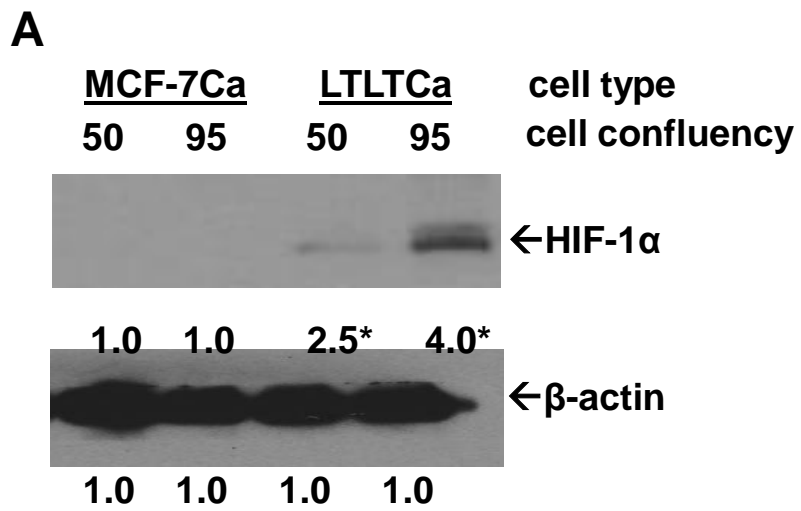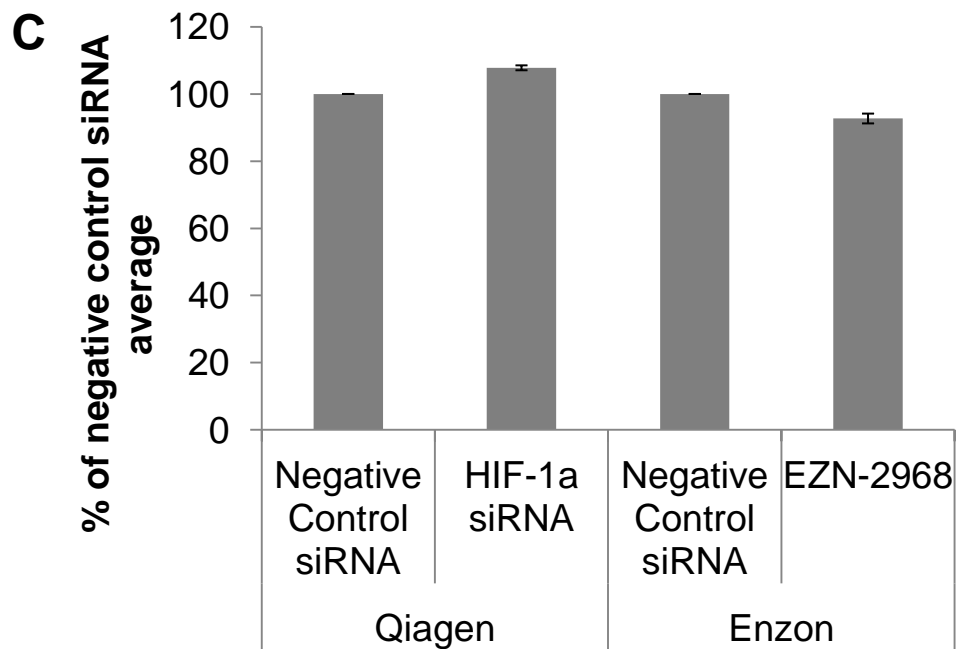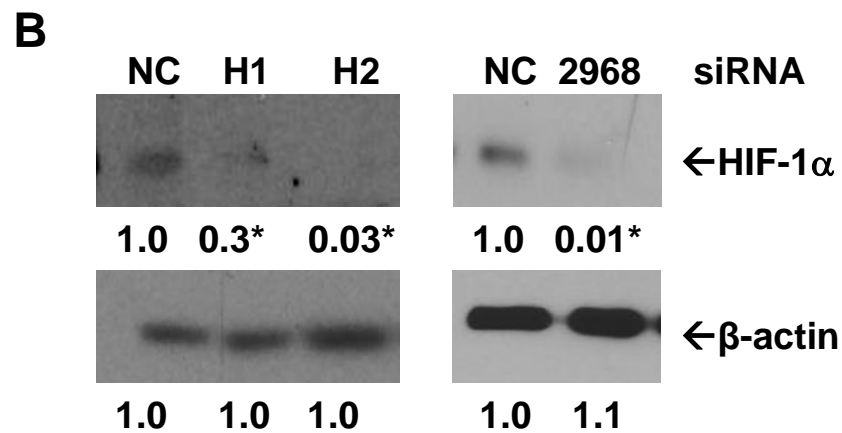
Figure 1

Figure 2

A

MCF-7Ca		LTLTCa		cell type
50	95	50	95	cell confluency

←HIF-1α

1.0 1.0 2.5* 4.0*

←β-actin

1.0 1.0 1.0 1.0

B

-    +   trastuzumab

←HER2

1.0   0.4*

←p-ERK1/2

1.0   0.5*

←ERK1/2

1.0   1.0

←ERα

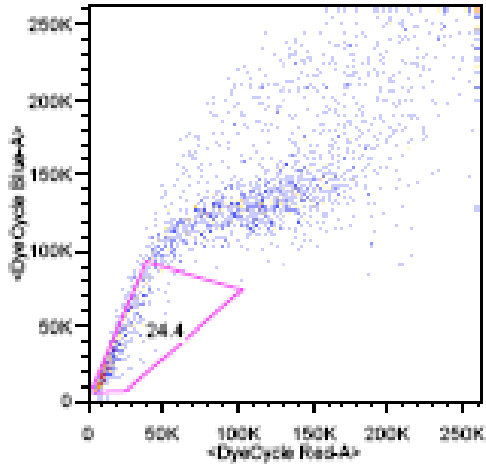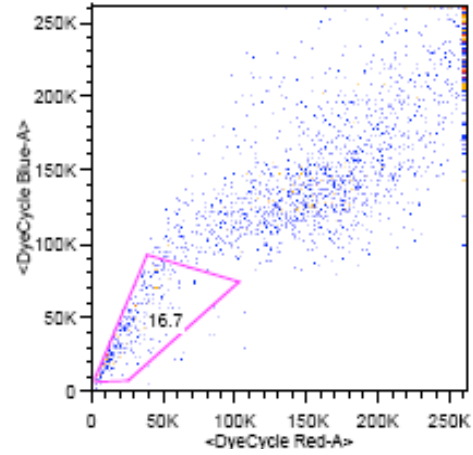1.0   1.0

