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AKT-aro and HER2-aro, models for de novo resistance to aromatase inhibitors; molecular characterization and inhibitor response studies

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Abstract

Aromatase inhibitors (AI) are currently the first line therapy for estrogen receptor (ER)-positive postmenopausal women. De novo AI resistance is when a patient intrinsically does not respond to an AI therapy as well as other targeted endocrine therapy. To characterize this type of resistance and to examine potential therapies for treatment, we have generated two cell models for de novo resistance. These models derive from MCF-7 cells that stably overexpress aromatase and Akt (AKT-aro) or HER2 (HER2-aro). Evaluation of these cell lines revealed that the activities of aromatase and ER were inhibited by AI and ICI 187280 (ICI) treatment, respectively; however, cell growth was resistant to therapy. Proliferation in the presence of the pure anti-estrogen ICI, indicates that these cells do not require ER for cell growth and distinguishes these cells from the acquired AI resistant cells. We further determined that the HSP90 inhibitor 17-DMAG suppressed the growth of the AI-resistant cell lines studied. Our analysis revealed 17-DMAG-mediated decreased expression of growth promoting signaling proteins. It was found that de novo AI resistant AKT-aro and HER2-aro cells could not be resensitized to letrozole or ICI by treatment with 17-DMAG. In summary, we have generated two cell lines which display the characteristics of de novo AI resistance. Together, these data indicate the possibility that HSP90 inhibitors may be a viable therapy for endocrine therapy resistance although additional clinical evaluation is needed.

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Keywords

HSP90 inhibitors; Breast cancer; De novo aromatase inhibitor resistance

Introduction

Breast cancer is typically thought to overproduce the hormone estrogen by the enzyme aromatase. Estrogen is the ligand for the estrogen receptor (ER). Estrogen-bound ER induces gene expression, including those involved in proliferation and survival. Approximately 70 % of breast cancers are ER/progesterone receptor (PR)-positive. Currently, the mode of treatment for ER/PR+ breast cancers include endocrine therapies, consisting of an anti-estrogen tamoxifen and aromatase inhibitors (AIs) [1, 2]. Tamoxifen binds to the ER, thereby preventing the binding of estrogen and subsequent activation of ER. AIs inhibit the activity of aromatase, thereby directly inhibiting the production of estrogen. While the use of these inhibitors has been effective, resistance is found in some patients. There are two categories of resistance: acquired and de novo resistance. Acquired resistance occurs when a patient initially responds to the treatment, but after a period of time, no longer responds. De novo resistance is observed when a patient is treated with an agent and intrinsically does not respond to treatment. The major mechanism thought to underlie both acquired and de novo endocrine therapy resistance is the crosstalk between hormonal signaling and growth factor signaling pathways. Growth factor receptors EGFR, HER2, IGF-1R, and kinases such as PI3K and Akt are indicated in endocrine resistance [3–6]. Activated kinases may cause the activation of ER in a ligand-independent manner [6]. Acquired resistance is thought to occur by the gradual selection of the cell toward an alternative method of ER induction to promote growth. In AI-resistant cells, low to no levels of estrogen is present and the cell eventually depends on ER activation in a ligand-independent manner by several growth factor signaling pathways such as HER2, Akt, and IGFR. One mechanism for de novo resistance is that these cancers have an upregulation/overexpression of the HER2 receptor and/or Akt protein. Clinical and in vitro studies have concluded that HER2 overexpression is associated with resistance to hormone therapy [7–10]. The luminal B subtype breast cancers display features of being ER+ and or PR+ with high Ki-67. However, the luminal B subtype can also overexpress HER2 and be further classified into two groups: luminal B (HER2 negative) and luminal B (HER2 positive) [11]. Approximately 30 % of luminal B tumors overexpress HER2 and respond poorly to therapy compared to their HER2 negative luminal B counterparts [12]. Furthermore, aberrantly activated Akt was reported in many tumors and associated with resistance to endocrine therapy. Activated Akt was associated with HER2-overexpressed breast cancers [7, 13–15]. These proteins may activate growth and anti-apoptotic pathways [15]. ER+ and HER2-overexpressed breast cancer cell lines, such as BT474 [9, 10, 16] and MCF-7HER2 [7, 10, 17] have been used as models to study tamoxifen resistance. However, since these cell lines do not express aromatase, physiologically relevant models are needed to better evaluate the mechanisms and to explore treatment of de novo AI resistance. To address this concern, we have generated two cell models. These cell lines are derived from ER positive, MCF-7 cells which overexpress aromatase, as well as Akt or HER2 and display features of de novo resistant breast cancer. These cell models allow us to test drugs for identifying potential agents for inhibiting de novo AI resistant breast cancer.

