Hormone-Re refractory Breast Cancer Remains Sensitive to the Antitumor Activity of Heat Shock Protein 90 Inhibitors


ABSTRACT

Purpose: The antiestrogen tamoxifen (Tam) has been used as therapy against estrogen receptor (ER)-positive breast cancer for decades. Most tumors respond initially, but resistance frequently develops. The ER exists in a multiprotein complex containing the molecular chaperone heat shock protein (Hsp) 90, which is known to regulate the stability and activity of this receptor. Therefore, we investigated a ligand-independent approach to hormonal therapy that depletes cellular levels of the receptor by inhibiting the function of Hsp90.

Experimental Design: The activity of the Hsp90 inhibitor geldanamycin (GA) and its clinically relevant derivative, 17-allylamino-17-demethoxygeldanamycin (17AAG), was examined at the molecular and cellular levels using Tam-resistant MCF-7 breast cancer cells both in vitro and in tumor xenografts.

Results: The ER was depleted by GA in several Tam-resistant cell lines, as were other Hsp90 client proteins such as Akt and Raf-1. Unexpectedly, Tam inhibited ER depletion by GA but had no effect on destabilization of Akt or Raf-1. When SCID mice supplemented with Tam were treated with 17AAG, their tumors also showed no decrease in ER levels as measured by immunofluorescent staining and laser scanning cytometry. In these same tumors, however, decreased Akt and Raf-1 levels were observed. Drug administration also led to inhibition of tumor xenograft growth. The mechanism by which Tam inhibits GA-mediated ER depletion is unclear, but immunoprecipitation experiments showed that Tam does not inhibit the ability of GA to alter the ER-chaperone complex.

Conclusions: Based on its ability to deplete the ER as well as other critical signaling molecules in Tam-resistant breast cancer, 17AAG may provide a useful alternative treatment for patients with recurrent, hormone-refractory breast cancer that should be explored further in Phase II trials. In this context, combined treatment with 17AAG and Tam should be avoided because Tam may inhibit the ability of 17AAG to deplete the ER, potentially reducing its anticancer activity.

INTRODUCTION

The ER has become an important target in the management of hormone-responsive breast cancer, and the antiestrogen Tam is now a standard component of front-line therapy for ER+ breast cancers, inducing remissions in over half of patients treated (1). Unfortunately, most tumors eventually become Tam resistant. The mechanisms that underlie this resistance are poorly understood but appear to include alterations in ER expression, structure, or the association of cofactors involved in ER transactivation (2, 3). Furthermore, Tam is known to exhibit partial agonist activity in breast cancer cell lines, which may compromise its antitumor activity (4). The antiestrogens ICI 164,384 and ICI 182,780 exhibit pure antagonist properties and are able to inhibit the growth of Tam-resistant cell lines (5, 6). However, resistance to these pure antiestrogens has also been observed (7). Thus, whereas it is possible to delay breast tumor progression with currently available hormone antagonists, the frequent development of resistance remains a major limitation to their overall effectiveness as anticancer agents.

To address the problem of resistance, we have been examining the feasibility of targeting Hsps to disrupt ER function in a manner that does not rely on the interaction of antagonist with the hormone-binding subunit of the receptor (8). Steroid hormone receptors exist in multiprotein complexes containing Hsp90 and other essential molecular chaperone proteins (reviewed in Ref. 9). Iterative, low-affinity interactions with these complexes are required to maintain the receptor in a mature conformation that is capable of binding ligand with high affinity (10, 11). Upon ligand binding, these interactions are altered, allowing the receptor to bind DNA tightly, recruit coactivators and/or corepressors, and regulate target gene expression (12). Over the past decade, we and others have shown that the antitumor

2 The abbreviations used are: ER, estrogen receptor; Tam, tamoxifen; Hsp, heat shock protein; GA, geldanamycin; 17AAG, 17-allylamino-17-demethoxygeldanamycin; LSC, laser scanning cytometry; E2, 17β-estradiol; DAPI, 4′,6-diamidino-2-phenylindole; SCID, severe combined immunodeficient; ERE, estrogen response element; IP, immunoprecipitation; MCF, median channel fluorescence.
antibiotics GA and radicicol act as selective Hsp90 inhibitors with the ability to alter the stability and activity of steroid hormone receptors (13) as well as numerous cancer-associated growth factor receptors, kinases, and transcription factors (reviewed in Refs. 14 and 15). Due to this novel mechanism of action, considerable enthusiasm exists for the development of Hsp90 inhibitors as anticancer agents. National Cancer Institute-sponsored Phase I trials of the GA derivative 17AAG are nearing completion (16–19).

