Heat Shock Protein 90 Inhibitors: New Mode of Therapy to Overcome Endocrine Resistance

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Abstract

Aromatase inhibitors are important drugs to treat estrogen receptor α (ERα)–positive postmenopausal breast cancer patients. However, development of resistance to aromatase inhibitors has been observed. We examined whether the heat shock protein 90 (HSP90) inhibitor 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) can inhibit the growth of aromatase inhibitor–resistant breast cancers and the mechanisms by which 17-DMAG affects proliferation. Aromatase inhibitor–responsive MCF-7aro and aromatase inhibitor–resistant LTEDarō breast epithelial cells were used in this study. We observed that 17-DMAG inhibited proliferation in both MCF-7aro and LTEDarō cells in a dose-dependent manner. 17-DMAG induced apoptosis and G2 cell cycle arrest in both cell lines. Although inhibition of HSP90 decreased the levels of ERα, the ERα transcriptional activity was not affected when cells were treated with 17-DMAG together with estradiol. Moreover, detailed mechanistic studies suggested that 17-DMAG inhibits cell growth via degradation of HSP90 client proteins AKT and HER2. Collectively, results from this study provide data to support that HSP90 inhibitors may be an effective therapy to treat aromatase inhibitor–resistant breast cancers and that improved efficacy can be achieved by combined use of a HSP90 inhibitor and an AKT inhibitor. [Cancer Res 2009;69(22):8670–7]

Introduction

Aromatase is the enzyme that converts androgen into estrogen. Estrogen is known to play an important role in breast cancer growth through its activation of the estrogen receptor α (ERα). Activated ERα can then translocate into the nucleus where it can bind to estrogen response elements on various gene promoters and subsequently transactivate these genes, which are involved in promoting tumor cell growth (1). Tamoxifen, an antagonist of ERα, and aromatase inhibitors (anastrozole, letrozole, and exemestane), which inhibit the synthesis of estrogens, have been effective therapies to combat estrogen-dependent breast cancer (2–4). However, resistance to these inhibitors has been observed. How resistance to these therapies develops as well as the mechanisms of how these cells survive and proliferate in the presence of these inhibitors are not completely understood. Studies of aromatase inhibitor and tamoxifen resistance have revealed an important role of the ERα in the acquisition of resistance. In the long-term estrogen-deprived (LTEDarō) cells, a model of aromatase inhibitor resistance, and other aromatase inhibitor–resistant cells, ERα was found to be constitutively active (5). Moreover, it is thought that this ERα activity is dependent on growth factor pathway signaling that is responsible for activation and influencing the levels of ERα in aromatase inhibitor–resistant breast cancers in a ligand-independent manner (6, 7). Growth factor–upregulated kinases can phosphorylate ERα and activate it, leading to transcriptional activation of target genes and signaling pathways involved in growth (6–12).