Heat shock protein 90 (HSP90) is a chaperone protein that functions to assist other proteins, termed “client proteins,” in their proper folding. Proteins which do not bind to chaperone proteins and those that are not folded properly are targeted for proteasomal degradation. Many HSP90 client proteins include those involved in cell proliferation and survival. HSP90 inhibitors are powerful due to their capability of targeting multiple proteins simultaneously.

We have previously shown that this class of inhibitor may be an effective therapy to treat acquired AI resistant breast cancers [18]. Here, we demonstrate that inhibition of HSP90 can effectively inhibit two cell models for de novo AI resistance and may be a viable treatment option for de novo resistant breast cancer.

Materials and methods

Meta-analysis

We estimated the effect size of AKT+ versus AKT− in de novo ER+ breast cancer patients by performing a fixed-effects meta-analysis on the limited number of published reports in the literature. The reports we considered were Stal et al. [19], Tokunaga et al. [14], and Tokunaga et al. [13]. Both Stal et al. [19] and Tokunaga et al. [13] provide point estimates for relapse-free survival from their figures with respect to AKT+ or − status in de novo ER+ breast cancers, including statistical significance levels. In Tables 4 and 5 of Tokunaga et al. [14], they provide information quantifying the relationship between the AKT status, ER, and HER2 status. We used these estimates for back-calculating the effect sizes for relapse-free survival in Tokunaga et al. [13]

We estimated the meta-analysis significance level by the Fisher method of pooling *p* values [20] of recurrence-free survival between Stål et al. [19] and Tokunaga et al. [13], and then we estimated the variance from the fixed-effects model. Our fixed-effects model assumed that the two studies' populations as relating to AKT+ were comparable. The baseline estimate for relapse-free survival was derived from the hazard rates. To estimate the hazard rates and corresponding variances, we fit an exponential distribution through the Kaplan–Meier curves presented in Stål et al. [19] and Tokunaga et al. [13]. We simulated a single relapse-free survival data set after estimating the hazard ratio and 95 % confidence intervals using the product-limit method. These simulations were performed in R [21] with the prodlm package [22]. In the SimSurv function, we specified the baseline recurrence-free rate and the approximate effect size for the hazard ratio; our estimate and standard error of the log hazard was −0.949 and 0.235, respectively (hazard ratio of 0.39, Fig. 1). The confidence interval width of the hazard ratio corresponded to a sample size of approximately 50 per AKT group. This in silico exercise is provided as an illustration of the difference expected of AKT+ expression in the absence of any published clinical data.

Cell lines and cell culture

MCF-7 derived cell lines MCF-7aro and LTEDaro were generated in this laboratory and reported [23, 24]. MCF-7AKT overexpressing cells were generated as described by Glaros et al. [25] and MCF-7HER2 cells were generously provided by Dr. Dihua Yu of the University of Texas MD Anderson Cancer Center. These cells were stably transfected with a pMG-H2 (InvivoGen, San Diego, CA) plasmid containing the aromatase gene and the hygromycin B resistance gene to generate AKT-aro and HER2-aro cells. Stable clones were selected with 50 µg/ML hygromycin B (Invitrogen, Carlsbad, CA). Single clones were picked after 2 weeks and the aromatase activity was assayed for each clone. The selected AKT-aro and HER2-aro clones displayed high aromatase activity and were used for subsequent experiments. AKT-aro and HER2-aro cells were cultured in MEM media supplemented with 10 % fetal bovine serum, 1 mM/L sodium pyruvate, 2 mM/L L-glutamine, 100 IU/ML penicillin, 100 µg/ML streptomycin, 0.1 mg/ML G418, and 50 µg/ML hygromycin B. MCF-7aroLTLTca (LTLTca) cells were provided by Dr. Angela Brodie and cultured according to Jelovac et al. [3]. BT474 cells were cultured in DMEM high glucose media supplemented with 10 % fetal bovine serum, 1 mM/L sodium pyruvate, 4 mM/L L-glutamine, and 1 % non-essential amino acids.