In the case of steroid receptors, drug-induced alterations in Hsp90 function clearly lead to rapid loss of hormone binding and enhanced proteasome-mediated degradation of the progestosterone (20), androgen (21, 22), and glucocorticoid receptors (23). Consistent with previous reports that the ER does not require ongoing Hsp90 interaction to maintain its high-affinity hormone-binding conformation (24, 25), we found that Hsp90 inhibitors do not immediately disrupt estrogen binding in whole cells. However, they do deplete cellular ER levels and impair receptor function both in vitro and in estrogen-supplemented mice bearing hormone-responsive breast tumor xenografts (4, 8, 26). In the current study, we now extend these observations to hormone-refractory breast cancer and assess the feasibility of disrupting hormone signaling in this setting using a chaperone-targeted, ligand-independent approach. We found that GA and its clinically relevant derivative, 17AAG, can destabilize the ER and inhibit its ability to activate gene expression in both Tam-sensitive and Tam-resistant breast cancer cells. Furthermore, 17AAG treatment of tumor-bearing mice inhibited the growth of Tam-resistant tumors. As expected, growth inhibition was associated with modulation of several well-recognized Hsp90 client proteins. Surprisingly, however, we found that Tam, unlike estrogen, inhibited ER destabilization by GA and 17AAG both in vitro and in vivo. The precise mechanism underlying this Tam-related effect is currently under investigation, but it does not appear to involve inhibition of GA-induced alterations in the composition of ER-associated chaperone complexes. Taken together, our results indicate that 17AAG can disrupt ER function in hormone-refractory breast cancer but that combined therapy with Tam and Hsp90 inhibitors may prove problematic.

MATERIALS AND METHODS

Cells and Reagents. MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA). TAMR-1, TAMR-7, and TAMR-8 cells were generated in the laboratory of A. E. Lykkefeldt and have been described previously (5, 27). Cells were cultured at 37°C under 6% CO2 in air using phenol red-free DMEM:Ham’s F-12 (Invitrogen) containing 1% fetal bovine serum (Irvine Scientific, Santa Ana, CA) and 6 mg/ml insulin. Cells were refed twice weekly and passaged 1:5 once weekly. To maintain high-level resistance in TAMR cell lines, medium was supplemented with Tam (1 μM). All cell lines tested negative for Mycoplasma by ELISA. The C3 luciferase reporter plasmid (C3-luc) was provided by D. P. McDonnell and has been described previously (28). All biochemical reagents were from Sigma unless otherwise specified. GA (NSC 122750) and 17AAG (NSC 330507) were provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute. Stock solutions were formulated in DMSO and maintained at −70°C in the dark before use within 1 month. For immunodetection of the ER, a mixture of monoclonal antibodies 1D5 and 6F11 was used at 1:1000 for immunoblotting and 1:100 for immunofluorescent cell staining (Neomarkers, Fremont, CA). For ER IP, clone AER310 was used (Neomarkers, 3 μg/reaction). Anti-Akt (catalogue number 9272) and phospho-Akt (Ser473; catalogue number 9271) were used at 1:1000 for immunoblotting (Cell Signaling; Beverly, MA). Additional immunoblotting antibodies included Anti-r-raf-1 (Santa Cruz Biotechnology, Santa Cruz, CA; clone E-10; 1:250), anti-Hsp72 (Stressgen, Victoria, British Columbia, Canada; clone C92F3A-5; 1:5000), and anti-Hsp90 (Stressgen; clone AC88; 1:500). Anti-p23 ascites were provided by D. O. Toft (clone J13; 1:1000).

Immunoblotting. To analyze protein levels, breast cancer cells were harvested by scraping into nonionic detergent buffer supplemented with protease and phosphatase inhibitors as described previously (29). Tumor lysates were prepared by grinding snap-frozen tissue into a powder over a dry ice/ethanol bath. Lysis buffer was then added directly to the powder followed by extraction on ice for 15 min. All lysates were clarified by centrifugation at 14,000 × g for 30 min at 4°C. Supernatants were collected, and equal amounts of protein [as determined by bicinchoninic acid assay (Pierce, Rockford, IL)] were resolved by SDS-PAGE using 7.5% or 12% gels. Proteins were electroblotted to nitrocellulose membranes, which were subsequently stained with Ponceau S to confirm that equal amounts of protein were loaded and transferred. Membranes were then blocked with 3% milk and immunoblotted for the protein of interest. Species-appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson Immunolabs; 1:100,000) and chemiluminescent substrate (Pierce) followed by exposure to Kodak XAR-5 film were used for detection. Multiple exposure times were evaluated for each blot to ensure that the band intensities were within the dynamic response range of the film.