Heat shock proteins (HSP) are chaperone proteins that correctly fold and assist proteins in the active, correct conformations. They are involved in stress response and also in assembly and transporation across different cell compartments (13, 14). HSP90 is the most abundant protein in cells, comprising ~1% to 2% of the total soluble cytosolic protein (15). HSPs are expressed in normal cells but are overexpressed in cancer cells (16). Many HSP client proteins are involved in processes such as proliferation, apoptosis, and cell cycle progression (17, 18). It is not surprising that cancer cells exploit these HSPs to correctly fold client proteins to further the growth and survival of the cancer cells. Due to the importance of these HSPs in the growth and survival of cancer cells, inhibitors against these proteins have been developed. Early version of inhibitors against HSP90 include geldanamycin and its synthetic derivative, 17-allylamo-17-demethoxygeldanamycin (19–22). Due to their toxicity and low solubility, another HSP90 inhibitor, 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG), was developed. 17-DMAG displays better water solubility and oral bioavailability and has been tested in phase I clinical trials for treatment of metastatic or unresectable tumors or lymphomas (23, 24). Although HSP90 is expressed in both normal and cancer cells, HSP90 inhibitors display preference for cancerous cells (25). Furthermore, due to the wide range of protein targets HSP90 affects, its inhibitors can be useful for treating cancer, such as aromatase inhibitor–resistant breast cancers that are thought to rely heavily on growth factor signaling pathways, which include many HSP90 client proteins. However, to date, the efficacy of 17-DMAG on aromatase inhibitor therapy–resistant breast cancers has not been examined. The purpose of this study is to determine whether 17-DMAG may be used as a therapy to treat aromatase inhibitor–resistant breast cancers and to study how it alters molecular properties of aromatase inhibitor–resistant breast cancer cells. Here, we show that nanomolar concentrations of 17-DMAG can inhibit both aromatase inhibitor–responsive and aromatase inhibitor–resistant breast cancer cell growth by inducing apoptosis as well as arresting the cell cycle at the G2 phase. We also show that 17-DMAG has no effect on the ERα transcriptional activity in the aromatase inhibitor–responsive and aromatase inhibitor–resistant breast cells in the presence of hormone. This suggests that the mechanism of 17-DMAG inhibition of breast cell...
proliferation functions independently of the ERα signaling pathway. Instead, we found that HSP90 client proteins, AKT and HER2, are involved in the growth of both aromatase inhibitor–responsive and aromatase inhibitor–resistant breast cancer cell lines, suggesting that 17-DMAG–mediated inhibition of growth may result from inhibition of these signaling pathways. The data suggest that HSP90 inhibitors will be a suitable therapy for breast cancers that have developed resistance to current aromatase inhibitor therapies.

Materials and Methods

Cell lines and cell culture. The human MCF-7 breast epithelial-derived cell lines MCF-7aro and LTEDaro were generated previously in this laboratory and have been reported (5, 26). Human mammary epithelial cells (Lonza) were cultured in MEGM medium supplemented with bovine pituitary extract, human epidermal growth factor, hydrocortisone, gentamicin sulfate and amphotericin B, and insulin.

Reagents and antibodies. 17-DMAG was from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute, and dissolved in DMSO. Exemestane was from Pharmacia & Upjohn. Triciribine was from Cayman Chemical and AG825 was from Calbiochem. MTT compound was from Sigma–Aldrich. The antibody to HSP90 was from Stressgen Bioreagents; the antibodies to phospho-HER2 and HER2 were from Upstate; the antibodies to phospho-ERα Ser118, cleaved poly (ADP-ribose) polymerase (PARP), cyclin D1, and AKT were from Cell Signaling Technology; the antibody to ERα was from Santa Cruz Biotechnology; and the antibody to glyceraldehyde-3-phosphate dehydrogenase was from Chemicon.

Cell proliferation assay. MCF-7aro cells were hormone-deprived for 1 day before plating. MCF-7aro, LTEDaro, and human mammary epithelial cells were cultured in 96-well plates at a concentration of 1.5 × 10^4 to 4 × 10^5 per well (200 μL/well). Cells were treated with DMSO or inhibitor for up to 3 days and MCF-7aro cells were additionally treated with 1 nmol/L testosterone. Cell viability was assessed by the MTT assay. Briefly, the MTT powder was dissolved in cell culture medium to a concentration of 0.5 mg/mL. MTT reagent (150 μL) was added to each well and incubated for 1 h at 37°C, 5% CO2. DMSO was added to solubilize the formazan product and formazan absorbance was measured at 570 nm on a SpectraMax M5 microplate reader (Molecular Devices). Three replicates were used for each measurement and the mean and SD were calculated.

Apoptosis analysis. Cells were plated at a density of 5 × 10^4 per 60 mm dish. After 24 h, either DMSO or 100 nmol/L 17-DMAG was added to the dishes, in addition to 1 nmol/L testosterone for MCF-7aro cells, and the cells were cultured for 48 or 72 h. To detect apoptotic cell death, DNA fragmentation was detected using Cell Death Detection ELISAPLUS (Roche Applied Science) according to the manufacturer’s instructions.