Additional materials and methods can be found in supplemental materials and methods.

Results

Elevated HER2 and Akt expression are correlated with poor AI response

HER2 overexpression has been linked to reduced response [26–28] and overall survival to endocrine therapies [29–32]. To confirm the significance of Akt overexpression on AI response in ER+ breast cancer and response to therapy, we performed a meta-analysis of available clinical data. We demonstrated that relapse-free survival from endocrine therapy treatment was reduced in breast tumors which overexpressed Akt, compared to low Akt expressing tumors (Fig. 1). Importantly, data from this and other laboratories demonstrate that tumors which overexpress HER2 or Akt, display less response to endocrine therapy, which indicates that elevated levels of HER2 or Akt in breast cancers may be an indicator for de novo AI resistance [7, 13, 14, 29, 31, 33]. Using ER-positive MCF-7 cells, either HER2 or Akt was overexpressed, in addition to aromatase, to generate two cell lines as models of de novo AI resistance. Resulting cell lines are referred to as HER2-aro or AKT-aro.

Molecular characterization of AKT-aro and HER2-aro cells

Using western blot analysis we confirmed that AKT-aro and HER2-aro cells overexpress Akt and HER2, respectively, compared to the parental MCF-7aro cells. These proteins were also highly phosphorylated, indicating that they are activated. HER2-aro expressed pAkt, which is likely a downstream target of overexpressed HER2 and may be activated as a consequence of HER2 overexpression. These results are consistent with previous reports [13]. Phosphorylated ERK was only detected in HER2-aro cells, suggesting HER2 overexpression mediates the activation of MAP kinase pathway. Overexpression of Akt or HER2 had no apparent effect on the levels of total ER, but HER2-aro cells expressed slightly increased levels of phosphorylated ER at S118 (Fig. 2a). It has been reported that MAP kinase is able to phosphorylate ER at S118 [34], supporting what we have found with HER2-aro cells.

De novo AI resistance is not due to changes in aromatase function

To demonstrate expression of functional aromatase in AKT-aro and HER2-aro cells, we performed the aromatase enzyme kinetic analysis and examined its response to letrozole. Kinetic studies indicated that MCF-7aro, AKT-aro, and HER2-aro have comparable catalytic activities (Supplementary Table 1). The aromatase activity response to letrozole was similar among the three cell lines, indicating that the aromatase in AKT-aro and HER2-aro cell lines are sensitive to letrozole (Fig. 2b). The V_{max} value for AKTaro is slightly lower than those for both MCF-7aro and HER2-aro, which may reflect differences in aromatase transfection efficiencies. However, the aromatase is inhibited by letrozole nearly identical for all three cell lines. By these results, we can conclude that aromatase function is not altered due to overexpression of Akt or HER2.

Evaluation of ER transcriptional activity in de novo AI resistant cells

Both AKT-aro and HER2-aro cells displayed low basal levels of ER activity by luciferase assay. E_2 treatment increased ER activity, as also observed in MCF-7aro cells. Treatment with ICI 182780 (ICI) abolished the E_2 induction of ER activity (Fig. 2c). To verify whether estrogenresponsive gene expression was altered, we examined the mRNA levels of pS2 and PgR. The expression of both genes were stimulated by testosterone and repressed by letrozole (Fig. 2d). Interestingly, HER2-aro cells displayed increased basal PgR mRNA levels compared to the parental MCF-7aro and AKT-aro cells. This may be the result of an

ER-activating signaling pathway found uniquely in HER2-aro cells. Together, these results indicate that the ER function in the AKT-aro and HER2-aro cells are unaltered. ER remains responsive to hormone and does not display significant deviation from the parental MCF-7aro cells.

