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Aromatase Inhibitors and Xenograft Studies

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Abstract

Aromatase inhibitors (AIs) have become the front-line choice for treatment of ER+ breast cancer. Nevertheless, although patients are responsive initially, they may acquire resistance and become unresponsive to further treatment. In addition, approximately 25% of breast cancers do not express the estrogen receptor (ER α) and consequently, are innately resistant to endocrine therapy. We have investigated the mechanisms associated with this lack of treatment response using xenograft models. We found that in cells and tumors that acquired resistance to the AI letrozole therapy, expression of the ER was reduced whereas growth factor signaling was enhanced, including a marked increase in HER2 expression. Treatment with trastuzumab (HER2 antibody) resulted in a significant down-regulation of HER2 and p-MAPK as well as restoration of ER α expression. Thus, when trastuzumab was added to letrozole treatment at the time of tumor progression, there was significantly prolonged tumor suppression compared to trastuzumab or letrozole alone. This suggests that inhibition of both HER2 and ER α signaling pathways are required for overcoming resistance and restoring treatment sensitivity. ER negative tumors are innately resistant to endocrine therapy. Repression of the ER α has been found to be due to epigenetic modifications such as increased methylation and histone deacetylation. We found that entinostat (ENT), a histone deacetylase inhibitor (HDACi), activated not only expression of ER α but also aromatase in MDA-MB-231 ER-negative breast cancer cells, resulting in their ability to respond to estrogen and letrozole. Treatment with ENT in combination with letrozole significantly reduced tumor growth rate in xenografts compared to control tumors ($p < 0.001$). ENT plus letrozole treatment also prevented the colonization and growth of MDA-MB-231 cells in the lung with a significant reduction ($p < 0.03$) in both visible and microscopic foci. These results provide a strong indication for possible use of AIs in combination with HDAC inhibitors for the treatment of ER-negative breast cancer.

Introduction

Endocrine therapy has to be very effective in aiding patients with breast cancer. Aromatase inhibitors have become the front-line choice for treatment of ER+ breast cancer, due to studies showing the greater efficiency of these drugs over tamoxifen [1]. Nevertheless,

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although patients may be responsive initially, they may eventually relapse and become unresponsive to further treatment. In addition, roughly one quarter of all breast cancer patients express neither the estrogen receptor alpha (ER α) nor the progesterone receptor (PR), both of which are important targets in drug therapies manipulating aromatase. Given that the United States alone is expected to report almost 1 cases of breast cancer for 2009 [2], the number of patients that are unresponsive to endocrine therapy and must be subjected to cytotoxic chemotherapy is significant. It is therefore clear that a greater understanding of aromatase and its inhibitors is required to improve our efforts to manage and treat breast cancer.

In our lab, we have investigated the underlying mechanisms of acquired resistance to AIs that allow tumors to adapt and survive the pressure of estrogen suppressive therapy. More recently, we have also investigated the novel strategy of reprogramming hormone receptor-negative breast cancer and re-sensitize them to AIs. In this article, mechanisms associated with acquired resistance to AIs and novel strategies to reverse both the acquired and *de novo* resistance to AIs are reviewed.

Materials and Methods

Cell culture

ER α -positive MCF-7Ca aromatase expressing cells were provided by Dr. S. Chen, City of Hope. ER α -negative MDA-MB-231, Hs578T, and SKBR3 cells were obtained from ATCC. These cell lines were authenticated by ATCC using Short Tandem Repeat (STR) profiling, Karyotyping and by monitoring cell morphology. *In vitro* assay conditions and data analysis are described previously.

Inhibitors

Letrozole was provided by Dr. Dean Evans, Novartis, Basel, Switzerland. Entinostat (ENT, MS-275) was supplied by Dr. Peter Odentlich, Syndax, Watham, MA.

Tumor growth rate analysis

All animal studies were performed according to the guidelines and approval of the Animal Care Committee of the University of Maryland, Baltimore. Tumor xenografts of MCF-7Ca cells or MDA-MB-231 cells inoculated into each flank of the female ovariectomized (OVX) athymic nude mouse as previously described [3–8]. Tumors were measured weekly for MCF7Ca xenografts and twice weekly for MDA-M-231 xenografts. Volumes were calculated from $\frac{4}{3} \pi r_1^2 r_2$ where $r_1 < r_2$.

Lung colonization assay

Mice received injections of 3×10^6 of MDA-MB-231 cells via the tail vein. Groups of mice were treated three weeks later with vehicle (control), ENT, letrozole, or ENT plus letrozole. Mice were treated for six weeks, and then euthanized.