←HIF-1α

1.0   0.6*

←β-actin

1.0   1.0
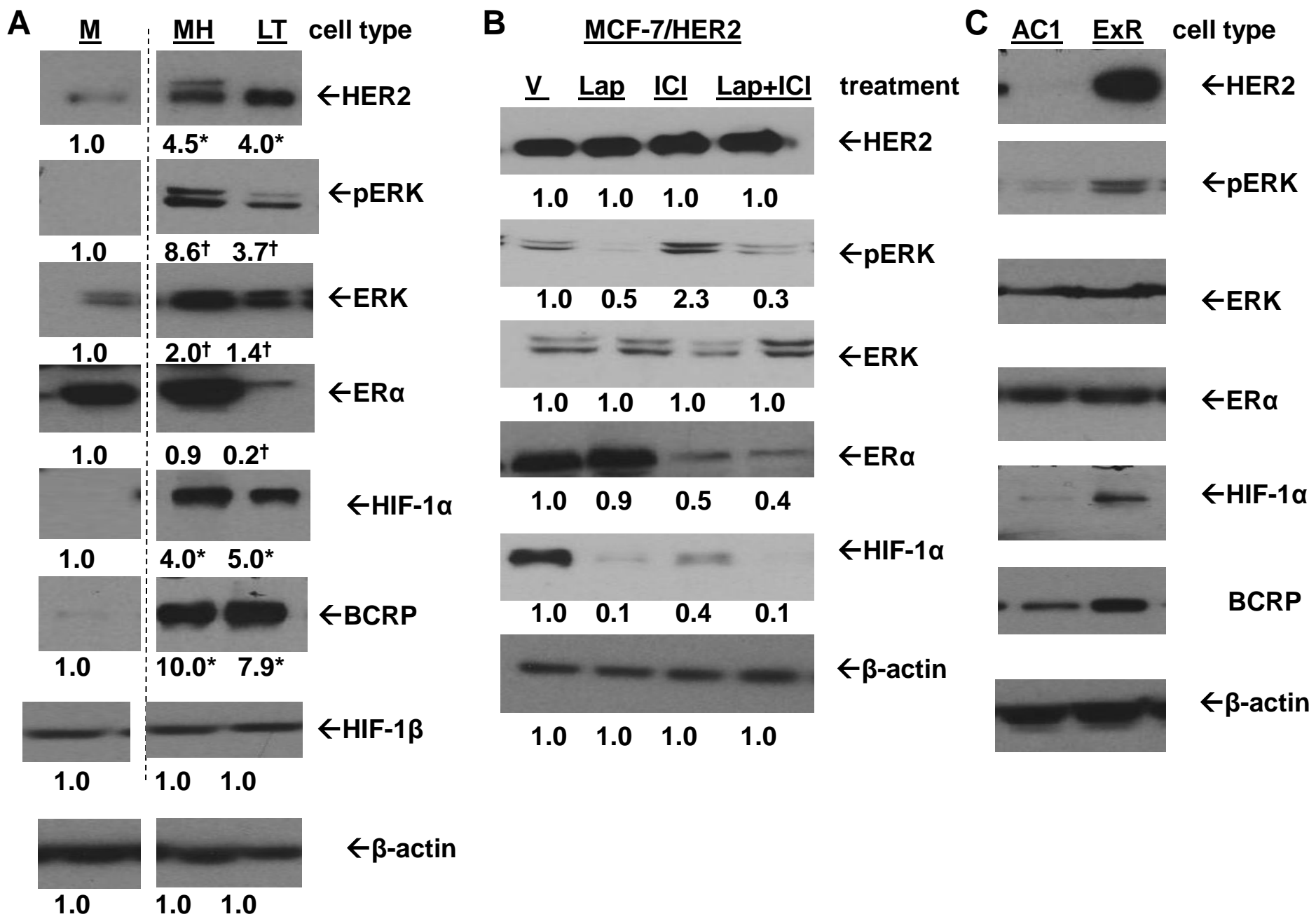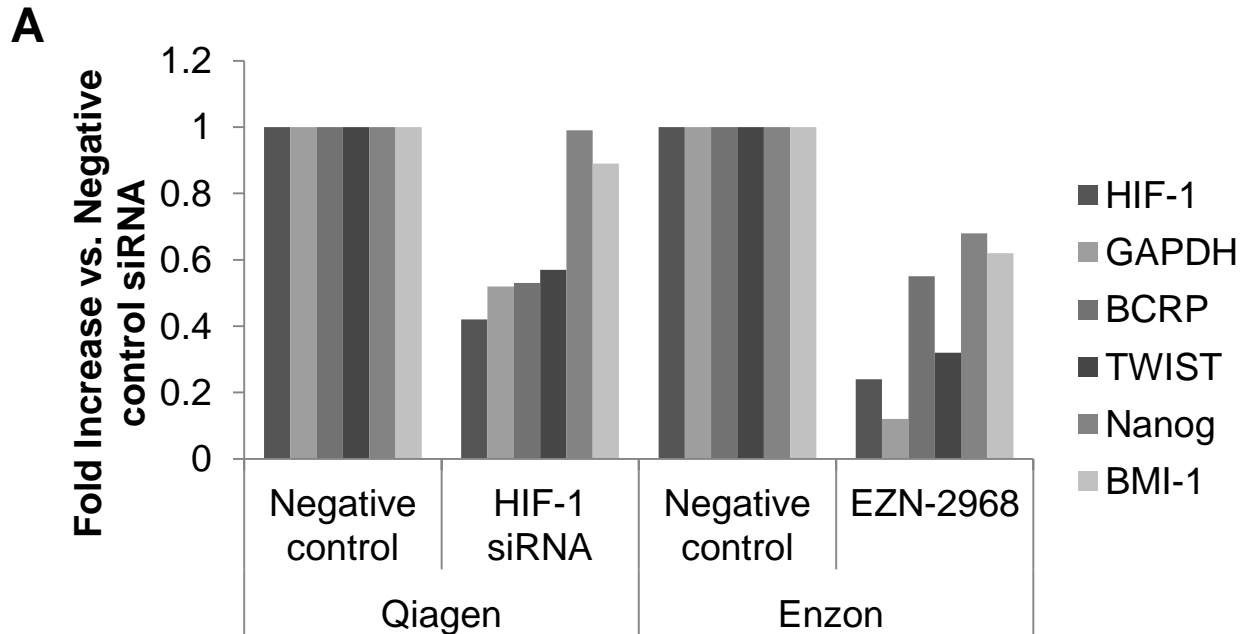
Figure 3

Figure 4
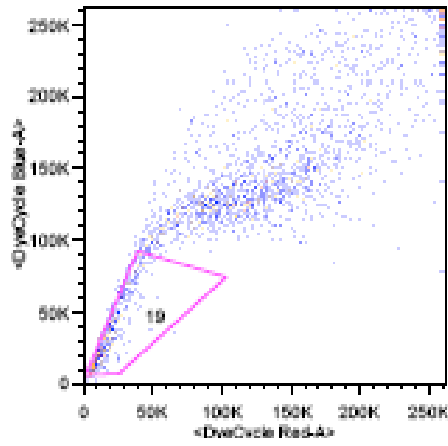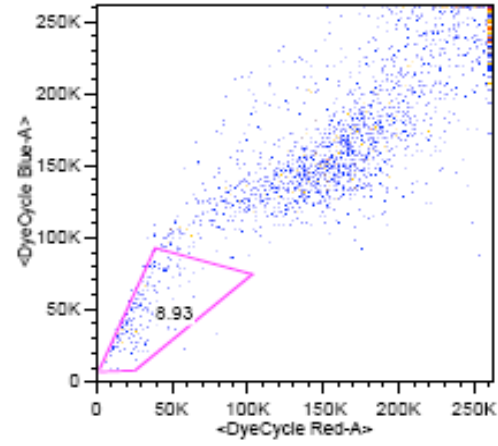
A

**Qiagen Negative control siRNA
(23.5±0.69)**

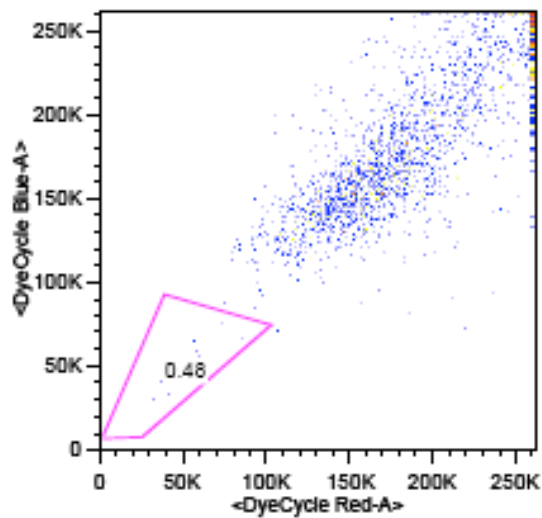**Enzon Negative control siRNA
(16.65±0.04)**
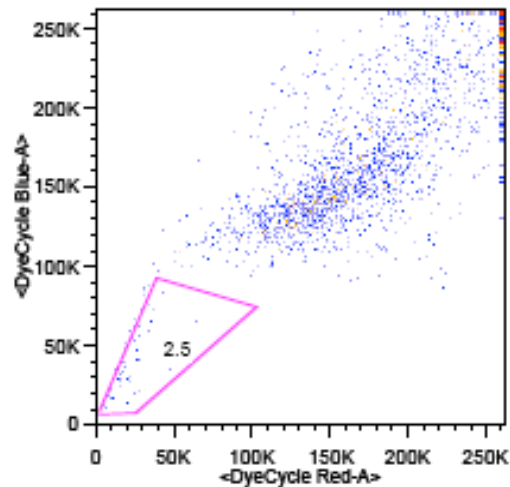
**Qiagen HIF-1α siRNA
(19.2±0.20)**

**EZN-2968
(9.39±0.37)**

Figure 5
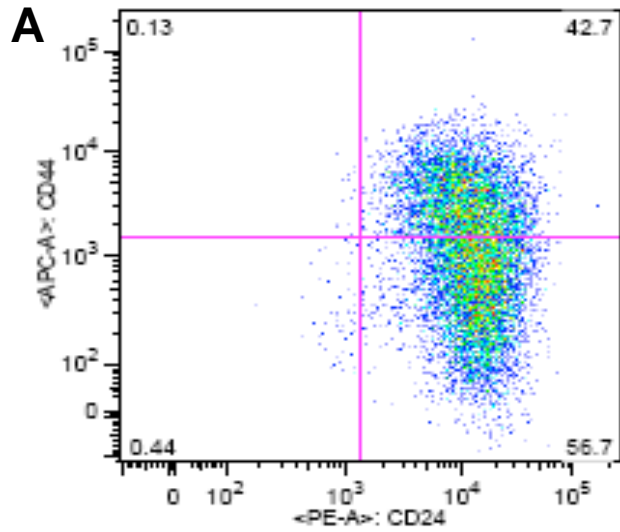
B

K0143
(2.2±0.04)

Qiagen BCRP siRNA
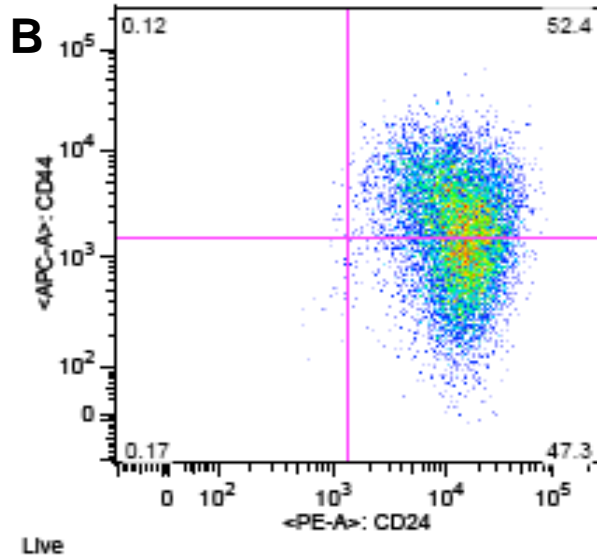**(2.4±0.12)**

C

PI- live
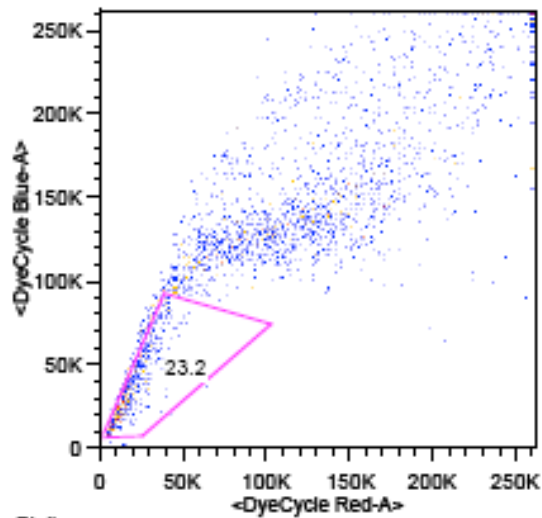
untreated
(21.4±1.7)