The proliferation of AKT-aro and HER2-aro is resistant to letrozole and ICI 182780

While aromatase and ER in AKT-aro and HER2-aro are responsive to the treatment of letrozole and ICI, respectively, we performed proliferation assays and treated MCF-7aro, long-term estrogen deprived cells overexpressing aromatase (LTEDaro), AKT-aro, and HER2-aro cells with testosterone, alone or with either ICI, or letrozole. LTEDaro cells have been extensively used as a model of acquired AI resistance and it has been demonstrated that these cells are capable of proliferating in the presence of AIs, including letrozole, but sensitive to ICI [24, 35, 36]. It has been suggested that LTEDaro represents a late stage of acquired AI resistance, ER is activated in an estrogen-independent manner, and the activated ER plays an important role in the proliferation of LTEDaro. We observed strong stimulation of growth by testosterone treatment in MCF-7aro cells (Fig. 3a), but not in either AKT-aro or HER2-aro cells (Fig. 3c, d). Similar to LTEDaro cells, AKT-aro, and HER2-aro cells are not growth inhibited by letrozole, regardless of whether testosterone is present or not (Fig. 3b – d). To further examine the role of ER in AKT-aro and HER2-aro cells, we tested the proliferative response of these cells to the pure anti-estrogen inhibitor ICI. HER2-aro cells were completely resistant to the anti-proliferative effect from ICI (Fig. 3d). AKT-aro cells demonstrated slight growth inhibition to ICI, while MCF-7aro and LTEDaro cells were potently growth inhibited by ICI (Fig. 3a – c). The additional treatment of testosterone had no effect on the growth response to ICI (Fig. 3). Their resistance to ICI indicates that AKT-aro and HER2-aro cells can proliferate even when the activity of ER is suppressed. This differs from both MCF-7aro and LTEDaro cells which do require ER for growth. These results demonstrate that AKTaro and HER2-aro cells have the features of and are models for de novo AI resistance. Furthermore, our findings strongly suggest that the mechanisms of acquired and de novo AI resistance are not identical.

17-DMAG can inhibit de novo AI resistant cell growth

De novo AI resistance may result from over-activation of HER2, Akt and/or other signaling pathways. Western blot analysis of protein expression in our HER2-aro and AKTaro cells indicate that a number of signaling proteins are not only over-expressed, but activated. Therefore, treatment with a single inhibitor agent may not be as effective as other pathways may still be active and bypass the inhibition. Thus, an agent capable of multiple routes of inhibition is needed. As a proof-of-principle study, we tested whether 17-DMAG, a HSP90 inhibitor, could provide a possible therapeutic option to treat de novo AI resistant breast cancer, based on our knowledge that many key players in signal transduction are HSP90 client proteins. Through the use of an HSP90 inhibitor, we may simultaneously target these proteins and their downstream effectors responsible for conferring AI resistance. We have previously shown that 17-DMAG effectively inhibits acquired AI resistant breast cancer growth as well as display some selectivity toward cancerous cells [18]. It is known that both Akt and HER2 are HSP90 client proteins [37, 38]. In this study, both AKT-aro and HER2-aro cell growth were inhibited in a dose-dependent fashion with 17-DMAG treatment. 5–30 nM of 17-DMAG was able to reduce growth by 50 % (Fig. 4a). Previously, we have shown that 17-DMAG concentrations up to 15 nM are not toxic to normal breast epithelial cells [18]. BT474, an ER+, HER2-overexpressing cell line which may also be considered as a de novo tamoxifen resistant cell model was growth suppressed by 17-DMAG at low nanomolar doses (Fig. 4a). LTLTCa cells, a model of acquired AI resistance generated in a laboratory independently of ours, also demonstrated strong growth inhibition at low nanomolar doses of 17-DMAG and were more susceptible to the inhibitor than the LTEDaro cells (Fig. 4a).