Cell cycle analysis. Cells were plated on 100 cm dishes at a density of 1 × 10^5 per dish. DMSO or 100 nmol/L 17-DMAG was added to the dishes the following day in addition to 1 nmol/L testosterone for MCF-7aro cells. At each time point, 2 × 10^6 cells were collected, resuspended in 1 mL cold PBS, and fixed by the addition of 4 mL of –20°C absolute ethanol. The fixed cells were resuspended in 500 μL PBS/0.1% bovine serum albumin and 200 μg/mL RNase-free RNase A (100 μL) was added. After incubation at 37°C for 30 min, 1 mg/mL propidium iodide (50 μL) was added to the cells and allowed to incubate for at least 1 h before analysis by a Cyan ADP 9 color flow cytometer (Becton Dickinson).

ER functional assays. MCF-7aro and LTEDaro cells were plated in 12-well plates at 1 × 10^5 per well. The following day, each well was transfected with 1 μg pGL3–EREβ reporter using Lipofectamine 2000 (Invitrogen). After 5 h, transfection medium was replaced with medium with or without 1 nmol/L E2 and 100 nmol/L 17-DMAG. After 48 h, the firefly luciferase activity was measured using the luciferase assay system (Promega) and a TD-20/20 luminometer (Turner Designs). Luciferase activity was normalized to protein concentration.

Western blot analysis. Cell monolayers were washed twice with ice-cold PBS and then lysed. The protein concentration was quantified by the Bradford’s method. Equal amounts of protein were resolved by SDS-PAGE, transferred to nitrocellulose membrane (Bio-Rad), and detected by using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology).

Results

LTEDaro is a model of aromatase inhibitor resistance. MCF-7aro cells were generated in this laboratory as a model of aromatase-positive and ER-positive breast cancer (26). MCF-7aro cells were continuously cultured in steroid-depleted medium to generate LTEDaro cells (5). Therefore, through the conversion to E2 by aromatase, testosterone is needed for the proliferation of MCF-7aro cells, but LTEDaro cells grow well in the absence of testosterone. To show that the LTEDaro cells are a model of aromatase inhibitor resistance, we treated both MCF-7aro and LTEDaro cells with 1 μmol/L exemestane, an aromatase inhibitor, and measured cell proliferation. MCF-7aro cells treated with 1 μmol/L exemestane showed significant growth inhibition after 72 h of treatment (Fig. 1). This marked sensitivity was not observed in the LTEDaro cells, which displayed growth levels similar to the DMSO vehicle control. This result confirms that MCF-7aro cells are aromatase–inhibitor–sensitive, whereas LTEDaro are aromatase–resistant. The LTEDaro cells have also been shown to be resistant to tamoxifen and two nonsteroidal aromatase inhibitors, letrozole and anastrozole (5). Clinically, lack of cross-resistance among different aromatase inhibitors is typically observed, that is, patients will respond partially to a second aromatase inhibitor after acquiring resistance to the first aromatase inhibitor. Thereafter, we believe the LTEDaro cell line is representative of the last stage of resistance because these cells no longer respond to any of the aromatase inhibitors. Thus, LTEDaro is valuable to test for new drugs that can overcome aromatase inhibitor resistance.