Reporter Construct Assays. MCF-7 and TAMR-1 cells were inoculated into 24-well plates at 1–1.5 × 105 cells/well. The following day, wells were transfected with 0.9 μg of C3-luc inducible reporter and 0.1 μg of pRLnull constitutive Renilla reporter (Promega) using LipofectAMINE Plus reagent (Invitrogen). After 3 h, transfection medium was replaced with prewarmed serum-free media ± 2 μM 17AAG. After an additional 3–5 h, wells were supplemented with E2 (5 μM) or E2 (5 μM) plus 17AAG (2 μM). After 24 h, the firefly and Renilla luciferase activities of cell lysates were quantitated using a dual luciferase assay system (Promega) and a Femtomaster FB 12 single tube luminometer (Zylux Corp.) per the manufacturer’s recommendations.

Immunostaining. Immunofluorescent staining was performed on monolayer cultures and on frozen tumor sections mounted on glass slides. Monolayer cultures were established using 1 × 105 cells/well in 8-well chamber slides and allowed to adhere overnight (Nalge Nunc International). After incubation with test compounds overnight in serum-free medium, cells were fixed in 4% freshly prepared paraformaldehyde for 20 min and permeabilized with 0.5% Triton X-100 for 30 min. Slides were then blocked for 30 min in PBS containing 10% goat serum and 0.3% BSA. Anti-ER primary antibody was applied in PBS containing 1% goat serum, 0.3% BSA, and RNase A (20
μg/ml), and incubation was carried out overnight at 4°C. After extensive washing, slides were incubated for 60 min with Alexafluor-conjugated goat antimouse F(ab')2 fragment secondary antibody (Molecular Probes, Eugene, OR). Cells were then incubated with DAPI (1 μg/ml) in PBS for 15 min followed by extensive washing and mounting with Cytoseal 60 under coverslips (VWR Scientific). The staining procedure for snap-frozen tumor sections was identical to that used for chamber slides, except that 1% Triton X-100 was used to permeabilize the tissue.

**ER Quantitation by Immunofluorescent Staining.**

LSC is a microscope-based cytofluorometric technique that combines the advantages of flow cytometry with image analysis (30, 31). To measure relative ER levels, immunostained chamber slides and frozen tumor sections were analyzed on a Model LSC-1 instrument using sequential violet and argon laser excitation and the appropriate filter sets (CompuCyte Corp., Cambridge, MA). Individual cells were identified by contouring on their DAPI-stained nuclei. The relative ER level for each cell identified was defined by maximum pixel intensity, and frequency histograms were acquired for each experimental sample in a manner analogous to standard flow cytometry. Appropriate size gates were used to omit multicell events. At least 3000 events/sample were analyzed per chamber slide, and at least 5000 events/sample were acquired to evaluate tumor sections. To complement the quantitative data generated by LSC, the same slides examined by LSC were also evaluated qualitatively by confocal microscopy (MRC 5000; Bio-Rad). All images were acquired using a ×20 objective with identical gain, black level, and iris settings.

**IP.** MCF-7 cells were plated in 10-cm dishes, grown to ~70% confluence, and then hormone deprived by incubation in medium containing 1% charcoal-stripped serum for 48 h. To examine drug effects on the composition of ER-chaperone protein complexes, cells were incubated for 2 h with various test compounds and then scraped into cold, molybdate-containing lysis buffer as described previously (20). After brief sonication on ice, lysates were cleared by centrifugation at 14,000 × g for 30 min at 4°C, and supernatants were incubated with ER-specific primary antibody for 3–4 h at 4°C. Protein G-Sepharose beads (GammaBind Plus; Pharmacia; 15 μl resin/precipitation) were added, and incubation was continued for an additional hour with gentle agitation in the cold. Beads were spun down and washed four times, and bound proteins were eluted into 1X Laemmli sample loading buffer by heating at 95°C for 5 min. Samples were then resolved on 7.5% or 12% SDS-PAGE gels and immunoblotted as described above.

**In Vivo Studies.** To examine the antitumor activity of 17AAG against hormone-refractory breast tumors, TAMR-1 cells (5 × 106) suspended in 200 μl of a 1:1 mixture of saline and Matrigel (Discovery Labware, Inc., Bedford, MA) were injected into the mammary fat pad of SCID mice (Arizona Cancer Center breeding colony; C.B-17/IcrACCscid). The following day, hormone supplementation was begun as daily s.c. injections of Tam (500 μg/dose) dissolved in peanut oil. Injections were continued 5 times/week for the duration of the experiment. Eleven days after cell inoculation, mice with palpable tumors were randomized to receive i.p. injections of 17AAG (100 mg/kg) or an equal volume of DMSO vehicle 3× weekly for 2 weeks. To assess drug effects on tumor progression, serial caliper measurements were performed for 77 days (8 mice/group), and tumor volume was calculated using the equation: length × width²/2. To assay for 17AAG-mediated target modulation, TAMR-1 tumor xenographs were established, and mice were treated as described above. Eighteen h after the second 17AAG injection, mice were sacrificed, and tumors were removed. Half of each tumor was snap-frozen in OCT embedding medium (Tissue-Tek, Torrance, CA), and cryotome sections were prepared for immunofluorescence staining. The other half was snap-frozen for preparation of lysates and immunoblot analysis. For quality control purposes, tumor lysates were routinely fractionated by 7.5% SDS-PAGE, followed by Coomassie Blue staining, to evaluate the overall integrity of the sample. Only those samples found to be free from extensive proteolysis or heavy contamination by serum albumin were evaluated by immunoblotting for specific protein levels. All in vivo experiments were performed under protocols approved by the University of Arizona Institutional Animal Care and Use Committee.