Western blotting

Cell lysates were prepared as described previously [3–7,9] and 50 μ g of protein from each sample was analyzed by SDS-PAGE. The densitometric values were corrected using β -actin as a loading control.

Aromatase activity

Aromatase activity in cells was determined using a radiometric assay by measuring $^3\text{H}_2\text{O}$ formed on conversion of [1- ^3H] androstenedione (aromatase substrate) to estrone [3, 10].

RNA Extraction and Reverse Transcription (RT) and PCR

RNA was extracted and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA) as per manufacturer's protocol. Analysis of *ESR1*, *CYP-19* and *TFF1* mRNA expression was carried out by conventional RT-PCR as described earlier [4,11].

Statistics

The tumor volumes were analyzed with S-PLUS (7.0, Insightful Corp.) to estimate and compare an exponential parameter (β_i) controlling the growth rate for each treatment groups. The original values for tumor volumes were log transformed. All p values less than 0.05 were considered statistically significant. The graphs are represented as mean $\pm$ SEM

Results

Acquired Resistance to Aromatase Inhibitors

To address the specific problem of acquired resistance, our lab has developed a xenograft mouse model that mimics postmenopausal women with hormone receptor-positive breast cancer. In this model, MCF-7, which is a human ER-positive breast cancer cell line, was stably transfected with aromatase gene (*CYP19*) (MCF-7Ca) and inoculated into oophorectomized, athymic nude mice. By supplementing with androstenedione which is a substrate for the aromatase enzyme, the growth of MCF-7Ca xenograft tumors is stimulated by estrogens. These are produced via aromatization of androstenedione within the tumor, thus modeling the postmenopausal condition. The mice were then treated with letrozole for approximately 56 weeks until tumors were actively growing despite administration of letrozole [8,11]. Letrozole resistant tumors were subsequently excised and cells isolated. The resistant cell line was designated as Long Term Letrozole Treated (LTLT). During these experiments, tumors were collected at different time points [8, 12]. Western blot analysis of collected tumors demonstrated that ER α expression initially increased while tumors are still responding to letrozole at week 4 but subsequently reduced as tumors became resistant. While the total ER α protein level was progressively reduced at week 28 and 56, the levels of phosphorylated ER α (p-ER α) were significantly higher than at baseline. Furthermore, the downstream effector of ER, PR, remained unchanged from the baseline which indicates that the ER signaling pathway continues to be activated in AI resistant tumors regardless of the loss of its expression (figure 1). This phenomenon that ER signaling pathway can be activated despite the lack of its ligand can be explained by the cross-talk between ER α and other signaling pathways like mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinases (PI3K)/Akt pathways [13–15]. Thus, besides loss of ER α expression, the AI resistant tumors appeared to alter their growth signaling and became dependent on growth factor receptor pathways, particularly human epidermal growth factor receptor 2 (HER2)/MAPK pathway. By 28 and 56 weeks of letrozole treatment, the expression of HER2 and its downstream effectors (p-Shc, Grb-2, p-Raf, p-Mek1/2, and p-MAPK) were all significantly increased by letrozole treatment compared to baseline (figure 1). In fact, the increase in the expression of HER2 can be observed as early as 4 weeks while tumors still responded to letrozole. Moreover, downstream targets of MAPK such as p90 ribosomal S6 kinase (p90RSK) and the ETS-domain containing protein (Elk) were also found to be activated 8

The AI resistant cells appeared to be more sensitive to the inhibition of the MAPK signaling pathway compared to the parental MCF-7Ca cells. Both MEK inhibitors U0126 and PD98059 significantly inhibited proliferation of LTLT in a dose dependent manner, while there was no effect on the parental MCF-7Ca cells [8]. The interplay between the MAPK and ER signaling pathways can be further demonstrated by the restoration of ER α expression when LTLT cells were treated with the MEK inhibitor PD98059. This suggests