17-DMAG inhibits cell growth in aromatase inhibitor–resistant epithelial breast cancer cells. It has been shown that breast cancer cells that become aromatase inhibitor–resistant no longer depend on estrogen production by aromatase for growth. Instead, the cells shift their dependence on growth factor signaling pathways for promoting aromatase inhibitor–resistant breast cancer growth (5). We reasoned that HSP90 inhibitors might be effective in suppressing the proliferation of aromatase inhibitor–resistant cells because many members of growth factor signaling pathways are HSP90 clients. To determine whether aromatase inhibitor–responsive and aromatase inhibitor–resistant cells are susceptible to the HSP90 inhibitor, 17-DMAG, cell proliferation assays were conducted. 17-DMAG inhibited hormone-independent LTEDaro and hormone-dependent MCF-7aro cell growth in a dose-dependent manner at nanomolar concentrations as well as a time-dependent manner. At low doses (5–15 nmol/L) of 17-DMAG, up to 70% growth inhibition was observed in both cell lines. Moreover, by 72 h of treatment with 100 nmol/L 17-DMAG, both LTEDaro (Fig. 2A) and MCF-7aro (Fig. 2B) cells were almost completely growth-inhibited compared with the DMSO control. Furthermore, 17-DMAG inhibited growth of exemestane-resistant cells in a dose- and time-dependent manner (data not shown). Normal human mammary epithelial cells were treated with 17-DMAG to test the selectivity of the drug for cancerous cells. The drug was ineffective at low doses (5–15 nmol/L) and growth inhibition was evident only at higher doses (≥20 nmol/L) (Fig. 2C). These results

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HSP90 Inhibitors and Breast Cancer

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confirmed that proliferation of aromatase inhibitor–resistant breast cancer cells can be attenuated by HSP90 inhibitors. Moreover, at lower doses, 17-DMAG is well tolerated by normal cells, but it can still inhibit cancer cell growth.

**17-DMAG induces apoptosis and a G2-phase arrest.** The inhibition of cell proliferation could possibly occur due to either induced apoptosis, an arrest in the cell cycle, or both. To determine whether the 17-DMAG–induced inhibition of cell growth was due to apoptosis, we decided to detect DNA fragmentation resulting from treatment with 100 nmol/L 17-DMAG. A 2- to 4-fold increase in the levels of DNA fragmentation was observed in both cell lines after 48 and 72 h of treatment (Fig. 3A and B). This result confirms that apoptosis is induced by inhibition of HSP90.

Next, to determine whether, in addition of apoptosis, 17-DMAG treatment causes alterations of the cell cycle, cells were treated with 100 nmol/L 17-DMAG and analyzed by flow cytometry to measure the population of cells in each phase of the cell cycle. LTEDaro cells treated with 17-DMAG showed significantly higher percentage (2- to 3-fold) of cells in G2 compared with DMSO-treated cells (Fig. 3C). Similarly, MCF-7aro cells treated with 17-DMAG displayed a decreasing population of cells in S phase and an increase in the number of cells in G2 with each day of treatment (Fig. 3D). These results indicate that 17-DMAG arrests cells at the G2-M phase transition.