**RESULTS**

**GA Depletes the ER and Other Hsp90 Client Proteins in Hormone-Refractory Cells.** To evaluate the effects of GA on Tam-sensitive and Tam-resistant breast cancer cells, we used MCF-7 and three Tam-resistant subclones of MCF-7 (TAMR-1, TAMR-7, and TAMR-8), which have been described previously (27). All TAMR cell lines express readily detectable amounts of ER protein, although at somewhat reduced levels compared with the parental MCF-7 cells. Studies with the TAMR-1 cell line have shown that it is E2 responsive and contains no ERα mutations (32). Cells were exposed to GA, Tam, E2, or combinations thereof overnight in low serum-containing media. Western blot analysis revealed that GA depleted ER (Fig. 1A, Akt (Fig. 1B), and Raf-1 (Fig. 1C) levels in all cell lines tested. As expected from previous reports, Tam exposure resulted in a slight increase in ER levels [Fig. 1A, Lane 2 (33, 34)], whereas there was a substantial decrease in ER protein upon E2 stimulation [Fig. 1A, Lane 3 (35, 36)]. Unexpectedly, Tam appeared to limit the ability of GA to deplete ER levels in MCF-7 and all TAMR cell lines (Fig. 1A, Lane 5). This effect was not seen with Akt or Raf-1 (Fig. 1, B and C, Lanes 5), suggesting that Tam selectively inhibits the enhanced ER degradation induced by GA. A similar effect was not seen with the GA + E2 combination (Fig. 1, A–C, Lanes 6). Because all TAMR cell lines responded similarly to GA treatment, we chose the TAMR-1 cell line for further study.

**Modulation of ER Levels by 17-AAG Is Detectable by Immunoblotting, Confocal Microscopy, and LSC.** It is problematic to monitor ER protein levels in tumor tissue by Western blotting because of interference by comigrating serum albumins present in tissue lysates. Therefore, we developed a method involving immunofluorescence staining and LSC as an alternative approach. To validate the method and prepare for in vivo studies, MCF-7 cells were grown as confluent monolayers in vitro and treated with ethanol vehicle (ethanol), Tam, 17AAG, or Tam + 17AAG. 17AAG is a clinically relevant GA derivative that retains activity in whole animals, whereas GA does not. After overnight exposure to drugs, lysates were pre-
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Tamoxifen treatment was markedly reduced after 17AAG treatment. ER staining after exposure to 17AAG was observed with ethanol vehicle, whereas the signal was readily apparent (Fig. 2A). Robust nuclear ER microscopy, and qualitative differences between treatment groups were demonstrated in Fig. 2B. DAPI counterstain was included in the procedure to identify cell nuclei during cytometry. We confirmed specific nuclear staining for ER under these conditions by confocal microscopy, and qualitative differences between treatment groups were readily apparent (Fig. 2B). Robust nuclear ER staining was observed with ethanol vehicle, whereas the signal was markedly reduced after 17AAG treatment. ER staining after Tam + 17AAG treatment was intermediate, consistent with the data in Fig. 2A. The no primary antibody control displayed very little background staining. To quantify these observations, the same chamber slides were scanned by LSC using a sequential violet and argon laser excitation protocol that keys on DAPI-stained nuclei to identify events and then acquires relative ER fluorescence intensity (as a measure of ER protein levels) were compared and immunoblotted for ER, Akt, or phospho-Akt (Ser473). As shown in Fig. 2A, there was a marked reduction in ER, Akt, and phospho-Akt levels after exposure to 17AAG (Lane 3), whereas treatment with Tam had little effect. Consistent with the GA data in Fig. 1, Tam inhibited depletion of ER levels by 17AAG but had no effect on down-regulation of Akt or phospho-Akt. To compare the changes in ER protein levels documented by immunoblotting with those detectable by LSC, we next grew cells to confluence in chamber slides and exposed duplicate wells to the identical drug treatment conditions used to generate immunoblotting lysates. Cell monolayers were then fixed and stained for ER using an indirect immunofluorescence technique. DAPI counterstain was included in the procedure to identify cell nuclei during cytometry. We confirmed specific nuclear staining for ER under these conditions by confocal microscopy, and qualitative differences between treatment groups were readily apparent (Fig. 2B). Robust nuclear ER staining was observed with ethanol vehicle, whereas the signal was markedly reduced after 17AAG treatment. ER staining after Tam + 17AAG treatment was intermediate, consistent with the data in Fig. 2A. The no primary antibody control displayed very little background staining. To quantify these observations, the same chamber slides were scanned by LSC using a sequential violet and argon laser excitation protocol that keys on DAPI-stained nuclei to identify events and then acquires relative ER immunofluorescence signal for that same event. As demonstrated in Fig. 2C, frequency distribution histograms of relative fluorescence intensity (as a measure of ER protein levels) were generated and compared between the ethanol, 17AAG, and Tam + 17AAG treatment groups. Consistent with immunoblotting and confocal microscopy data, ER levels were markedly decreased after 17AAG treatment, and this effect was substantially inhibited when Tam and 17AAG were combined.