De novo AI resistant cell protein expression is modulated by 17-DMAG

Next, we determined whether inhibition of AKT-aro and HER2-aro cell growth may result from altered expression of various key proteins involved in cell proliferation, apoptosis, and cell cycle regulation. 17-DMAG treatment resulted in drastically decreased levels of HER2 and phosphorylated HER2 by 24 h, while potent downregulation of Akt levels were observed by 48 h (Fig. 4b, c). HSP90 levels remained unaltered as expected because 17-DMAG inhibits the activity rather than the protein expression of HSP90. We also observed decreased levels of ERα in both cell lines. Furthermore, E₂-induced ER transcriptional activity was decreased with 17-DMAG treatment (Supplementary Fig. 2). Notably, we detected an increase in the levels of cleaved PARP, which is an indicator of apoptosis induction, with 17-DMAG treatment in AKT-aro and HER2-aro cells (Fig. 4b, c). Levels of Bcl-2, an anti-apoptotic protein, decreased with 17-DMAG treatment in HER2-aro cells (Fig. 4c). This suggests that 17-DMAG induces apoptosis in these cells. Cyclin D1 regulates the cell progression from G₁ to S phase. Cyclin D1 levels decreased with 17-DMAG treatment in both cell lines (Fig. 4b, c), and cell cycle analysis show an increased population of cells in G₂ indicating cell cycle arrest by 17-DMAG (Supplementary Fig. 1).

17-DMAG cannot resensitize de novo AI resistant cells to endocrine therapy

Often times, cells rely on a particular pathway for growth. When treated with an inhibitor to this pathway, cells may switch to an alternative pathway and develop resistance to therapeutic agents. Cells which have developed resistance to endocrine therapy have been shown to be resensitized to the same therapy after treatment with a different type of inhibitor that targets the pathway the cells have switched their dependence on [9, 39]. Therefore, we tested whether treatment of our acquired AI resistant cells with 17-DMAG could restore cell sensitivity to letrozole. LTEDaro cells were growth suppressed by 17-DMAG, as were the parental AI-responsive MCF-7aro cells (Fig. 5a, b). Combinatorial treatment with both 17-DMAG and letrozole drastically enhanced suppression of MCF-7aro cell growth, but did not in LTEDaro cells (Fig. 5a, b). Both MCF-7aro and LTEDaro cells were growth inhibited by ICI. Cotreatment with 17-DMAG and ICI increased growth suppression for both MCF-7aro and LTEDaro cells (Fig. 5c, d) compared to the treatment with an individual inhibitor. These results indicate that LTEDaro cells cannot be resensitized to letrozole treatment when simultaneously inhibiting a number of pathways by 17-DMAG treatment. Furthermore, 17-DMAG and AI co-treatment potently suppressed MCF-7aro cell growth, indicating that the use of both of these inhibitors can achieve improved suppression of growth and may be an efficient method of therapy in the clinic to delay or prevent resistance. Next, we tested whether treatment of our de novo AI resistant cells with 17-DMAG could produce cell sensitivity to both letrozole and ICI. Our proliferation assay results recapitulated our previous results that 17-DMAG could inhibit cell growth and letrozole or ICI alone, could not (Figs. 3c, d; 4a). Additional treatment of letrozole or ICI, with 17-DMAG, did not result in any additional inhibition of growth (Fig. 6). In summary, our results demonstrate that LTEDaro cells cannot be resensitized to letrozole with concomitant HSP90 inhibitor treatment. Furthermore, based on our AKT-aro and HER2-aro results we have provided new support that cells intrinsically resistant to an agent cannot be made sensitive to those agents.

Discussion

De novo AI resistance is defined as tumors which express ER and aromatase, but do not respond to AI treatment. To our knowledge, there are no cell models for de novo AI resistance, nor reports documenting their mode of growth. As a proof-of-principle study, we have developed two MCF-7 cell lines to overexpress either Akt or HER2. These cells, named AKT-aro and HER2-aro, overexpress a functional aromatase enzyme that responds to the AI letrozole (Fig. 2b). However, treatment of these cells with letrozole does not inhibit