**17-DMAG–mediated inhibition of growth does not target the ERα pathway.** Our proliferation, apoptosis, and cell cycle studies revealed that 17-DMAG is effective on both hormone-dependent and independent cell lines in a similar fashion. This suggests that the mechanism by which 17-DMAG inhibits growth does not involve ERα. To confirm this hypothesis, we analyzed the effect of 17-DMAG on ERα levels and activity. Total ERα levels decreased with 17-DMAG treatment in a dose- and time-dependent manner (Fig. 4A and B). These results indicate that ERα is degraded with 17-DMAG treatment and confirm that ERα is a HSP90 client protein. Next, we examined whether 17-DMAG can inhibit the ERα transcriptional activity, a result of constitutive ligand-independent ERα phosphorylation in hormone-independent cells or a result of ligand activation of ERα in hormone-dependent cells. We transfected both LTEDaro and MCF-7aro cells with a reporter plasmid encoding three ERE sequences, in tandem, upstream of the firefly luciferase gene. After transfection, the cells were treated with medium containing either DMSO or 17-DMAG along with or without 1 nmol/L E2. Our analysis revealed that 17-DMAG abolished ligand-independent ERα activity in LTEDaro cells as well as the basal ERα activity in MCF-7aro cells compared with the DMSO control (Fig. 4C and D). The basal ERα activity was high in the LTEDaro cells and was not affected by the treatment of 1 nmol/L E2 (Fig. 4C). However, cotreatment with E2 and 17-DMAG was unable to completely abolish the ERα transcriptional activity (Fig. 4C). In MCF-7aro cells, as expected, E2 stimulated transcriptional activation of ERα (Fig. 4D). Surprisingly, treatment with both E2 and 17-DMAG further enhanced the transcriptional activity of the MCF-7aro cells. These results show that whereas 17-DMAG can abolish ERα transcriptional activity in the absence of hormone, it is unable to inhibit this transcriptional activity in the presence of ligand. Additional studies by Western blot analysis corroborate these results. Basal phosphorylation of ERα at S118 was observed in DMSO and 1 nmol/L E2–treated LTEDaro cells (Supplementary Fig. S1). Phosphorylation was abolished by 17-DMAG and total levels of ERα also decreased indicating degradation by 17-DMAG.
treatment. However, phosphorylation was restored by cotreatment with 1 nmol/L E2 and 100 nmol/L 17-DMAG (Supplementary Fig. S1). Phosphorylation of ERα at S118 was detected in MCF-7aro cells treated with 1 nmol/L E2 but was not detected with DMSO or 100 nmol/L 17-DMAG treatment. In addition, total ERα was degraded by 17-DMAG treatment. These results confirm that ERα is a HSP90 client protein in both hormone-dependent and independent cells. However, 17-DMAG does not affect ERα activity in the presence of ligand, confirming that 17-DMAG–mediated inhibition of growth does not occur by targeting of the ERα pathway.

Inhibition of HSP90 alters protein expression in epithelial breast cancer cells. To understand the molecular mechanisms of growth defect in 17-DMAG–treated cells and to identify other pathways that may be affected by 17-DMAG treatment, we performed Western blot analysis to detect levels of HSP90 and its

Figure 3. Induction of apoptosis and G2-M phase arrest by 17-DMAG. The Cell Death Detection ELISAPLUS kit was used to quantify the number of LTEDaro (A) and MCF-7aro (B) cells undergoing apoptosis in the presence of either DMSO or 100 nmol/L 17-DMAG for 48 or 72 h. *, P < 0.05, Student's t test when comparing with the DMSO control. Bars, SE. LTEDaro (C) and MCF-7aro (D) cells were treated with either DMSO or 100 nmol/L 17-DMAG for 24, 48, or 72 h. After each time point, cells were stained with propidium iodide and analyzed by flow cytometry.

Figure 4. ERα protein levels and activity in the LTEDaro and MCF-7aro cell lines after 17-DMAG treatment. LTEDaro (A) and MCF-7aro (B) cells were treated with either DMSO or 17-DMAG for 24, 48, or 72 h. MCF-7aro cells were additionally treated with 1 nmol/L testosterone. ERα protein expression was determined by Western blot. The pGL3-(ERE)3 reporter plasmid was transiently transfected into LTEDaro (C) and MCF-7aro (D) cell lines. Both cell lines were treated with either DMSO or 100 nmol/L 17-DMAG and with or without 1 nmol/L E2 for 48 h. Mean ± SE. Representative of three independent experiments done in triplicate.
client protein expression. HSP90 expression was similar in both MCF-7aro and LTEDaro whole-cell lysates (Fig. 5A and B). To analyze whether apoptotic markers were activated, we checked the expression of PARP, which is cleaved due to caspase-3 and -7 activation (28). Levels of cleaved PARP increased with increasing concentration of 17-DMAG and with time, indicating induction of apoptosis resulting from 17-DMAG treatment and confirming our data that 17-DMAG induces apoptosis (Fig. 5A and B). In addition, cyclin D1 levels also decreased with increasing concentration, indicating that fewer cells are in the G1 phase of the cell cycle (Fig. 5A and B). These results also corrobore our cell cycle analysis (Fig. 3C and D), which indicate an increased population of cells in the G2 phase after 17-DMAG treatment.