E2-Stimulated Gene Expression Is Inhibited by 17AAG in MCF-7 and TAMR-1 Cells. To determine whether depletion of ER protein by 17AAG had a functional effect on ER transcriptional transactivating activity, we used cells transiently transfected with a reporter plasmid encoding an ERE upstream of the firefly luciferase gene. Previous work had already demonstrated that a reporter plasmid containing the vitellogenin ERE upstream of the CAT gene could be activated upon E2 stimulation of the cells. Cotransfection with the plasmid pRLnull that constitutively expresses Renilla luciferase was used to normalize for transfection efficiency. After transfection, cells were treated with media ± 17AAG for 3 h and then stimulated overnight with E2 or E2 + 17AAG. Analysis of the lysates revealed a 3- to 4-fold induction of luciferase activity by E2 in both cell types. Not only was E2 stimulation of luciferase activity inhibited by 17AAG, reporter activity was actually reduced below basal levels in both cell types. This reduction was not due to nonspecific cytotoxicity because 17AAG treatment had little effect on the absolute level of reporter activity generated by the cotransfected constitutive Renilla reporter plasmid (data not shown). Of note, TAMR-1 cells displayed lower basalERE reporter activity than parental MCF-7 cells. This may be due to the lower level or altered composition of these complexes, most notably by inhibiting the

Tam Does Not Interfere with GA-Induced Alterations in Chaperone Complexes Physically Associated with the ER. Mature steroid hormone receptors exist in multiprotein complexes containing Hsp90, the cochaperone p23, and one of several large immunophilins (9). GA is known to alter the composition of these complexes, most notably by inhibiting the

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Fig. 1 Hsp90 client proteins are depleted by GA treatment in Tam-sensitive and Tam-resistant breast cancer cells. MCF-7, TAMR-1, TAMR-7, and TAMR-8 cells were cultured in media with 1% charcoal-stripped serum for 24 h. Subsequently, cells were exposed overnight to ethanol vehicle control (Lane 1), 1 μM Tam (Lane 2), 1 μM E2 (Lane 3), 1 μM GA (Lane 4), a combination of 1 μM Tam + 1 μM GA (Lane 5), or a combination of 1 μM E2 + 1 μM GA (Lane 6). Lysates were prepared, and equal amounts of cellular protein were analyzed by immunoblotting for ER level (A) and Akt (B). In C, the same blot presented in A was reprobed for Raf-1 without stripping. The Raf-1 band is indicated by an arrow, and the persistent ER band is represented by an asterisk.
participation of p23. It is GA-induced alterations in chaperone associations with receptor protein that are thought to enhance receptor ubiquitination and subsequent proteasome-mediated degradation (23). Because ER antagonists such as Tam are known to induce distinct conformational changes in the receptor protein (37), we wondered whether Tam might alter the composition of the chaperone complexes that associate with the ER and thus alter its stability and sensitivity to GA. To examine this possibility, we treated MCF-7 cells for 2 h with E2, Tam, GA, or Tam/H11001 GA and then lysed the cells and immunoprecipitated the ER. In Fig. 4, precipitates were analyzed by immunoblot to assess levels of the ER and its associated Hsp90 and p23.