that the sensitivity to endocrine therapy can be reestablished by disruption of the MAPK signaling cascade. Inhibition of the MAPK signaling pathway can also be achieved by interruption of HER2 signal transduction. Trastuzumab, which is a monoclonal antibody against the extracellular domain of HER2, is currently used in the clinic for patients with HER2-positive breast cancer [16]. We further investigated the effect of trastuzumab in these letrozole resistant cells and found that trastuzumab can drastically inhibit the proliferation of LTLT cells. By inhibiting p-HER2 and p-MAPK expression with trastuzumab, restoration of ER α expression and E₂ sensitivity in LTLT cells were observed after pre-treatment with trastuzumab for 72 hours [17]. Although LTLT were shown to be insensitive to growth stimulation of E₂ [8], LTLT cells exhibited a marked stimulation of proliferation with relatively low concentration of E₂ when pre-treated with trastuzumab (100 μ g/mL) for 72 hours. Transcriptional activation of ER α was induced in LTLT cells to similar extent as in parental MCF-7Ca stimulated with E₂. Taken together, these results suggested that HER2 is a negative regulator of ER α and inhibition of HER2 may be utilized as a strategy to overcome the acquired resistance to AI therapy. Furthermore, our lab also confirmed this result *in vivo* by adding trastuzumab at week 16 when the tumors started to double in size while treated with letrozole [17]. We found that an addition of trastuzumab to letrozole at the time of tumor progression resulted in significantly prolonged tumor suppression when compared to trastuzumab or letrozole alone (figure 2A). Addition of trastuzumab resulted in significant down-regulation of HER2 and p-MAPK as well as restoration of ER α expression (figure 2 B). This suggests that inhibition of both HER2 and ER α signaling pathways are required for prolonged tumor suppression. Notably in this experiment, trastuzumab alone did not have anti-tumor activity in the parental endocrine responsive MCF-7Ca and the upfront combination of trastuzumab and letrozole could not prolong or avert the resistance [17].

Multiple emerging clinical data have supported our preclinical findings. Lipton *et al.* demonstrated that approximately 26% of patients treated with letrozole converted from serum Her2 negative to positive at the time of disease progression [18]. The extracellular domain of Her2 protein detected in peripheral blood has been demonstrated to correlate with the overexpression of Her2 protein in tumor cells [19]. Several clinical trials have confirmed the benefit of targeting both ER and HER2 pathways. A phase II trial of letrozole and trastuzumab in ER-positive/Her2-negative metastatic breast cancer patients demonstrated that the combination was well tolerated with a clinical benefit rate of 50% [20]. A subsequent phase III trial (TANDEM trial) of anastrozole in combination with trastuzumab demonstrated a significant improvement in PFS with an addition of trastuzumab (3.8 vs. 5.6 months; $p = 0.0059$) [21]. Moreover, recent randomized phase III trial of letrozole in combination with lapatinib, an oral dual tyrosine kinase inhibitor of HER2 and EGFR, also demonstrated a significant benefit of adding lapatinib to letrozole with the PFS of 8.2 versus. 3.0 months (HR of 0.71; $p = 0.019$) [22]. However, the benefit only appears to be in the group of patients with HER2 overexpression. Nonetheless, the preplanned Cox regression analysis of patients with Her2-negative tumors who relapse less than 6 months after tamoxifen discontinuation demonstrated a non-significant trend toward improvement in PFS for the combination (HR 0.78; $p = 0.117$) [22]. This result supports our preclinical finding that the upfront combination of AI with anti-Her2 therapy does not prolong or avert the resistance but the combination is more beneficial where HER2 is expressed and at the time of the resistance

De Novo Resistance to Aromatase Inhibitors in ER-Negative Tumors

It has long been known that breast cancers that do not express either ER or PR would not respond to endocrine therapy such as AIs. Several studies have suggested that patients with higher ER or PR levels have better outcomes when treated with endocrine therapy [23–25]. The recent American Society of Clinical Oncology (ASCO)/ College of American

Pathologists (CAP) recommended that ER and PR tests should be considered being positive if there are at least 1% positive tumor nuclei in the breast cancer tumors ([23]. Nevertheless, approximately a third of breast cancers do not express both ER and PR and this type of breast cancer is intrinsically resistant to endocrine therapy including AIs.

Previous studies have demonstrated that the loss of ER α expression occurs via epigenetic mechanisms. Unlike normal breast tissue, the CpG island within the ER α promoter is hypermethylated in all of the ER-negative breast cancer cell lines and tumors [26,27]. Furthermore, the expression levels of functional ER α can be restored in ER-negative breast cancer cells treated with the DNA methyltransferase (DNMT) inhibitor, 5-azacytidine [28]. Similarly, the expression of ER α transcription is increased by 300–400 fold when combining the histone deacetylase inhibitor (HDACi), Trichostatin A, with 5-azacytidine [29]. More recently, we have shown that HDACi can be used to induce responsiveness of ER-negative tumors to aromatase inhibitors *in vivo* [30, 31]. Thus, our recent study as well as that of others demonstrated that ER/PR-negative cancer cells can be re-programmed by epigenetic modulators which render them to be sensitive to endocrine therapies.