Figure 5

A

Qiagen Negative control siRNA
(44.0±0.78)
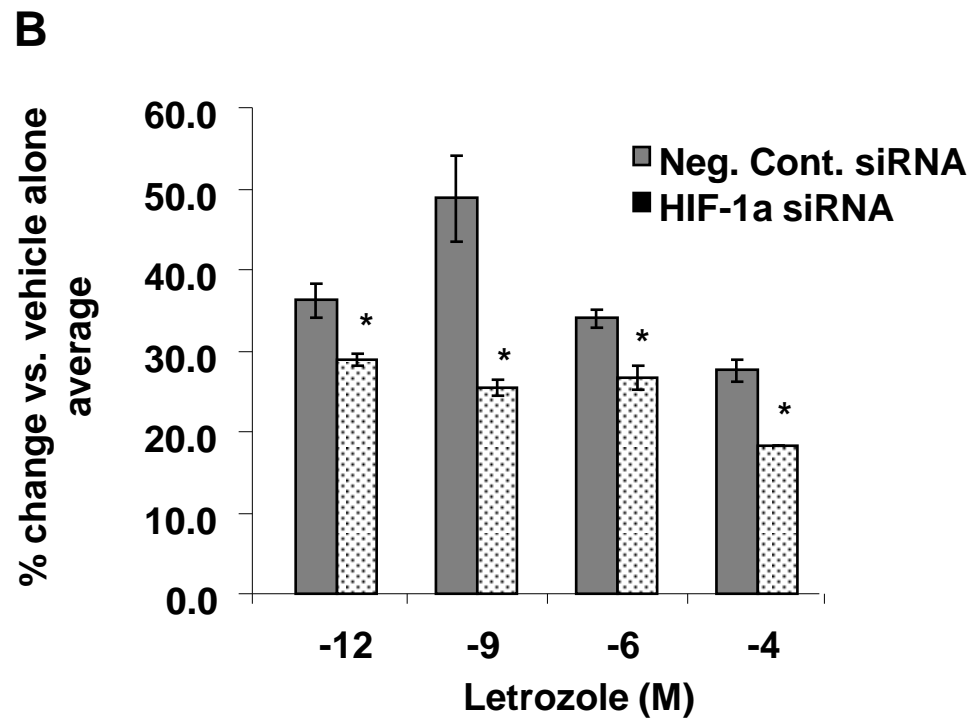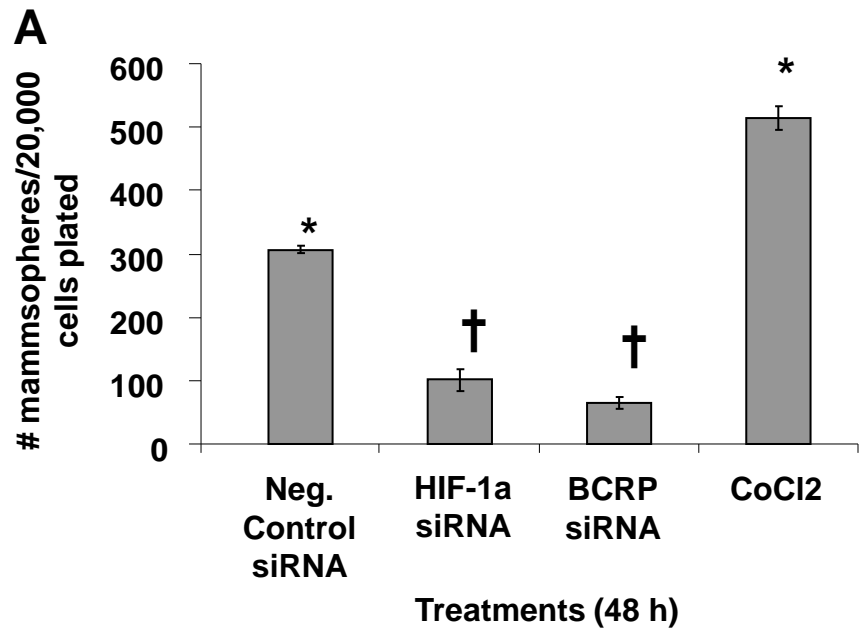
B

Enzon Negative control siRNA
(46.1±0.15)
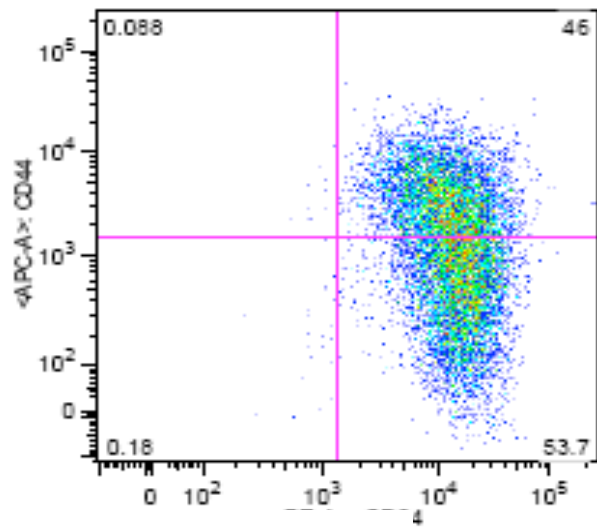
Qiagen HIF-1α siRNA
(52.0±0.50)
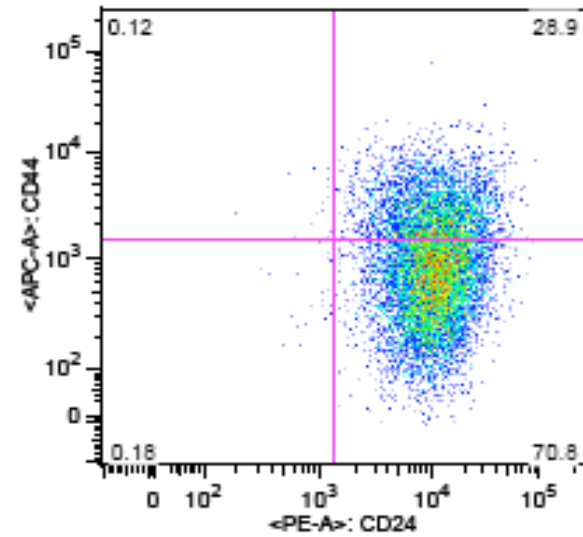
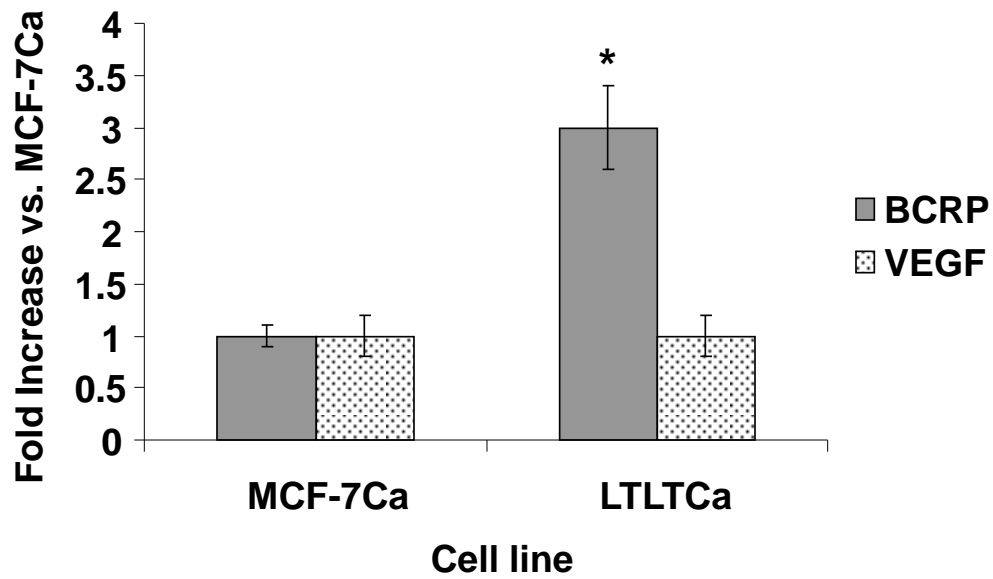**EZN-2968**
(27.4±0.75)

Figure 6

Figure 7

Figure 8

The importance of HER2 signaling in the tumor-initiating cell population in aromatase inhibitor-resistant breast cancer

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Amanda J. Schech · Saranya Chumsri ·
Gauri Sabnis · Anil K. Jaiswal · Angela H. Brodie

Abstract Aromatase inhibitors (AIs) are an effective therapy in treating estrogen receptor-positive breast cancer. Nonetheless, a significant percentage of patients either do not respond or become resistant to AIs. Decreased dependence on ER-signaling and increased dependence on growth factor receptor signaling pathways, particularly human epidermal growth factor receptor 2 (EGFR/HER2), have been implicated in AI resistance. However, the role of growth factor signaling remains unclear. This current study investigates the possibility that signaling either through HER2 alone or through interplay between epidermal growth factor receptor 1 (EGFR/HER1) and HER2 mediates AI resistance by increasing the tumor initiating cell (TIC) subpopulation in AI-resistant cells via regulation of stem cell markers, such as breast cancer resistance protein (BCRP). TICs and BCRP are both known to be involved in drug resistance. Results from in vitro analyses of AI-resistant versus AI-sensitive cells and HER2-versus HER2+ cells, as well as from in vivo xenograft tumors, indicate that (1) AI-resistant cells over-express both HER2 and BCRP and exhibit increased TIC characteristics compared to AI-sensitive cells; (2) inhibition of HER2 and/or BCRP decrease TIC characteristics in letrozole-resistant cells; and (3) HER2 and its dimerization partner EGFR/HER1 are involved in the regulation of BCRP. Overall, these results suggest that reducing or eliminating the TIC subpopulation with agents that target BCRP, HER2, EGFR/HER1, and/or their downstream kinase pathways could be effective in preventing and/or treating acquired AI resistance.