Fig. 2  Modulation of ER levels by 17AAG as detected by immunoblotting, confocal microscopy, and LSC. MCF-7 cells were exposed to ethanol vehicle control, Tam (1 μM), 17AAG (2 μM), or Tam (1 μM) + 17AAG (2 μM) overnight as indicated. A, cell lysates were subjected to immunoblot analysis with antibodies specific for ER, Akt, or phospho-Akt (Ser473) as indicated. B and C, cells growing in chamber slides were fixed and stained for ER using an indirect immunofluorescent technique. B, representative confocal images from each treatment group were obtained using identical magnification, gain, and iris settings. Con refers to an ethanol-treated well that was stained without addition of primary antibody to assess nonspecific binding of the fluorescence-labeled secondary antibody. C, the same chamber slides were analyzed by LSC. Independent replicate wells for each treatment condition were scanned, and frequency distribution plots of the relative fluorescence intensity of individual cells within each well are depicted as open traces. The treatment conditions used to generate each pair of histograms are indicated in the figure. Filled trace, no primary antibody control.
actions with the ER and did not interfere with at least one of GA’s expected effects on Hsp90 function. As controls, Lane 1 contains an aliquot of total cell lysate before IP, and Lane 2 contains an aliquot of the same lysate after IP was performed. As expected, total Hsp90 and p23 levels did not decrease after IP because only a small fraction of the total cellular pools of these proteins are associated with the ER. The ER levels in lysate before and after IP are not visible on the exposure presented because the ER is a much less abundant protein than the chaperones seen in Lanes 1 and 2. Longer exposures did reveal depletion of the ER in the sample run in Lane 2 (data not shown). Lane 3, in which no primary antibody was added to the IP, is a control to identify proteins that bind protein G beads nonspecifically. A prominent nonspecific band (NS) was detected that migrates just below the p23 band. This same experimental design was repeated twice more with similar results.

17AAG Does Not Deplicate ER Levels in Breast Tumors Growing in Tam-Supplemented Mice But Does Modulate Other Hsp90 Clients. To examine the activity of 17AAG against Tam-resistant breast cancer in vivo, we developed a xenograft model that used the same TAMR-1 cells used for in vitro studies. Consistent tumor engraftment and reproducible growth kinetics were achieved by injecting cells suspended in Matrigel into the mammary fat pad of female SCID mice. Like parental MCF-7 cells (5), we found that TAMR-1 cells displayed an absolute requirement for hormone supplementation to support their tumorigenicity (Fig. 5). Unlike MCF-7 cells, however, Tam supplementation (500 μg/mouse/day) was much more effective than standard E2 supplementation (data not shown). To examine alterations in Hsp90 client protein levels in this model, mice bearing well-established TAMR-1 tumors were treated twice with 17AAG (4 mice) or DMSO vehicle control (4 mice) and sacrificed 18 h after the final treatment. Cryostat sections and protein lysates were prepared from each tumor. Sections were stained for ER. Specific staining was verified by confocal microscopy, and levels were quantitated using LSC. Confocal microscopy showed that tissue integrity was preserved sufficiently to achieve specific ER staining in four DMSO-treated tumors but in only three 17AAG-treated tumors (Fig. 6A). Visually, there appeared to be no decrease in ER levels with 17AAG treatment. LSC histograms were then generated for each of these DMSO-treated (Fig. 6B, top panel) or 17AAG-treated tumors (bottom panel). Included in each panel is a tumor where no primary antibody was added to control for nonspecific binding of the fluorescent secondary antibody to the tumor tissue (filled trace). Each trace represents a single tumor, and the average of the MCF determinations for each tumor is indicated within the relevant panel. We observed the highest variability in ER staining within the DMSO-treated group, with one tumor exhibiting a noticeably higher level of fluorescence intensity than the other three. The histograms for the 17AAG-treated tumors tracked more closely, with two tumors nearly superimposable, and a third displaying only slightly less fluorescence intensity. Comparison of the average MCF for each group showed no statistically significant difference (4197 for DMSO-treated group and 4800 for 17AAG-treated group; two-tailed t test, $P = 0.38$). The staining and scanning of additional sections from each of these tumors were repeated once with similar results.

Because TAMR-1 tumors required Tam supplementation for in vivo growth, it seemed likely that Tam antagonized ER depletion by 17AAG in vivo, just as we had observed with GA and 17AAG in cell culture (Figs. 1 and 2). To evaluate this hypothesis, we examined whether other 17AAG target proteins had been modulated in TAMR-1 tumors by drug treatment as expected. Lysates from three DMSO-treated tumors and three 17AAG-treated tumors were immunoblotted to determine relative levels of Akt, Raf-1, and Hsp72. An equal amount of
TAMR-1 cell lysate was included as a positive control for each of the immunoblots (Fig. 7). 17AAG treatment clearly down-regulated Raf-1 levels in two of three tumors tested compared with DMSO control, whereas Akt levels were decreased in all three tumors, but to a varying extent. Interestingly, the tumor in which the Raf-1 level was not modulated by 17AAG still showed marked depletion of Akt. An increase in Hsp72 was observed with 17AAG treatment in all three tumors tested, consistent with our previous findings in a different xenograft model (8).