To identify other pathways that may be affected by 17-DMAG treatment, we examined the expression levels of HSP90 client proteins after 17-DMAG treatment. We determined the levels of AKT, phospho-HER2, and HER2, proteins that have growth-promoting and prosurvival effects (29–31). Client proteins AKT and HER2 as well as the phosphorylated, activated form of HER2 protein levels decreased with increasing dose of 17-DMAG treatment and in a time-dependent manner in the LTEDaro (Fig. 5A) and MCF-7aro (Fig. 5B) cell lines. These results confirm that AKT and HER2 are targeted by 17-DMAG.

**AKT and HER2 pathways are important for cell growth in the MCF-7aro and LTEDaro cell lines.** Our Western blot data suggested that the pathways by which AKT and HER2 function may be targeted by 17-DMAG. To determine whether these pathways play an important role in the growth of the cells, we treated both LTEDaro and MCF-7aro cells with the AKT inhibitor triciribine and the HER2 inhibitor AG825 and measured cell proliferation. Triciribine and AG825, individually, inhibited both LTEDaro and MCF-7aro cell growth in a dose-dependent manner and were similarly potent in both cell lines (Supplementary Fig. S2A and B). Inhibition of growth by the AKT or HER2 inhibitors indicates the importance of these proteins for cell growth in the aromatase inhibitor–resistant breast cell lines and suggests that these pathways may be targeted by 17-DMAG.

To test this hypothesis, we treated both LTEDaro and MCF-7aro cells with 17-DMAG along with triciribine or AG825 and measured growth of these cells. Triciribine, 17-DMAG, and the combination of both inhibited LTEDaro cell growth in a dose-dependent manner (Supplementary Fig. S3A). Interestingly, when both inhibitors were used in combination, statistical analysis indicated synergistic reduction in cell growth (Table 1; Supplementary Fig. S3B). Similarly, the effect of 17-DMAG on MCF-7aro cells was synergistically enhanced with increasing concentration of triciribine (Table 1; Supplementary Fig. S3C and D).

Cell growth was inhibited with increasing concentration of HER2 inhibitor, AG825, 17-DMAG, as well as the combination of the two in both LTEDaro and MCF-7aro cell lines (Supplementary Fig. S4A and C). Although increasing concentrations of both inhibitors resulted in increased suppression of growth, statistical analysis of these results indicated that these inhibitors work in an antagonistic manner in both cell lines (Table 1; Supplementary Fig. S4B and D). These results show that both AKT and HER2 pathways play a role in the growth of aromatase inhibitor–resistant breast cancer cells, and all these pathways are targeted by 17-DMAG. However, because AKT inhibitor, triciribine, and 17-DMAG function synergistically, they can be used in a combinotorial manner to treat aromatase inhibitor–resistant breast cancer.

**Discussion**

Aromatase inhibitors are currently first-line therapies for the treatment of postmenopausal ER-positive breast cancer patients. However, acquired resistance to the inhibitors does develop. In the clinical setting, patients who develop resistance to aromatase inhibitors do not respond to current available endocrine therapies. This creates a need for new therapies or treatment regimens that can be used to treat these aromatase inhibitor–resistant breast cancer patients. HSP90 inhibitors have been shown to be effective at inhibiting the growth of various different cancers, including breast cancer. 17-allylamino-17-demethoxygeldanamycin, an early-generation HSP90 inhibitor, has been tested on hormone-refractory breast cancers and was shown to be quite effective at inhibiting cell proliferation.
proliferation (32) as well as inducing apoptosis (33, 34). The HSP90 inhibitor 17-DMAG has been reported to inhibit proliferation and angiogenesis in pancreatic and gastric cancers by interfering with growth factor signaling (35, 36).