This information presents new possibilities in terms of breast cancer therapy. In a recent study, using a mouse xenograft model of ER-negative breast cancer cells, we investigated the combined use of, the HDACi MS-275 (entinostat) (ENT), and the AI letrozole. Notably, entinostat raised the mRNA and protein expression levels of aromatase as well as the enzyme activity, in addition to ER α expression. Consequently, upregulation of ER α and aromatase resulted in sensitization of the ER-negative MDA-MB-231 cells to estrogen and letrozole. When mice with ER-negative tumors were administered ENT and letrozole, treatment resulted in almost total suppression of tumor proliferation (Figure 3) [30]. Similar tumor growth inhibition was also observed in a second ER-negative breast cancer cell line, Hs578T [31].

Other studies have also examined the roles that HDACs have in breast cancer cells, which may help elucidate the mechanism by which HDAC inhibitors effect breast tumors. ER α is also known to function in pathways not related to transcriptional regulation. ER α was recently shown to interact with HDAC6 to deacetylate tubulin, allowing breast cancer cells greater mobility [32]. This suggests that it may be possible for HDACi and AI combinational therapy to extend the benefit of aromatase inhibitors not only to those with ER-negative breast cancer, but it may also work to prevent the motility of cellular migration and subsequent metastasis. This may support our observation that mice receiving both entinostat and letrozole exhibited a markedly lower frequency of metastasis to the lungs [31]. Investigation of a different HDACi, panobinostat, also indicates potential for this class of drugs. Researchers have recently published information that shows panobinostat can increase the response of cancer cells to aromatase inhibition by letrozole, reducing the IC₅₀ of letrozole by 80% [33].

Conclusions

Our preclinical data as well as that of others demonstrates that the crosstalk between ER and other signaling pathways particularly MAPK and PI3K/Akt is the key resistant mechanism contributing to acquired resistance of ER-positive tumors. Perturbing these signaling pathways is an attractive strategy to circumvent the resistance to AI therapy in breast cancer. Several clinical trials are ongoing to evaluate the role of these novel targeted therapies to reverse resistance to AIs. These agents include HER2 inhibitors, MEK inhibitors, Raf inhibitors, PI3K inhibitors, mTOR inhibitors, and Akt inhibitors.

In ER-negative tumors, our studies demonstrate that HDAC inhibitor, ENT, increases both ER α and aromatase expression and activity, thereby converting ER-negative tumors to ER α -positive tumors. This results in tumors that are now sensitive to the growth stimulatory effects of estrogens synthesized locally by the aromatization of androstenedione in the tumor. Thus, when the HDAC inhibitor ENT was combined with AI letrozole, the ER α expressing tumors were deprived of estrogen preventing ER driven gene transcription and resulting in suppression of tumor growth and tumor cell colonization in the lungs. This novel strategy may provide a new approach to treat ER α -negative breast cancer.

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Figure 1.

A. Effect of letrozole treatment on ERα, PGR, erB-2, and p-Shc expression in MCF-7Ca tumour xenografts. B. Effect of letrozole treatment on Grb2, p-MAPK, and p-ERα expression in MCF-7Ca xenografts. Letrozole-treated tumors were collected at 4 weeks (when they were responding to letrozole), 28 and 56 weeks (when they were growing on letrozole), analysed by Western immunoblotting, and were compared with vehicle-treated tumors collected at week 4 (control). Numbers below the blots represent fold change in protein expression compared with the control obtained by densitometric analysis.

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A, Effect of trastuzumab alone or in combination with letrozole on the growth of MCF-7Ca xenografts: Tumor growth rate of the mice in the combination group was significantly lower than control ($p=0.004$), single agent ENT ($p=0.009$) or letrozole ($p=0.049$)

MS-275 sensitizes ER- MDA-MB231 xenografts to letrozole

Figure 3.

Effect of ENT alone or in Combination with Letrozole on the Growth of ER negative MDA-MB-231 Xenografts: The mice bearing MDA-MB-231 xenografts were treated with ENT alone or in combination with letrozole. Tumor volumes were measured twice a week. Tumor growth rate of the mice in the combination group was significantly lower than control ($p=0.004$), single agent ENT ($p=0.009$) or letrozole ($p=0.049$)

Figure 4.

Effect of Treatment with ENT and Letrozole Alone or in Combination on Colonization of MDA-MB-231 Cells in the Lungs of Mice: MDA-MB-231 cells were injected into the tail vein. The mice were treated with after 3 weeks with vehicle, ENT+ Δ^4 A, Δ^4 A+ letrozole or combination for 6 weeks. Visible or micrometastatic foci were quantitated at autopsy. (A) The combination of ENT (ENT) plus letrozole produced significantly fewer visible lung foci compared to control (*p=0.002) and ENT ($\dagger$ p=0.02). (B) Mice treated with a combination of ENT plus letrozole had significantly fewer micrometastases compared to control (*p=0.0269) and ENT ($\dagger$ p=0.038).