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Introduction

Inhibition of estrogen synthesis via aromatase inhibitors (AIs, i.e., letrozole, anastrozole, and exemestane) is highly effective in treating estrogen receptor-positive (ER+) breast cancer and preventing contra-lateral breast cancer [1]. A significant percentage of ER+ patients, however, either do not respond to AIs or become resistant to them during treatment [2, 3]. Previous work by our laboratory indicates that acquired AI resistance involves a switch from

the dependence on ER signaling to the dependence on growth factor-mediated pathways, such as human epidermal growth factor receptor 2 (EGFR2)/HER2 and insulin-like growth factor receptor (IGFR) [2–4]. However, the mechanism by which growth factor signaling confers AI resistance remains unclear.

One possibility is that HER2 mediates expansion of the tumor initiating cell (TIC) population, as suggested in previous studies [5, 6]. TICs are a small distinct subset of cells within a heterogeneous tumor which have the ability to initiate and maintain tumor growth and to promote metastasis [7–12]. Pertinent to acquired AI resistance, TICs are known to play a role in radio- and chemo resistance [7–9], as the residual tumor cell population remaining after treatment with conventional chemotherapy or AIs exhibit a high proportion of TICs [7–9, 13–15]. The expansion of TICs or inability to eliminate them may contribute to acquire AI resistance in breast cancer.

HER2 may maintain and/or expand the TIC subpopulation within an AI-resistant tumor by regulating certain TIC effector genes, such as the breast cancer resistance protein (BCRP). BCRP maintains TICs in an undifferentiated state [13, 15]. It is also a membrane-associated transporter protein, the function of which is to efflux out various molecules from the cell, and has been linked to multi-drug chemoresistance of breast cancer cells [13, 15–17]. Furthermore, other studies have shown a correlation between BCRP and HER2 [6, 18–20] and ERα [21, 22]. Thus, the purpose of this current study was to (1) determine if the TIC subpopulation is increased in AI-resistant versus-sensitive cells; (2) analyze the TIC subpopulation in letrozole-resistant cells; (3) investigate the importance of HER2 and BCRP on the TIC phenotype in letrozole-resistant cells; (4) determine whether HER2 regulates BCRP; and (5) determine if EGFR/HER1 is also involved.

Materials and methods

Cell lines and reagents

Cell lines

Cell lines (and their ER/HER2 status) used are listed in Table 1. MCF-7Ca cells are MCF-7 cells stably transfected with the human aromatase gene (supplied by Dr. Chen, City of Hope, Duarte, CA) and maintained in DMEM 1× high glucose (Invitrogen) supplemented with 5 % fetal bovine serum (FBS), 1 % penicillin/streptomycin (P–S), and 700 µg/mL G418. Long-term letrozole-treated (LTLT-Ca) cells are letrozole-resistant cells isolated from MCF-7Ca mouse xenograft tumors treated for 56 weeks with letrozole, and maintained in phenol red-free (PRF)

Table 1 Molecular profile and aromatase inhibitor-sensitivity of cell lines used

Cell Line	ERα status	HER2 status	AI-sensitivity
MCF-7Ca	+	−	Yes
LTLT-Ca	−	+	No (to letrozole)
MCF7	+	−	Yes
MCF7/HER2	+	+	ND
SUM149	−	−	ND
AC1	+	−	Yes
AC1-ExR	+	+	No (to exemestane)

ND not determined

modified IMEM (Invitrogen) supplemented with 5 % charcoal dextran-treated FBS (CDT-FBS), 1 % P–S, 750 µg/mL G418, and 1 µM letrozole. MCF-7 cells (ATCC) were maintained in DMEM 1 × high glucose (Invitrogen) supplemented with 5 % FBS and 1 % P–S. MCF-7/HER2 cells are MCF-7 cells transfected with the HER2 gene (supplied by Ann Hambruger, University of Maryland Baltimore, UMB) maintained in DMEM 1× high glucose (Invitrogen) supplemented with 5 % FBS, 1 % P–S, and 500 µg/mL hygromycin. AC1 cells are MCF-7 cells stably transfected with the human aromatase maintained in DMEM 1× high glucose (Invitrogen) supplemented with 5 % FBS, 1 % P–S, and 800 µg/mL G418. AC1-exemestane resistant (AC1-ExR) cells are a exemestane-resistant cells isolated from AC1 mouse xenograft tumors treated for ∼10 weeks with exemestane maintained in PRF modified IMEM (Invitrogen) supplemented with 5 % CDT-FBS, 1 % P–S, 800 µg/mL G418, and 5 µM exemestane.

Reagents

The following drugs were used: letrozole (Novartis); lapatinib (GlaxoSmithKline Pharmaceutical); trastuzumab (Genentech); BIBX1382 (Calbiochem); and BCRP inhibitors Ko143 and fumitremorgen C (FTC) were kindly provided by Dr. Douglas Ross (UMB). Use of Ko143 and FTC depended on availability from Dr. Ross' laboratory. The following antibodies were used in western blot analyses: HER2 (EMD Millipore); BCRP (EMD Millipore); ERα (Santa Cruz Biotechnology); and phosphorylated and total ERK1/2 or Akt, phosphorylated and total EGFR/HER1, and β-actin (all obtained from Cell Signaling Technology).

Side population (SP) analysis

SP analysis was performed either by Hoechst 33342 assay [6, 23] or using Vibrant DyeCycle Violet stain (Invitrogen). Briefly, after drug treatment, 1×10^6 cells/mL were suspended in prewarmed IMEM with 2 % FBS and 10 mM HEPES containing 5 µg of Hoechst 33342 (Sigma) or 1 µL

of DyeCycle Violet stock. Cells were incubated at 37 °C for 40 min (Dye Cycle) or 90 min (Hoechst). Cells were acquired using BD-LSRII. Data was analyzed by means of FlowJo software. Cells treated with BCRP inhibitor Ko143 were included in each SP analysis set as a control to demonstrate inhibition of dye efflux.

Aldehyde dehydrogenase (ALDH) assay

The Aldefluor assay was performed using the Aldefluor Assay kit from Stem Cell Technologies. ALDH stained cells were identified in cells by comparing the same sample with and without the ALDH inhibitor DEAB (diethylaminobenzalde-hyde). Cells were acquired using BD LSRII and FACS CANTO. Data were analyzed by means of FlowJo software.

Mammosphere assay

The mammosphere assay was performed using reagents from Stem Cell Technologies according to the manufacturer's instructions. Single cells were suspended in complete Mammocult media as per the manufacturer's instructions and plated in ultra-low-attachment plates (Corning) at a density of 10,000–20,000 cells/mL. Media was replenished every 3 days. Mammospheres were counted after at least 7 days and up to 3–4 weeks. Spheres with a colony count of at least 50 cells were considered mammospheres.

Immunofluorescence (IF)

IF staining for Oct-4 was performed on sorted LTLT-Ca cells kept under non-adherent conditions for 2–3 days prior to cytospinning. Pelleted cells were fixed with 4 % para-formaldehyde and incubated with OCT4 antibody (Santa Cruz Biotechnology) and the corresponding fluorochrome-tagged secondary antibody (Invitrogen). Images were obtained and analyzed by means of Image J software.

RT-PCR

RNA extraction and reverse transcription (RT)

RNA was extracted and purified using the RNeasy Mini Kit (Qiagen). RNA was reverse transcribed to complementary DNA (cDNA) using 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen) and incubating at 37 °C for 1 h.

Real-Time PCR

mRNA expression analyses were carried out by real-time PCR using a DNA Opticon system (MJ Research) and using DyNAmo SYBR green qPCR mix (New England

Biolabs). Standard curves were generated by serially diluting the sample expected to have the most amount of PCR product. The yield of product for each unknown sample was calculated by applying its threshold cycle, or C(T), value to the standard curve by means of the Opticon Monitor analysis software (version 1.01, MJ Research). Values were normalized to corresponding 18S rRNA values and expressed as the fold increase relative to controls. Primers for HER2, BCRP, BMI-1, Nanog, and Twist were obtained from Sigma or Qiagen.

Growth and treatment of MCF-7Ca mouse xenograft tumors

All animal studies were performed according to the guidelines and approval of the Animal Care Committee of UMB. MCF-7Ca tumor xenografts of MCF-7Ca cells were grown in ovariectomized athymic nude mice as previously described [24]. Briefly, each mouse received subcutaneous (sc) inoculations in one site per flank of 100 μL of cell suspension containing $\sim 2.5 \times 10^7$ MCF-7Ca cells. All mice were supplemented throughout the experiment with 100 μg/day androstenedione (Δ4A) sc 5 days/week. Δ4A is converted to estrogen by aromatase expressed in MCF-7Ca cells. Tumor growth was monitored weekly and treatments began when the tumors reached ~ 300 mm^3. Mice were then randomly divided into either control (100 μg/day Δ4A) or letrozole (100 μg/day Δ4A + 10 μg/day letrozole) treatment groups. Letrozole and Δ4A were prepared using 0.3 % Hydroxypropylcellulose in saline solution. Mice were injected sc five times weekly with the indicated drugs. Tumors were collected at necropsy either at previously designated times (weeks 4, 8, and 16), or when tumors reached 2,000 mm^3 in size.