cell proliferation (Fig. 3c, d). Further analysis revealed that ER protein is functional and its transcriptional activity is repressed by ICI (Fig. 2c). However, both of our AKT-aro and HER2-aro cell lines have demonstrated resistance to the pure antiestrogen ICI (Fig. 3c, d). These results indicate that de novo AI resistance may not be the result of defects of either aromatase or ER, as these proteins function normally as in the AI responsive cells; rather, the ER pathway is not required for growth in these cells. It has been demonstrated that clinical de novo AI resistance is not correlated with the molecular response of the cell to an AI treatment. Expression levels of proliferation markers and ER regulated genes decreased with AI treatment, however, the overall cellular response of the cell remained unaffected [40]. Our findings are in agreement with the results from this study. Treatment of de novo resistant breast cancer is problematic as this type of resistance cannot be treated by an alternative endocrine therapy agent since these cells also do not respond to those agents. Although AKT-aro and HER2-aro are artificially generated, our results support the important concept that de novo resistance is mainly due to the activation of signal transduction pathways, not to the loss of the molecular responses to AIs or anti-estrogens. Furthermore, since this type of resistance relies on different pathways, it is difficult to be resensitized to AIs, as demonstrated in our studies. These cell lines are very valuable to test hypotheses and to evaluate new treatment strategies to overcome de novo AI resistance.

HSP90 inhibitors have the potential for targeting multiple proteins simultaneously. Since AKT-aro and HER2-aro cells overexpress Akt and HER2 proteins which are also HSP90 client proteins, we hypothesized that HSP90 inhibitors, could be used as an effective therapy to inhibit the growth of these cells. We have shown that 17-DMAG can suppress the growth of our de novo AI resistant cell lines (Fig. 4a). Further studies using 17-DMAG revealed that it may suppress growth via induction of apoptosis and arrest of the cell cycle (Fig. 4b, c; Supplementary Fig. 1). It was reported that overexpression of Akt led to an increase in the Bcl-2 protein [33]. Bcl-2 protein is an antiapoptotic protein that dimerises with the pro-apoptotic Bax protein. More Bax protein leads to apoptosis, while more Bcl-2 protein protects the cell from apoptosis [33]. We observed a prominent induction of apoptosis with 17-DMAG treatment, likely due to the degradation of the Akt protein (Fig. 4b, c). Decreased levels of Akt lead to decreased Bcl-2 expression, thereby swinging the balance of the cell toward apoptosis. HER2 effects may be more distributed such that it involves other pathways besides the downstream Akt and anti-apoptosis pathway, such as ERK. We have observed a drastic increase in phosphorylated ERK, indicating that this protein is activated and likely resulting in the activation of ERK downstream pathways (Fig. 2a). In addition, the role of MAPK/ERK in HER2-induced endocrine resistance has been suggested. Phosphorylated ERK was detected in tamoxifen-resistant MCF-7 cells stably overexpressing HER2. Treatment of these cells with U0126, a non-competitive inhibitor of MEK-1/MEK-2, the enzymes that activate ERK, restored tamoxifen sensitivity by restoring the interaction between ER and nuclear corepressor (N-CoR), and decreased colony formation in the presence of inhibitor [9]. The notion that the MAPK pathway plays a role in HER2-induced endocrine resistance is consistent with our results. It is possible that HER2-overexpression results in the activation of ERK via the activation of the Ras/MAPK signaling pathway which may contribute to de novo AI resistance. MEK is also an HSP90 client protein, further supporting the use of HSP90 inhibitors as a therapy to treat de novo AI resistant breast cancers. By targeting a multitude of proteins selectively in cancer cells, we may effectively inhibit resistant cancer cell growth as well as block numerous alternative pathways possibly chosen by breast cancer cells to evade therapy, thus allowing us to delay resistance to the therapy.

Currently, cell models used to study acquired resistance mainly include LTED and LTEDaro cells [35, 41, 42]. While the LTED model mimics the hormone-free environment and provided much information about tamoxifen resistance, it may not be suitable for AI-

resistance study due to the lack of aromatase activity. In order for a cell line to be a model for AI resistance, it should express aromatase as clinical breast tumors do, for both acquired and de novo AI resistant breast cancer models. As a result, our lab has developed a MCF-7 derived LTED cell line which stably expresses aromatase (LTEDaro) [24] as a model for acquired AI resistance and our MCF-7-derived de novo AI resistance models AKT-aro and HER2-aro stably overexpress aromatase as well.