Aromatase inhibitor–resistant breast cancers do not rely on hormone-mediated signaling, but growth factor signaling is important for their growth. It has been found that the ERα can be phosphorylated and activated in a ligand-independent manner. This activation is mainly due to the cross-talk between the ERα and growth factor signaling pathways, such as insulin-like growth factor-I receptor and ErbB2-mediated signaling pathways. Because these growth factor signaling proteins are important for resistance to aromatase inhibitors and are also HSP90 client proteins, we were interested in studying whether the HSP90 inhibitor 17-DMAG would be an effective mode of treatment for aromatase inhibitor–resistant breast cancer.

In this study, 17-DMAG is shown to effectively suppress cell growth of both the aromatase inhibitor–responsive MCF-7aro cells and the aromatase inhibitor–resistant LTEDaro cells. The dosages used are in a submicromolar range, showing that the inhibitor is very potent in these cells. A previous report indicated that HSP90 inhibitors display specificity toward cancerous cells (25). Our data show that the inhibitor is ineffective at low doses in normal breast epithelial cells. This is expected because noncancerous cells do not overexpress HSP90 as cancerous cells do (Supplementary Fig. 55). Thus, cancerous cells would be more susceptible to the effects of the inhibitor at low doses. However, at higher doses, an excess of the inhibitor would affect all HSP90 client proteins necessary for growth, even in normal cells. This suggests that, at a certain concentration range, 17-DMAG could inhibit cancer cell growth, without affecting normal cells.

Further analysis as to how treatment with 17-DMAG leads to inhibition of growth indicated DNA fragmentation. Confirmation of 17-DMAG–mediated induction of apoptosis was observed by Western blotting for cleaved PARP, which facilitates cellular disassembly and is an indicator of cells undergoing apoptosis. 17-DMAG treatment also led to a decrease in the levels of HSP90 client proteins, HER2 protein levels decreased as well as its active phosphorylated state. Following the decreased HER2 levels, the downstream AKT protein levels also decreased. AKT plays an important role in the suppression of apoptosis and promotes growth. AKT has been shown to suppress apoptosis through the phosphorylation of proapoptotic proteins, transcription factors, and regulators of transcription factors, which results in blockage of transcriptional repression of proapoptotic genes and survival (37–39). HER2 and AKT proteins promote growth and decreased levels of these proteins would facilitate apoptosis in the cell.

In addition to inducing apoptosis, 17-DMAG also causes cell cycle arrest at the G2-M phase. This phenomenon has been observed in some studies (40), whereas others have detected arrest at the G1-S phase (40, 41) depending on the cell line. The levels of HSP90 client proteins may differ in each cell line; thus, treatment with the inhibitor may affect certain proteins more readily than others, which may explain the differences among the cell lines. Cyclin D1 is needed for the progression from G1 to S phase. Although cyclin D1 is not a HSP90 client protein, it is indirectly regulated by client proteins. One study has described a phosphoinositide 3-kinase/AKT regulatory mechanism to control cyclin D1 expression posttranslationally (42). AKT degradation by 17-DMAG treatment might affect cyclin D1 expression. Decreased cyclin D1 expression might allow cells to bypass the G1-S checkpoints and continue through the cell cycle in an unregulated manner. We observed a reduction in the cyclin D1 levels after 17-DMAG treatment in both MCF-7aro and LTEDaro cells, suggesting this could be a mechanism for higher number of cells in G2 stage. Our results show that 17-DMAG inhibits cell proliferation by blocking cells at G2 stage while also inducing cell death of the remaining cells by inhibiting the signaling of growth promoting pathways.