17AAG Inhibits Tumor Growth of MCF-7 and MCF-7/TAMR-1 Xenografts. Given the demonstrated ability to modulate several Hsp90 client proteins, but not ER, in Tam-resistant tumors, we also evaluated the effects of 17AAG on tumor growth. Tam-supplemented SCID mice bearing established TAMR-1 tumors were treated with 17AAG (100 mg/kg)
The antiestrogen Tam has been used as front-line hormonal therapy for ER+ breast cancer for decades (1). Acquired resistance, however, limits its long-term efficacy. Although the mechanisms of Tam resistance are not completely understood, they include alterations in cofactor association, mutations in the ER itself, and a variety of compensatory changes in other growth factor signaling pathways (1, 38). Loss of ER expression, however, is not commonly observed in Tam-resistant tumors (3). A recent study has shown that 77% of patients who developed contralateral breast cancer despite Tam treatment had ER+ tumors (39). These findings suggest that the ER may continue to play a key role in tumor progression despite Tam therapy. In recent years, efforts have been made to develop alternative ER antagonists for treatment of Tam-resistant breast cancer (4). The pure antiestrogens ICI 164,384 and ICI 182,780 have been shown to inhibit the growth of Tam-sensitive breast cancer both in vitro (5) and in patients (40), but acquired resistance to these agents has been reported (7). Thus combinations of antiestrogens may delay tumor progression more effectively than single-agent therapy, but the growth of resistant tumors remains likely. An alternative hormonal approach to antagonist therapy for breast cancer is to reduce levels of circulating E2. Aromatase inhibitors reduce E2 biosynthesis, whereas luteinizing hormone-releasing hormone analogues such as SB-75 (Cetrorelix) pharmacologically mimic ovarian ablation. These hormone deprivation strategies have shown some activity against Tam-resistant breast cancer (41). Recent evidence indicates, however, that during the development of resistance to E2 deprivation (and Tam), cross-talk between critical signal transduction pathways occurs via the ER protein, and this essential cross-talk facilitates tumor survival and proliferation (42). In light of these observations, therapies that do not depend on receptor-ligand interactions or that deplete cellular levels of the ER could prove particularly useful in dealing with the problem of resistance.

We have previously described (8) a novel strategy for the disruption of ER signaling in breast cancer cells, making use of drugs that bind to the chaperone Hsp90. Hsp90 is a critical component of the multiprotein chaperone complexes that have been shown to be required for stabilization of hormone receptors including ER (reviewed in Ref. 10). GA binds to the ATP-binding pocket of Hsp90, altering its function and the chaperone complexes associated with steroid hormone receptors. As a result of these alterations, the receptor undergoes ubiquitination and is targeted for proteasomal degradation (43). We now show that ER levels can be decreased in both Tam-sensitive and Tam-resistant breast cancer cells as a result of exposure to Hsp90 inhibitors in vitro. Unlike the ER itself, however, Hsp90 provides a stable target for pharmacological intervention. Whereas overexpression of certain chaperone proteins has been documented in a variety of cancers, mutation of Hsp90 has never been reported. Furthermore, Hsp90 is known to be involved in the regulation of greater than 100 different client proteins, many of which are involved in mitogenic and survival signaling pathways critical to hormone-refractory breast cancer. Depletion of several of these client proteins may simultaneously contribute to the antitumor activity of Hsp90 inhibitors (44) and make the emergence of resistance less likely. The fact that Hsp90 inhibitors affect multiple signaling pathways also makes them attractive for the treatment of breast cancer, given the evidence that several signaling pathways other than the ER pathway are involved in the development of Tam resistance and breast cancer progression (6, 42). Because Akt and Raf-1 have been shown to be important for the survival and proliferation of breast cancer cells (45, 46) and because these proteins are also known to associate with Hsp90 (47, 48), we evaluated the effects of GA/17AAG on Akt and Raf-1. We found that the levels of these proteins as well as that of the ER were decreased after exposure to GA in Tam-sensitive and Tam-resistant cells. We also found that levels of Akt and Raf-1 were decreased in TAMR-1 tumors from mice treated with 17AAG compared with levels in tumors from control animals. Although no changes in ER protein levels in these tumors were observed (see below), a significant inhibition of tumor growth in 17AAG-treated animals was nonetheless observed, suggesting that modulation of other targets, independent of the ER, contributes to the antitumor activity of 17AAG.