Western blot analysis

Cells

Plated cells were washed with ice-cold PBS and then lysed with radioimmunoprecipitation (RIPA) buffer containing protease and phosphatase inhibitors (Roche) by sonication and incubation for 20 min at 4 °C. Lysed samples were centrifuged at 14,000 rpm for 20 min at 4 °C to collect protein lysates (supernatant). 10–40 μg of protein underwent 10 % SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Fisher Scientific). The resulting blots were probed with specific mouse or rabbit primary antibodies and either goat anti-mouse or -rabbit secondary antibodies conjugated to horseradish peroxidase (Biorad), respectively. Blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Blots that were to be re-
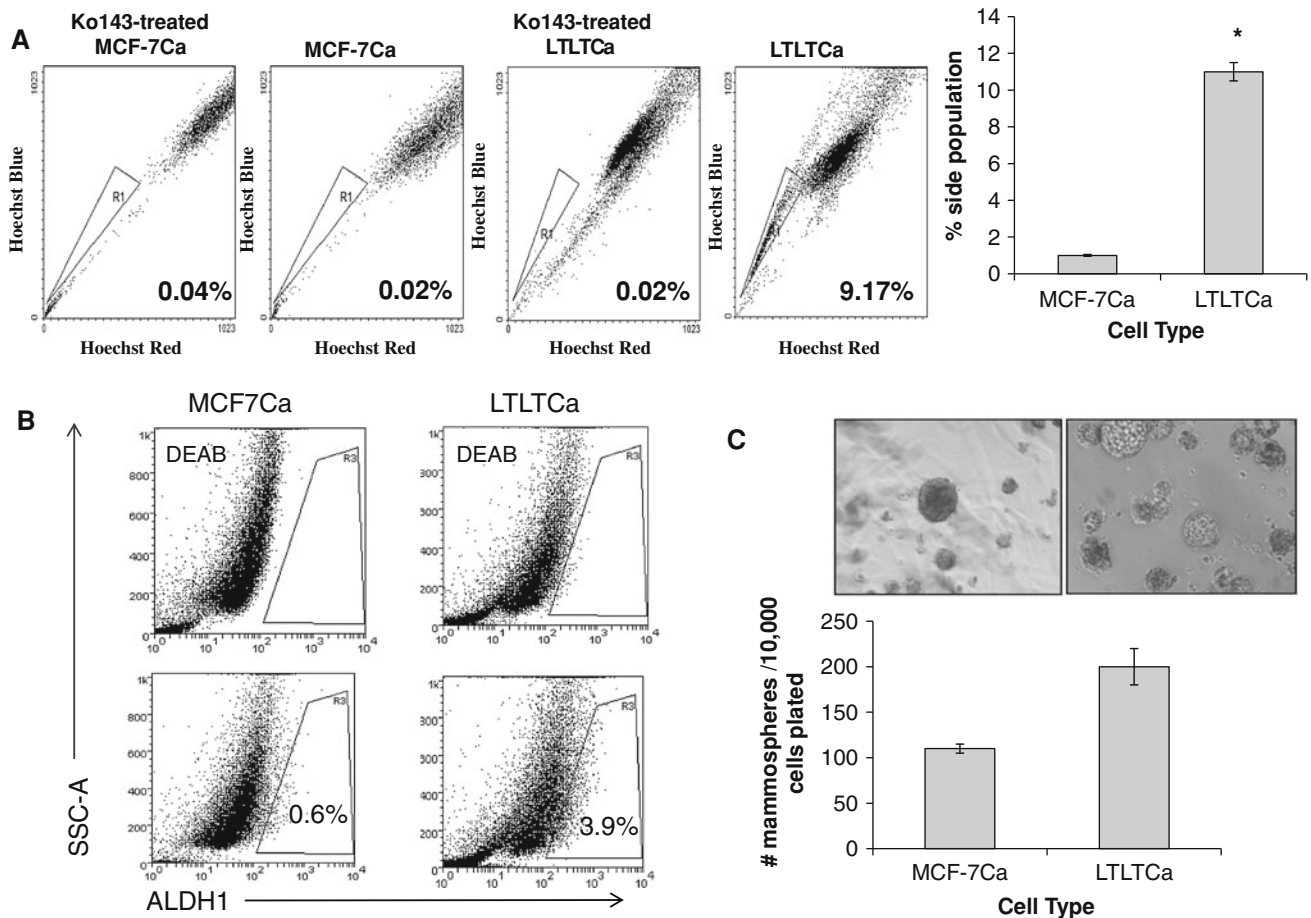
Fig. 1 Analysis of SP and ALDH in MCF-7Ca and LTLT-Ca cells. **a** Percentage of side population (SP) cells were analyzed in MCF-7Ca and LTLT-Ca cells by Hoechst staining and flow cytometry in the absence or presence of BCRP inhibitor Ko143. Plots show representative SP fractions as designated by *polygons* and quantified as percentages ($n = 3$ independent cell samples/group; $p < 0.01$). **b** Analysis of aldehyde dehydrogenase 1 (ALDH) by measuring the cellular fluorescence of bodipy-aminoacetate (BAAA) in the presence or absence of specific ALDH inhibitor DEAB. Plots show representative ALDH1-expressing cells, as designated by *polygons* and quantified as percentages ($n = 3$ independent cell samples/group; $p < 0.05$). **c** MCF-7Ca and LTLT-Ca cells were plated onto ultra-low-attachment plates containing mammosphere culture media. After 8 days in culture, formed mammospheres were imaged (*top panel*) and counted (*bottom panel*). Shown are representative images (*top panel*) and mean mammosphere counts. (Mean ± SEM, $n = 9$ independent cell samples/group, $p < 0.05$.)

probed were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific) for 40 min at room temperature before incubation with another primary antibody.

MCF-7Ca xenograft tumors

Tumor samples were first homogenized in ice-cold DPBS containing protease inhibitors and centrifuged. The resulting tumor homogenate pellets were lysed with RIPA buffer. Tumor protein lysates were then collected and subjected to the same western blot analyses as cells.

Statistical analysis

All experiments were carried out at least 2–3 times each with replicates. Statistical analyses were performed by

Student's *t* test (two samples), ANOVA (3+ samples) by means of Graph Pad Prism software.

Results

Letrozole-resistant LTLT-Ca cells exhibit higher percentage of TICs compared to letrozole sensitive MCF-7Ca cells

The TIC subpopulation can be distinguished from other cells within a cancer cell population by several characteristics: (1) exclusion of Hoechst dye from cells and identification as "side population" (SP) cells [25–27]; (2) increased ALDH1 expression [28]; (3) ability to form mammospheres in culture [29]; and (4) expression of
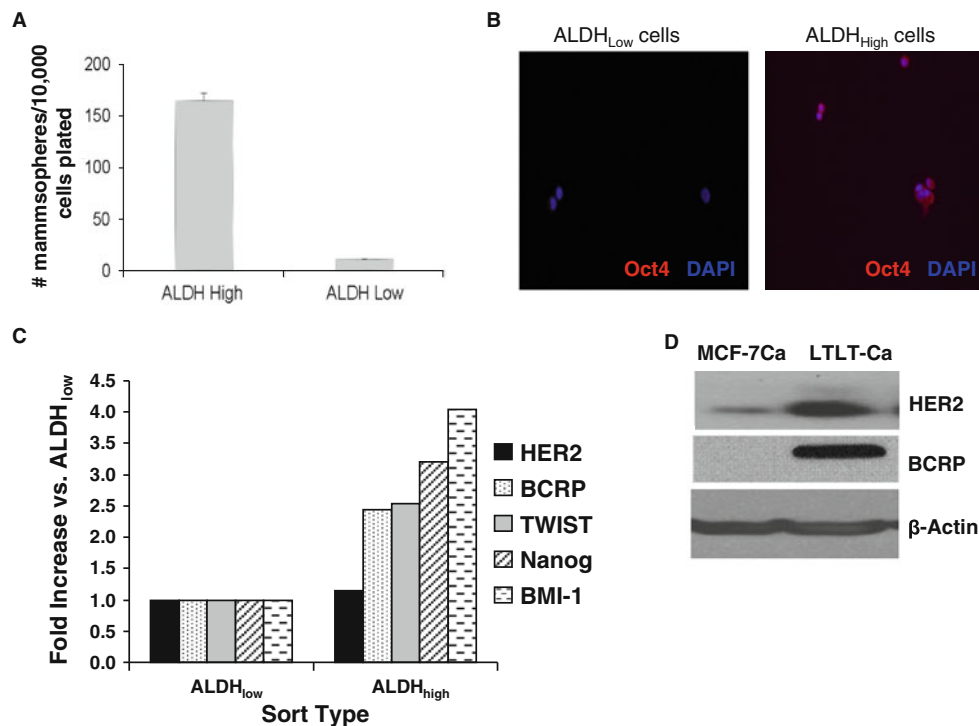
Fig. 2 mRNA expression in $ALDH_{low}$ and $ALDH_{high}$ LTLT-Ca cells and protein expression in LTLT-Ca versus MCF-7Ca cells. **a** LTLT-Ca cells were treated with aldefluor and then $ALDH1_{low}$ and $ALDH1_{high}$ cells (3–4 % lowest and highest) were sorted by fluorescence-activated cell sorting (FACS). Sorted cells were then plated under ultra-low-attachment conditions at a density of 10,000 cells/well. After ∼3 weeks, formed mammospheres were counted. A representative graph of the average number of formed mammospheres ($n = 3$; $p < 0.01$) is shown. **b** $ALDH1_{low}$- and $ALDH1_{high}$-expressing LTLT-Ca cells were subjected to immunofluorescence with Oct-4 antibody (*red*) and DAPI nuclear stain (*blue*).