Our results comparing the acquired AI resistant cell line, LTEDaro, and the de novo AI resistant cell lines, have indicated that the mechanisms of resistance between the cell lines are different. In the case of acquired resistance, hormone-dependent ER pathways are suppressed by treatment. However, growth factor pathways become activated. These growth factor pathways then activate ER through a cross-talk mechanism in a ligand-independent manner. Therefore, ER remains important and can be suppressed by ICI as demonstrated by our LTEDaro cell line. It has been demonstrated that de novo resistant tumors contain functional ER and aromatase, however, growth is predominantly driven by different growth factor pathways independent of ER. Thus, ICI and AIs are ineffective at suppressing growth of de novo AI resistant cell lines.

Typically, multiple signaling pathways are up-regulated in resistant tumors. Therefore, different pathways implicated in resistance were reported by different laboratories. Studies from these laboratories have focused on the use of inhibitors that target a single pathway in these tumors, which over time, do not work well. These tumors acquire secondary resistance and become dependent on an alternative signaling pathway to escape inhibition and promote growth. Due to this phenomenon, administering drugs, which target different pathways, in combination, or using drugs with multiple targets, such as HSP90 inhibitors, should be employed. Currently, the HSP90 inhibitors available are too toxic. However, new HSP90 inhibitors are currently being developed for improved efficacy and less toxicity.

Our research provides critical preclinical evidence that HSP90 inhibitors may be used as a therapy to treat both de novo and acquired AI resistant breast cancers. Moreover, HSP90 inhibitors enhance growth suppression with AIs in responsive cancers, suggesting that combinatorial treatment using both types of inhibitors may prevent AI resistance from developing. These exciting findings may provide a promising new type of treatment regime for AI-responsive breast cancers, although further clinical evaluation of this data is essential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
A simulated data set that shows the approximate hazard ratio and significance level of AKT + versus AKT- from our meta-analysis in the ER+ subgroup

Fig. 2.

Characterization of AKT-aro and HER2-aro cells. **a** Protein expression profile of MCF-7aro, AKT-aro, and HER2-aro determined by western blot. **b** Aromatase activity in MCF-7 cells overexpressing aromatase and Akt or HER2 treated with 0.032, 0.16, 0.8, 4, 20, and 100 nM letrozole. **c** ER functional assays: ER transcriptional activity, and **d** expression of ER regulated genes. The pGL3-(ERE)₃ reporter plasmid was transiently transfected into cells. Cells were then treated with 1 nM E₂, 100 nM ICI, or both. Quantitative real-time PCR analysis of ER-responsive genes: pS2 and PgR were conducted from cells treated with 1 nM testosterone, with or without 200 nM letrozole

Fig. 3. AKT-aro and HER2-aro cells are resistant to endocrine therapy. **a** MCF-7aro, **b** LTEDaro, **c** AKT-aro, and **d** HER2-aro were treated with DMSO, 1 nM testosterone, 100 nM ICI, 200 nM letrozole, or a combination of inhibitor and testosterone

Fig. 4. Effect of HSP90 inhibitor 17-DMAG on AI responsive and resistant breast cancer cell growth and AKT-aro and HER2-aro protein expression. **a** Cells were treated with DMSO or 17-DMAG for 7 days. Cell viability was assessed by the MTT assay. **b** AKT-aro, and **c** HER2-aro cells were treated with either DMSO or 17-DMAG for 24, 48, or 72 h. Protein expression was determined by western blot

Fig. 5.

Co-treatment of 17-DMAG and letrozole enhance growth suppression in MCF-7aro but do not resensitize LTEDaro cells to letrozole. (a, c) MCF-7aro, (b, d) LTEDaro cells were treated with DMSO; 1 nM testosterone; 17-DMAG, with or without 1 nM testosterone; 100 nM letrozole, with 1 nM testosterone; 100 nM ICI with 1 nM testosterone; or a combination of inhibitors and testosterone

Fig. 6.

AKT-aro and HER2-aro cells cannot be resensitized to letrozole or ICI 182780. (a, c) AKT-aro, (b, d) HER2-aro cells were treated with DMSO, 1 nM testosterone, 17-DMAG, 100 nM letrozole, 100 nM ICI, or a combination of inhibitors and testosterone