To evaluate effects of Hsp90-binding agents on ER levels in tumor tissues, we used LSC to overcome a methodological problem. Although the ER is readily detectable in cultured cells using immunoblotting, serum albumins from tumor tissue migrate very closely with the Mr 67,000 ER during SDS-PAGE electrophoresis. It is therefore difficult to detect ER in tumor homogenates using this technique. LSC permits highly sensitive, reproducible quantitation of ER levels with minimal manipulation of the experimental sample because tumor homogenization and electrophoresis are not required for analysis. We validated the technique by comparing our ability to detect 17AAG-mediated ER modulation in MCF-7 cells by using immunoblot and LSC (Fig. 2). LSC findings corresponded well with immunoblot results in these experiments. To confirm the ER-specific staining of tissue culture cells detected by LSC, every slide evaluated by LSC was also examined using confocal microscopy. LSC and confocal results were concordant in each case. We then used LSC to analyze tumors from in vivo experiments. As shown in Fig. 6, we were able to quantitate relative ER levels in TAMR-1 xenograft sections after exposure to 17AAG or DMSO. As might be expected, there was variation in ER-specific staining among tumor samples within the same treatment group, likely due to a number of factors including degree of vascularization, interaction between tumor cells and supporting stromal tissue, and systemic levels of growth factors in different animals. Nonetheless, we were able to clearly demonstrate that 17AAG had little effect on ER protein levels in tumors from Tam-supplemented mice. This result was concordant with our in vitro finding that Tam antagonizes the ability of 17AAG to destabilize the ER.
It is not yet clear why Tam inhibits modulation of ER levels by Hsp90-binding drugs, but there is evidence to suggest that Tam itself may decrease the turnover of the ER protein. We have shown that the half-life of unliganded ER in wild-type MCF-7 cells is 2–4 h (8). In contrast, other investigators have demonstrated that only 10% of the ER is turned over in 6 h if it is covalently labeled by a Tam aziridine derivative (34). Consistent with this finding, we and others have also shown that, unlike E2, Tam treatment does not cause a decrease in the level of ER in breast cancer cells (Fig. 1; Ref. 49). Tam clearly induces a unique change in the conformation of the ER protein (37, 50), and it is possible that this change in turn alters the interaction of ER with Hsp90 and/or other chaperone proteins. If this were the case, the Tam-bound ER could become less sensitive to Hsp90-binding drugs. To begin investigating this possibility, we performed coimmunoprecipitation experiments designed to detect changes in the binding of critical chaperone proteins to the ER in the presence of GA and Tam. We did not find any Tam-induced changes in the association of Hsp90 or p23 with the ER protein. As expected, we did find that GA alone caused the dissociation of p23 from the ER-chaperone complex. If Tam induced a conformational change that effectively negated GA effects on the receptor-chaperone complex, a failure to dissociate p23 would have been observed. Instead, as seen in Fig. 4, the presence of both Tam and GA still led to dissociation of p23 from the ER-associated protein complex. At this point, alterations in the ER-chaperone complex do not explain Tam-induced stabilization of the ER in the presence of GA. Interestingly, a recent report has shown that helix 12 of the ER protein is critical for its stability (51). The position of this helix within the receptor protein’s structure appears to vary in a ligand-specific manner, leading to differential association of coregulator proteins and the distinct transcriptional responses induced by various selective estrogen response modifiers. Consistent with this model, these investigators found that a point mutation at Asp538 that alters the conformation of this domain results in enhanced ER turnover in the presence of Tam rather than stabilization as seen with its wild-type counterpart. Likewise, another group has shown that accumulation of wild-type ER in the presence of Tam results from reduced ubiquitination of the protein, apparently as a result helix 12 alterations (52). Further work examining changes in ER ubiquitination and turnover in the presence of Tam and GA is in progress and should provide insights into the mechanism of the ER stabilization observed in our experiments.

Our findings with regard to the effects of the combination of Tam and GA have several important therapeutic implications. Whereas ER levels in breast tumors did not decrease after Hsp90 inhibitor exposure in the presence of Tam, 17AAG treatment nonetheless resulted in decreased growth of these Tam-resistant tumors. This finding, together with the observation that 17AAG modulates the levels of other proteins known to be important in breast cancer, including Raf-1 and Akt (Fig. 7), as well as IGF-IR3 and erbB2 (53), suggests that 17AAG may be effective in the treatment of recurrent breast cancer.

Based on our in vitro results (Figs. 1–3), it is likely that in the absence of Tam, ER levels would be depleted by 17AAG, and its antitumor activity could be even more pronounced. Preliminary work in our laboratory indicates that raloxifene, a selective estrogen response modifier in current clinical use, also antagonizes GA-induced ER depletion, indicating that the effect is not Tam specific. The pure antiestrogen ICI 182,780 (fulvestrant) stimulates degradation of the ER and is effective in about 20% of patients with Tam-resistant breast cancer in clinical trials (54, 55). Experiments in our laboratory are under way to examine the effects of combining GA and fulvestrant on ER stability and to see whether fulvestrant can enhance the antitumor activity of 17AAG in mice bearing TAMR-1 tumors.

Data from ongoing Phase I trials have already indicated that 17AAG is well tolerated in adults with refractory solid tumors at doses that modulate Hsp90 client protein levels in the lymphocytes of patients receiving the drug (16, 17). Our findings now provide strong preclinical support for the pursuit of Phase II trials of 17AAG in patients with recurrent, hormone-refractory breast cancer. Furthermore, our findings suggest that discontinuation of hormonal therapy, especially Tam, before study entry may be important.

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REFERENCES


29. Schirr, R., Massarweh, S., Shou, J., and Osborne, C. K. Breast cancer endocrine resistance: how growth factor signaling and estrogen...
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