Shown are representative immunofluorescence images. **c** Total mRNA was extracted from $ALDH1_{low}$- and $ALDH1_{high}$-expressing LTLT-Ca cells, and real-time RT-PCR analyses were done for HER2, BCRP, TWIST, Nanog, and BMI-1 mRNA and 18S rRNA. Real-time results are expressed as the fold change in mRNA levels compared with ALDH low after normalization to 18S rRNA (mean ± SEM, $n = 2$ pooled samples/group). **d** MCF-7Ca and LTLT-Ca cells were plated in their respective passage media. Total protein was extracted and HER2, BCRP, and β-actin protein were analyzed by western blot. Representative blots ($n = 6$ independent cell samples/group) are shown

known stem cell markers, such as BCRP [13, 15, 30], Nanog [31], and BMI-1 [32]. Letrozole-resistant LTLT-Ca, derived from long-term letrozole treated MCF-7Ca xenograft tumors, and letrozole-sensitive parental MCF-7Ca cells were, therefore, compared to each other based on these TIC parameters. LTLT-Ca cells were found to have higher percentage of SP cells (9.17 ± 2.0 % LTLT-Ca vs. 0.02 ± 0.01 % MCF-7Ca, $p < 0.01$) and ALDH-expressing cells (3.9 % LTLT-Ca vs. 0.6 % MCF-7Ca, $p < 0.01$) than MCF-7Ca cells (Fig. 1a, b). LTLT-Ca cells also formed significantly more mammospheres in culture than MCF-7Ca cells (Fig. 1c, $p < 0.05$). Thus, letrozole-resistant LTLT-Ca cells express more TIC characteristics than parental, letrozole-sensitive MCF-7Ca cells.

The TIC subpopulation in LTLT-Ca cells was further analyzed through isolation of the highest and lowest 3–4 % ALDH1-expressing cells (Fig. 2). These were designated as $ALDH1_{high}$ (i.e., TICs) and $ALDH1_{low}$ cells, respectively, and their ALDH expression levels were confirmed by immunofluorescence (data not shown). These

subpopulations were then compared for their ability to form mammospheres and their expression of known stem cell markers and HER2. As expected, $ALDH1_{high}$ cells formed significantly more mammospheres (164.7 ± 7.5 vs. 11 ± 1, $p < 0.05$; Fig. 2a) and they expressed higher levels of stem cell markers OCT-4 (as shown by immunofluorescence, Fig. 2b), BCRP, BMI-1, and Nanog (>2.5– >3.3-fold as shown by RT-PCR, Fig. 2c) compared to $ALDH1_{low}$ cells. In addition, $ALDH1_{high}$ cells expressed higher mRNA levels of TWIST, an epithelial–mesenchymal transition marker and transcription factor known to regulate BMI-1 and of HER2 (>1.3 and >2.5-fold vs. $ALDH1_{low}$, Fig. 2c). HER2 overexpression in $ALDH1_{high}$ versus $ALDH1_{low}$ cells was additionally confirmed by flow cytometry (data not shown). Both HER2 and BCRP proteins were also increased in LTLT-Ca cells when compared overall to MCF-7Ca cells (Fig. 2d). These results demonstrate that the TIC population can be isolated from LTLT-Ca cells and that they express factors known to be involved in their regulation.
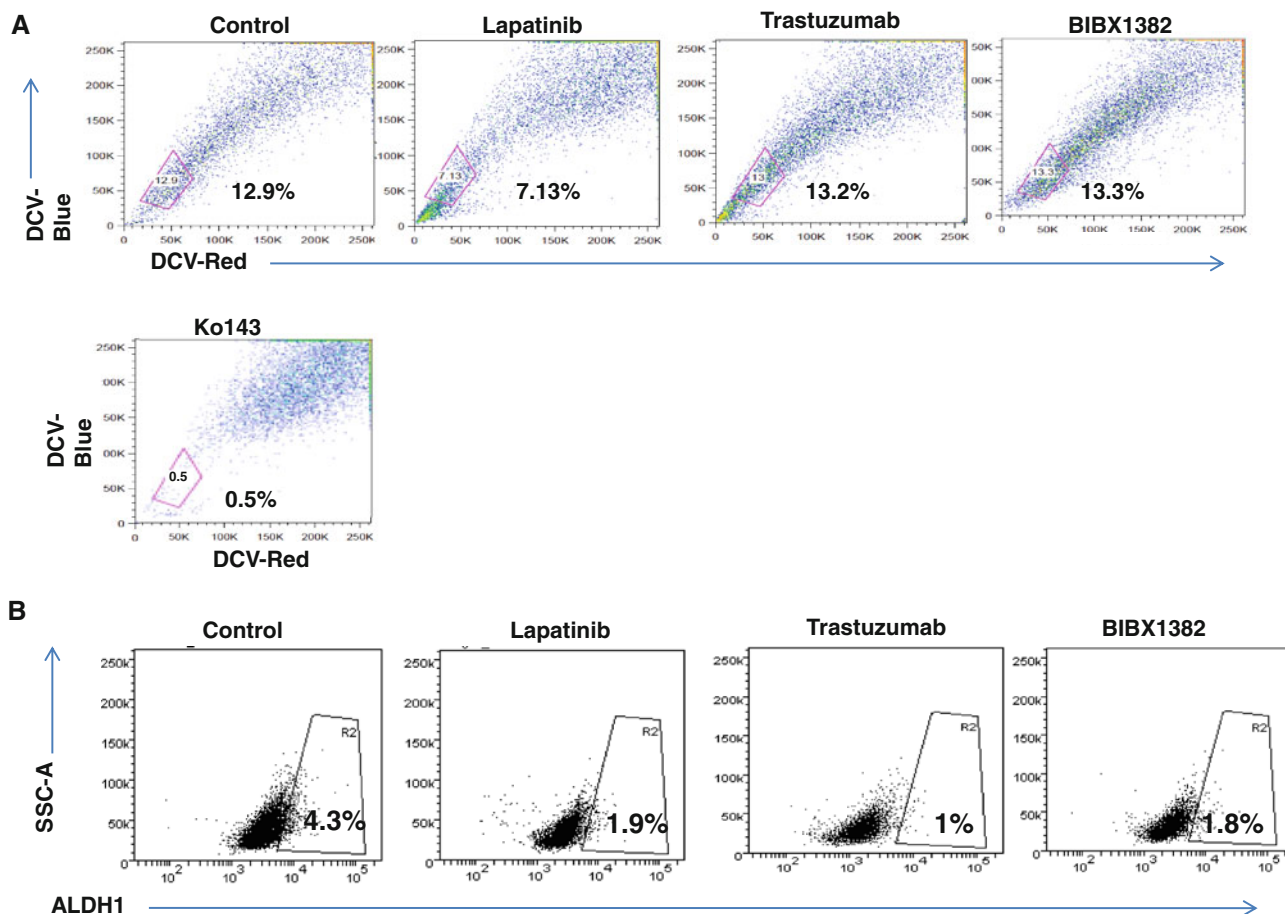
Fig. 3 Effect of HER2, BCRP, and/or EGFR inhibitors on percentage of side population (SP) cells and ALDH-expressing LTLT-Ca cells. **a** Percentage of side population (SP) cells were analyzed in MCF-7Ca and LTLT-Ca cells by Hoechst staining by flow cytometry after 48 h treatment with vehicle (*control*), 1 μM Ko143, 1 μM lapatinib, 500 μg/mL trastuzumab, or 10 nM BIBX 1382. Plots show representative SP fractions, as designated by *polygons* and quantified as percentages ($n = 3$ independent cell samples/group; $p < 0.01$). **b** Analysis of aldehyde dehydrogenase 1 (ALDH) by measuring the cellular fluorescence of bodipy-aminoacetate (BAAA) after 48 h treatment with vehicle (*control*), 1 μM lapatinib, 500 μg/mL trastuzumab, or 100 nM BIBX 1382. Plots show representative ALDH expressing cells, as designated by *polygons* and quantified as percentages ($n = 3$ independent cell samples/group; $p < 0.05$)

EGFR/HER1, HER2, and BCRP are involved in regulating the TIC phenotype in LTLT-Ca cells

HER2 and BCRP are examples of TIC-regulating factors [9, 13, 15, 18, 30, 33, 34]. To assess the importance of HER2 in regulating the TIC phenotype, the effects of HER2 inhibitors lapatinib (a EGFR/HER1-HER2 tyrosine kinase inhibitor), and trastuzumab (a HER2-specific monoclonal antibody) [35] were determined. Lapatinib treatment, in particular, has been previously shown to reduce TIC characteristics in breast cancer cells obtained from patients [9]. Since HER2 can be ligand-dependently activated through dimerization with EGFR/HER1 [36], and lapatinib can inhibit both HER2 and EGFR/HER1, the effect of highly specific and potent (at nM concentrations) EGFR/HER1 kinase inhibitor BIBX1382 [37] was also analyzed. The percentage of SP cells in LTLT-Ca remained unchanged by HER2-specific trastuzumab or EGFR/HER1-specific BIBX1382, but was decreased by dual inhibitor lapatinib (7.3 vs. 12.9 % control, Fig. 3a). ALDH1 expression, however, was decreased in LTLT-Ca cells by lapatinib, trastuzumab, and BIBX1382 (1.87, 0.99, and 1.8 vs. 4.34 % of vehicle-treated, respectively; Fig. 3b). Finally, 48 h treatment with either lapatinib or trastuzumab before plating in ultra-low-attachment conditions significantly decreased LTLT-Ca mammosphere formation ($p < 0.05$; Fig. 4), with greater effects observed in lapatinib-treated cells. The inhibitory effects of these drugs on TIC characteristics was not due to induction of cell death in the overall LTLT-Ca cell population, as trypan blue cell viability assays showed no significant differences between vehicle- and drug-treated cells within the same 48 h time frame (data not shown).