17-DMAG treatment also degrades the HSP90 client protein ERα, which is implicated in aromatase inhibitor resistance (5). Notably, total ERα protein levels and its ligand-independent phosphorylation are elevated in LTED cells compared with the aromatase inhibitor–responsive MCF-7 cells (6, 9). However, our data show that 17-DMAG affects both hormone-dependent MCF-7aro and hormone-independent LTEDaro cells in a similar fashion. This suggests that 17-DMAG does not inhibit both cell lines by targeting of the ERα pathway. ERα phosphorylation and activity were unaffected when cells were treated with 17-DMAG in the presence

### Table 1. Affected fractions and combination indices with 17-DMAG and triciribine or AG825, in combination, on LTEDaro or MCF-7aro at 48 h

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<th>Cell line</th>
<th>17-DMAG (nmol/L)</th>
<th>Triciribine (nmol/L)</th>
<th>AG825 (μmol/L)</th>
<th>Affected fraction</th>
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of E2. These results indicate that the presence of hormone has a protective effect against the inhibition of ERα transcriptional activation by 17-DMAG in both LTEDaro and MCF-7aro cell lines. A mechanism described to link transcription and degradation by the ubiquitin-mediated proteolysis may explain the results (43).

In the MCF-7aro cells, binding of the E2 ligand to ERα signals activation of EROs and dissociation from the HSP90 complex, thereby bypassing 17-DMAG-mediated degradation of EROs. EROs can then induce transcriptional activation. However, EROs are ubiquitinated and targeted for degradation simultaneously as RNA polymerase II elongates transcription through a transcription-coupled degradation mechanism. In the LTEDaro cells, the EROs is not stimulated by E2; however, it may retain its ability to bind. Thus, hormone binding may prevent ERα from being degraded with 17-DMAG treatment, thereby restoring its activity. Despite this postulated mechanism, how EROs transcriptional activity is elevated in the MCF-7aro cells with 17-DMAG and hormone treatment is unclear. More studies are required to fully understand this effect. Although EROs is a HSP90 client protein, the reporter assay results confirm that the EROs pathway is not a target by which 17-DMAG inhibits cell growth.

Our results show that 17-DMAG also targets growth factor pathways to inhibit breast cancer cell proliferation. We observed degradation of both AKT and HER2 by 17-DMAG treatment in LTEDaro and MCF-7aro cells. Additionally, AKT inhibitor triciribine and HER2 inhibitor AG825, alone, were able to inhibit growth of both LTEDaro and MCF-7aro cells. Moreover, we found that AG825, along with 17-DMAG, functions in an antagonistic manner, whereas triciribine and 17-DMAG work in a synergistic manner in both cell lines. Our data show that a combination of both 17-DMAG and an AKT inhibitor may be more effective than 17-DMAG alone in the treatment of aromatase inhibitor–resistant breast cancers.

Aromatase inhibitors are specific and effective inhibitors for treating aromatase inhibitor–responsive breast cancer. It is in breast cancers that have developed resistance to aromatase inhibitors that no good treatment is available. Our results show that the aromatase inhibitor–resistant LTEDaro cells and the aromatase inhibitor–sensitive MCF-7aro cells are growth-inhibited by the HSP90 inhibitor 17-DMAG. 17-DMAG treatment displays potent efficacy in inhibiting breast cancer cell growth. It displays selectivity toward cancer cells at lower doses and displays some cytotoxicity toward normal cells only at higher doses. Currently, HSP90 inhibitors are continually being developed for improved potency and selectivity and these drugs remain to be tested. However, the importance of this study is to show the utility of HSP90 inhibitors as a potential therapy for the treatment of aromatase inhibitor–resistant breast cancers.

The major finding from this study is that HSP90 inhibitors may be used as a therapy to treat aromatase inhibitor–resistant breast cancers. Their ability to target multiple client proteins involved in the different growth-promoting signaling pathways makes HSP90 inhibitors a valuable and promising novel treatment option for aromatase inhibitor–resistant breast cancer patients. This study provides a clinical basis for future clinical trials using HSP90 inhibitors in aromatase inhibitor–resistant breast cancers. In addition, treatment regimens using both a HSP90 inhibitor and a signal transduction inhibitor may also be considered for improved efficacy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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