BCRP's importance was similarly assessed using specific and potent BCRP inhibitors FTC or FTC's less toxic, more potent analog Ko143 [38]. Consistent with studies in other breast cancer cell lines [6] and with BCRP's known function as a membrane-associated transporter protein that effluxes from cells a variety of molecules (i.e., Hoechst dye and chemotherapeutic drugs) [16], Ko143 treatment significantly reduced the percentage of SP cells (0.02 % Ko143 vs. 9.17 % in Fig. 1a; 0.24 % Ko143 vs. 7.91 % vehicle in Fig. 3a). Both FTC and Ko143 significantly decreased mammosphere formation by ~75 % compared to vehicle-treated LTLT-Ca cells ($p < 0.05$, Fig. 4). Similar reduction in mammosphere formation was observed after treatment of LTLT-Ca cells with BCRP siRNA to reduce BCRP expression (Fig. 5). Neither Ko143 nor FTC had an effect on ALDH1 expression (data not shown). Overall, these results demonstrate that both HER2 and BCRP are involved in regulating the TIC phenotype of LTLT-Ca cells. BCRP is important in SP and mammosphere formation, whereas HER2 is required for ALDH1, SP, and mammosphere formation in LTLT-Ca cells. These results indicate that EGFR/HER1 may also have a role in TICs.

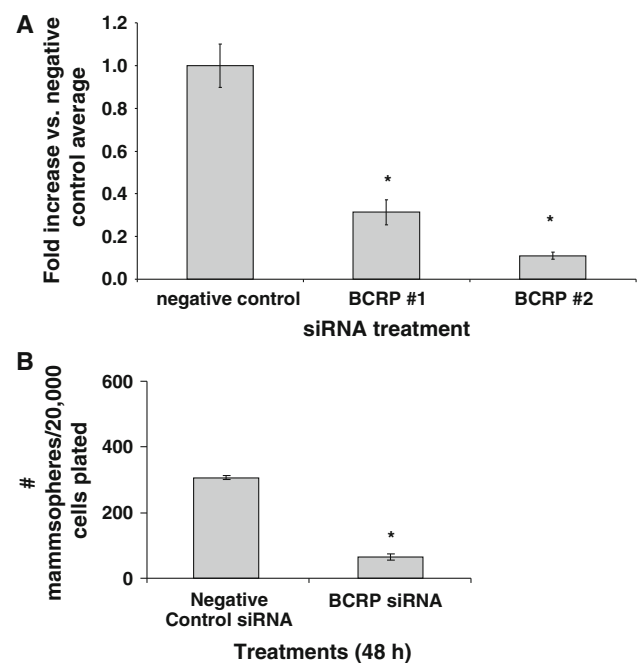### EGFR/HER1 and HER2 are involved in regulation of BCRP

Since HER2 and BCRP are both overexpressed in LTLT-Ca cells (Fig. 2) and are important in the TIC phenotype (Figs. 3, 4), another objective of this study was to determine whether HER2 regulates BCRP expression in LTLT-Ca cells and whether EGFR/HER1 was also involved. The role of HER2 is supported by several pieces of evidence.

First, other HER2-expressing cell lines also had elevated BCRP levels. HER2-transfected MCF-7 (MCF-7/HER2) cells overexpressed BCRP protein compared to HER2-/low expressing cells (i.e., MCF-7 and SUM149), and at comparable levels to LTLT-Ca cells (Fig. 6a). In addition, compared to exemestane-sensitive, HER2-, parental AC1 cells, another AI (exemestane)-resistant cell line (AC1-ExR) had elevated levels of both HER2 and BCRP protein (Fig. 6b) and increased mammosphere formation (Fig. 6c). Even though ERα is also known to regulate BCRP [22], BCRP protein was elevated in both ERα-/HER2 + LTLT-Ca cells and in ERα+/HER2 + MCF-7/HER2 and AC1-ExR cells (Fig. 6a, b). It was also elevated in cell lines with (AC1-ExR cells) or without (LTLT-Ca) increased EGFR/HER1 phosphorylation compared to their parental cell lines (Fig. 6a, b). Thus, upregulation of BCRP occurs independently of ERα expression and EGFR/HER1 activation.

Second, inhibition of HER2 signaling decreased BCRP expression. LTLT-Ca cells were treated for 24 h with lapatinib alone, trastuzumab alone, BIBX1382 alone, or

Fig. 4 Effect of HER2, BCRP, and/or EGFR inhibitors on mammosphere formation in LTLT-Ca cells. After 48 h treatment with either vehicle (control), HER2 inhibitors (1 μM lapatinib or 500 μg/mL trastuzumab), or BCRP inhibitors (1 μM of either Ko1453 or FTC), LTLT-Ca cells were plated onto ultra-low-attachment plates containing mammosphere culture media. After 14 days in culture, formed mammospheres were imaged and counted. The mean mammosphere counts. (mean ± SEM, $n = 3$ independent cell samples/group, $p < 0.01$.) are shown

Fig. 5 Effect of BCRP siRNA on BCRP mRNA expression and mammosphere formation in LTLT-Ca cells. a LTLT-Ca cells were plated in passage media and then treated with two siRNAs for BCRP for 48 h. Total mRNA was extracted and BCRP mRNA and 18S rRNA were analyzed by real-time RT-PCR. Real-time results are expressed as the fold change in mRNA levels compared with negative control after normalization to 18S rRNA (mean ± SEM, $n = 6$ samples/group; asterisk vs. vehicle, $p < 0.01$). b LTLT-Ca cells were plated in passage media and then treated with negative control siRNA or BCRP siRNA for 48 h. Cells were then collected and resuspended in mammosphere media on low-attachment cell culture wells. Results are expressed as number of mammospheres counted per 40,000 cells plated (mean ± SEM, $n = 6$ samples/group; asterisk vs. negative control, $p < 0.01$)
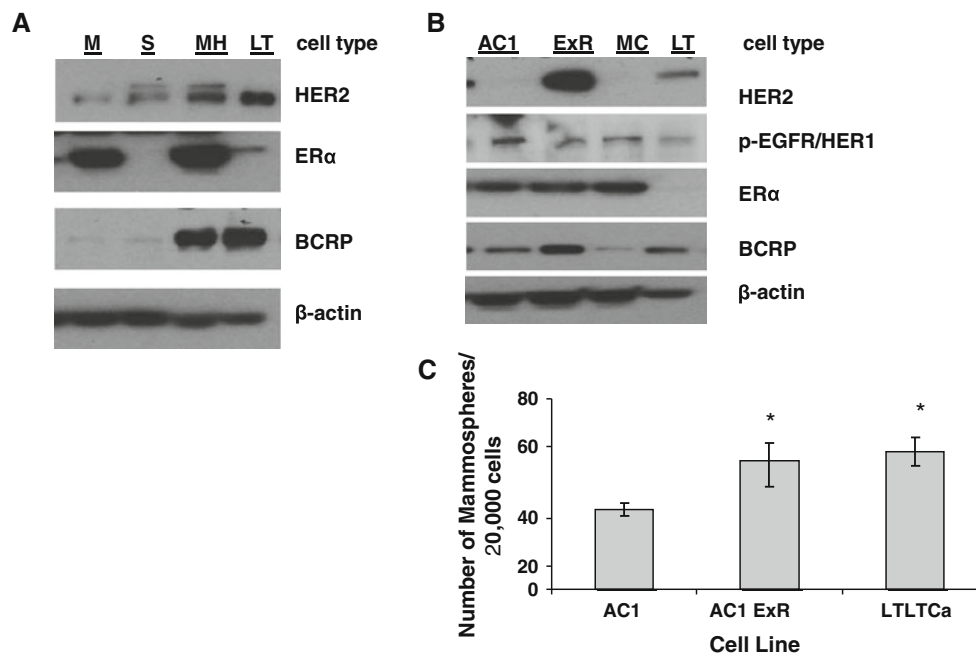
Fig. 6 Protein expression in HER2- and HER2+ cells and mammosphere formation in AC1-ExR cells. **a** MCF-7Ca (*MC*), SUM149 (*S*), MCF-7/HER2 (*MH*), and LTLT-Ca (*LT*) cells were plated in their respective passage media. Total protein was extracted and HER2, ERα, BCRP, and β-actin protein were analyzed by western blot. Representative blots (*n* = 6 independent cell samples/group) are shown. **b** AC1 (*AC1*), AC1-ExR (*ExR*), MCF-7Ca (*MC*), and LTLT-Ca (*LT*) cells were plated in their respective passage media. Total protein was extracted and HER2, phosphorylated EGFR/HER1 (p-EGFR/HER1), ERα, BCRP, and β-actin protein were analyzed by western blot. Representative blots (*n* = 3 samples/group) are shown. **c** AC1, AC1-ExR, and LTLT-Ca cells were resuspended and plated in mammosphere media onto ultra-low-attachment cell culture wells. After 8 days, formed mammospheres were counted. Results are expressed as number of mean mammospheres counted per 20,000 cells plated (mean ± SEM, *n* = 3 samples/group; *asterisk* vs. vehicle, $p < 0.05$ vs. AC1)
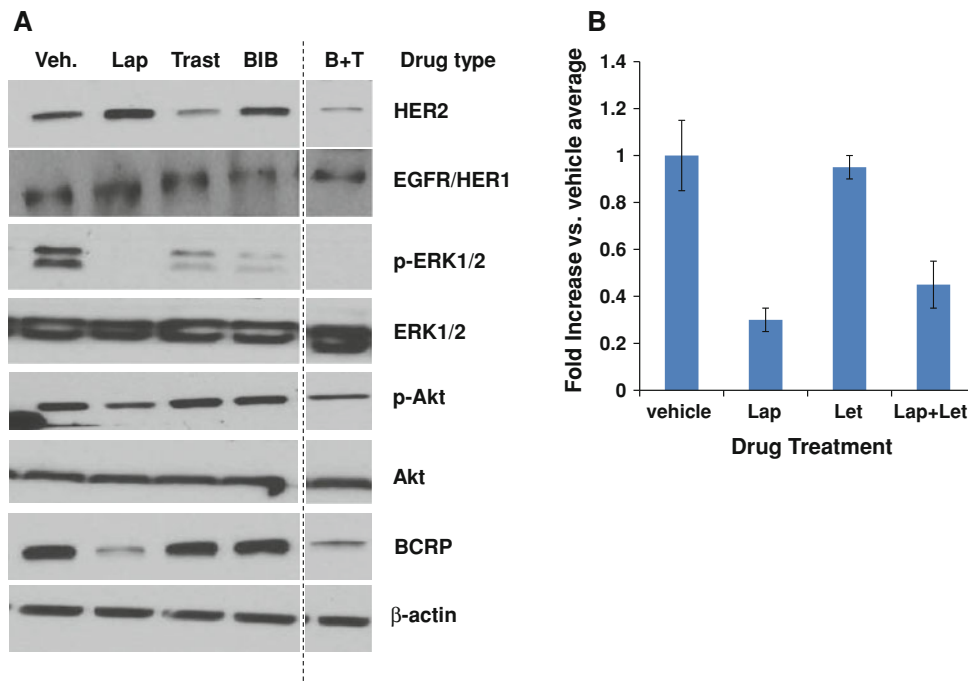
BIBX1382 and trastuzumab in combination (B + T). Cells were then subjected to western blot analyses to confirm efficacy and specificity of each treatment and to determine the effect of each on BCRP expression. Drug treatments affected HER2 and/or EGFR/HER1 expression and their downstream activation of MAPK (evidenced by p-ERK1/2) as expected: only cells treated with trastuzumab reduced HER2 protein expression; none affected EGFR/HER1 expression; and all decreased p-ERK1/2 protein expression with lapatinib alone and B + T having the greatest effects (Fig. 7a). Lapatinib and B + T were also the most effective treatments for reducing PI3K/Akt kinase pathway activation (as evidenced by p-Akt protein) and BCRP protein expression (Fig. 7a), consistent with other studies [20], RT-PCR analyses further showed that lapatinib's inhibitory effects on BCRP expression also occurred at the mRNA level and regardless of letrozole treatment (Fig. 7b).

Third, HER2 and BCRP upregulation occurred concurrently and before tumors became resistant to letrozole's growth inhibitory effects. Our previous studies have demonstrated that letrozole-treated MCF-7Ca xenografts acquire resistance to letrozole after 16–18 weeks of letrozole treatment [24]. This is preceded by upregulation of HER2 protein expression in tumors beginning at ∼4 weeks of letrozole

treatment [24]. In this current study, analysis of tumor samples acquired after 4 and 14 weeks of control treatment (Δ4A) demonstrated that BCRP protein levels remained unchanged (Fig. 8) despite significant growth of the tumor at this time (data not shown). In contrast, 4 weeks of letrozole treatment increased BCRP expression by 1.9- (not normalized) or 4.9-fold (normalized) compared control-treated tumors. BCRP expression increased even more compared to control after 8 and 24 weeks (3.5 or 4.9-fold and 3.7 or 4.1-fold vs. 4 weeks of control, respectively). Altogether, in vitro and in vivo results of this current study support a role for HER2, as well as EGFR/HER1 in the regulation of BCRP expression in LTLT-Ca cells.

Discussion

It has been previously established that resistance of ERα+ breast cancer cells to AIs involves a switch from ERα-dependent growth to growth factor-dependent growth [2, 3, 24, 39]. Particularly, in letrozole- and exemestane-resistant breast cancers, this switch involves overexpression of HER2 (Figs. 2, 7). How HER2 contributes to resistance to AIs, however, was unknown.

Fig. 7 Effect of HER2 and EGFR inhibitors on protein and/or mRNA expression in LTLT-Ca cells. **a** LTLT-Ca cells were treated for 24 h with either vehicle (*Veh*), 1 µM lapatinib, 500 µg/mL trastuzumab (*Trast*), 100 nM BIBX 1382 (*BIB*), or BIBX 1382 and trastuzumab in combination (*B + T*). Total protein was extracted and HER2, EGFR/HER1, phosphorylated- (p-ERK1/2) and total-ERK1/2, phosphorylated- and total-Akt, BCRP, and β-actin were analyzed by western blot (*n* = 3 independent cell samples/group). Representative blots are shown. *Dashed line* indicates omitted lane in between BIB and B + T lanes of the same blots. **b** LTLT-Ca cells were treated with either DMSO/ethanol vehicle or 1 µM lapatinib and/or 1 µM letrozole or 24 h. Total RNA was extracted and BCRP mRNA and 18S rRNA were analyzed by real-time RT-PCR analysis. Real-time results are expressed as the fold-change in mRNA levels compared with vehicle after normalization to 18S rRNA (mean ± SEM, *n* = 6 samples/group)
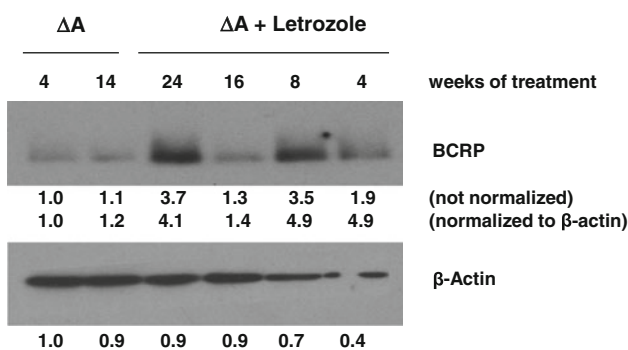
Fig. 8 Protein expression in xenograft MCF-7Ca tumors. Xenografts of MCF-7Ca cells were grown in mice as described in the Materials and Methods. After the tumors reached 300 mm^3, mice were treated with either control (100 µg/day Δ4A) or letrozole (100 µg/day Δ4A + 10 µg/day letrozole). At various pre-designated times or when the tumors reached 2,000 mm^3, tumors were collected at necropsy and BCRP and β-actin protein expression was analyzed by western blot

One possibility explored in this study is that HER2 confers resistance by regulating stem cell markers that, in turn, increase TICs within the overall cancer cell population. TICs have been linked to tumor relapse, metastasis,

and resistance to chemotherapy in several cancer types, including breast cancer [40]. However, their role in endocrine therapy resistance, particularly AI resistance, has not been well studied. Dubrovska et al. [41] has recently demonstrated that tamoxifen-resistant cells have higher BCRP and ALDH expression, and higher clonogenic potential and tumourigenicity in vivo compared to tamoxifen-sensitive MCF7 cells. Our current study provides support for the relevance of TICs in AI resistance. LTLT-Ca and/or AC1-ExR cells exhibited higher levels of TIC characteristics, including expression of known stem cell markers, than their AI-sensitive parental cell lines (Figs. 1, 2, 3, 6).

Expression of stem cell marker and drug efflux transporter BCRP [42] in letrozole and exemestane-resistant cells is of notable significance. LTLT-Ca cells expressed higher BCRP levels and SP percentage compared to letrozole-sensitive MCF-7Ca cells, and inhibition of BCRP function decreased LTLT-Ca cell mammosphere formation and SP % (Figs. 2, 3). While non-TIC cells can also express BCRP and be designated as SP cells, these characteristics tend to be enriched in the TIC subpopulation [43, 44]. Thus, acquired resistance to letrozole or

exemestane may occur via increased effluxing of these AIs out of breast cancer cells, particularly TICs, and via increased presence of TICs in general.

Increased TICs and BCRP in AI-resistant cells appear to be regulated by HER2, as well as EGFR1/HER1. This is consistent with studies demonstrating EGFR/HER1 and/or HER2 overexpression in tamoxifen-resistant and long-term estrogen-deprived cells, as well as in breast TICs [40]. Patients with EGFR1/HER1- and HER2-overexpression tumors also have poorer clinical outcomes with tamoxifen treatment [36]. EGFR/HER1's role in AI-resistant breast cancer, however, appears to be secondary to that of HER2 and/or only in association with HER2 via dimerization. Its expression and activation was not consistently associated with letrozole- or exemestane-resistance or with BCRP expression (Fig. 6). Nevertheless, it cannot be ruled out that EGFR/HER1 may independently regulate stem cell markers and other TIC characteristics in AI-resistant cells not investigated in this study.

Taken together, the results of this study suggest that reducing or eliminating the TIC subpopulation with agents that target BCRP, HER2, EGFR/HER1, and/or their downstream kinase pathways (i.e., MAPK and PI3K/Akt pathways) could be effective in preventing and/or treating acquired AI resistance. Clinical studies have shown that treating patients with AIs in combination with either lapatinib or trastuzumab [45–47], or gefitinib (an EGFR tyrosine kinase inhibitor) [48] resulted in better clinical outcomes than treating with AIs alone. The number of such clinical studies are limited, however, and even fewer have directly compared these drugs to each other. One study has shown that trastuzumab combined with neoadjuvant anthracycline-taxane-based chemotherapy elicits a better pathological response in untreated ER-/HER2+ breast cancer patients than lapatinib combined with chemotherapy [49]. However, results of our current study suggests that the dual EGFR/HER1-HER2 kinase inhibitor lapatinib may be more effective than either HER2-specific trastuzumab or EGFR-specific gefitinib, or in treating ER+ breast cancer patients that have relapsed from AI treatment.

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